Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- you will use the copy only for the purposes of research or private study
- you will recognise the author's right to be identified as the author of the thesis and due acknowledgement will be made to the author where appropriate
- you will obtain the author's permission before publishing any material from the thesis.
Introgression of root and shoot characteristics in *Trifolium repens* x *Trifolium uniflorum* interspecific hybrids

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy

at
Lincoln University
by
Shirley Naina Nichols

Lincoln University
2012
Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

Introgression of root and shoot characteristics in *Trifolium repens* x *Trifolium uniflorum* interspecific hybrids

by

Shirley Naina Nichols

A series of experiments were conducted to determine the effect of hybridisation with *Trifolium uniflorum* L. on root and shoot characteristics of *Trifolium repens* L. (white clover). In a grazing experiment, dry matter (DM) yield scores combined over the experimental period were higher for white clover (5.3) than for BC1 (backcross 1) (4.4) and BC2 (backcross 2) (4.3) hybrid generations. The proportion of nitrogen from fixation (85–96%) was not affected by hybridisation, and there were generally no differences among clover types in shoot %N. In 13 month old plants, tap root survival was higher for *T. uniflorum* (50%), and BC1 (31%) than for BC2 (13%) and white clover (11%). Some tap roots of *T. uniflorum* and the BC1 generation survived up to 19–20 months, but those of BC2 and white clover did not.

In contrast to the field experiment, shoot dry weight (DW) of BC1 hybrids was 2–24 times higher than white clover over four harvest times in a glasshouse root tube experiment using sand culture with a low ionic strength (LIS) nutrient solution. Shoot P concentrations (925–1716 mg kg⁻¹) were below critical levels for white clover growth. In a pot experiment with the same LIS treatment, these effects were confirmed. Over a range of nutrient treatments, some hybrid families were less affected by decreases in nutrient solution strength than others. In all treatments combined, shoot DW of Kopu II x 900-4 was 2.8 times higher than that of the Kopu II parent, and Kopu II x 487-9 was 1.5 times higher. Crusader hybrids did not differ to the Crusader parent. In some treatments, P- and P₂-use efficiency of Kopu II x 900-4 was higher than the other clover types.

Some root characteristics of *T. uniflorum*, inherited by BC1 hybrids, may affect water and nutrient interception. In a glasshouse tube experiment, BC1 hybrids and *T. uniflorum* had more root mass in the upper part of the profile than white clover. In a hydroponics
experiment, the roots of *T. uniflorum* were thicker than BC₁ hybrids and white clover, which may contribute to drought resistance. Topological indices of *T. uniflorum* (0.91–0.94) were higher than for white clover and some hybrid families (0.76–0.92), as were those of Kopu II x 900-4 (0.85–0.98). This herringbone root architecture may be adaptive to low soil fertility.

In a rain shelter experiment, shoot DWs of white clover and BC₂ were 95% and 26% higher, respectively, than that of BC₁ in the Watered treatment. However, shoot DW decreased less under water stress for the BC₁ generation (47%) compared with BC₂ and white clover (nearly 70%). There were no differences in shoot DW among clover types in the Stressed treatment. Stolon morphological characteristics of BC₁ (-31–68%) also decreased less under water stress than those of BC₂ (-44–73%) and white clover (-38–74%). Kopu II BC₁ was able to maintain photosynthesis and transpiration under lower leaf water potentials than Kopu II BC₂ and Kopu II. Net photosynthesis of Kopu II BC₁ did not change under water stress, but in Kopu II BC₂ and Kopu II it decreased by 48% and 44%, respectively. There was also no change in transpiration with water stress for Kopu II BC₁, but in Kopu II BC₂ and Kopu II it decreased by 60%, resulting in a higher mean transpiration rate for Kopu II BC₁ in the Stressed treatment. Under water stress, the leaf water potential of Kopu BC₁ decreased more (-47%) than that of Kopu II BC₂ (-28%) and Kopu II (-31%). Root DW of Kopu II BC₁ increased by 59% under water stress, but that of Kopu II BC₂ and white clover did not change significantly. In the Stressed treatment, root DW of Kopu II BC₁ was 72% higher than that of Kopu II BC₂, and mean cross-sectional area of the thickest nodal root of Kopu II BC₁ was 2.6 times higher than Kopu II.

In addition to improvements for some traits in broad hybrid generations, there was also evidence of differences among hybrid families for many parameters, which should enable selection of superior families and identification of segregating populations.

**Keywords**: *Trifolium repens*, *Trifolium uniflorum*, white clover, interspecific hybrid, introgression, roots, drought, dry matter production, photosynthesis, nitrogen fixation, $^{13}$C, quercetin, soil fertility, water potential, chlorophyll fluorescence, hydroxycinnamic acids, phosphate
Acknowledgements

I have been fortunate to have a supervisory team whose wealth and breadth of knowledge have contributed hugely, not just to this thesis, but to my own learning and professional development. To my principal supervisor, Dr Rainer Hofmann, thank you for your enthusiasm, support and commitment. To my associate supervisor, Dr Derrick Moot, thank you for all your input, especially your comments on the manuscript which have taught me a lot. And finally, Dr Warren Williams from AgResearch, Grasslands, who provided a fascinating PhD topic just as I was looking for one, and has taught me so much about clover interspecific hybrids.

Dr Jim Crush from AgResearch, Ruakura, set it all in motion. Thank you so much for your support. A special thanks also to Keith Widdup at AgResearch, Lincoln, for his advice and assistance. And to Isabelle Verry from AgResearch, Grasslands, for her undying enthusiasm, and hours of painstaking work to produce those precious packets of seed.

Plant and Food Research provided access to their rain shelter, without which a huge part of this project would not have been possible. Thanks especially to Richard Gillespie, Shane Maley and Andrew Fletcher for their advice and assistance.

My thanks go to Stephen Stilwell, Wouter Ballizany, Keith Pollock, Diane Monson, Roger Cresswell, Jenny Zhao, Lynn Clucas, Lydia Beth Disseveld, Jackie Sammonds and the students from PLSC325 2010 at Lincoln University; and Lily Ouyang, Bridget Wise, Ben Harvey, Ben Vlaming, Ivan Baird, Lee Sutherland, Daniel Williams and Gary Arnold at AgResearch for assistance and advice. Roger Orchard and Craig Gibson, at the AgResearch Lincoln farm are thanked for assistance with animals for grazing. To the residents of the smoko hut – thanks for bringing some light relief to long days in the field.

Chikako van Koten, Vanessa Cave and John Waller, of AgResearch, and Richard Sedcole, of Lincoln University, provided statistical advice and assistance – I have learnt a lot from you! Thank you also to Robyn Dynes for discussions on feed quality and comments on parts of the manuscript.

I would like to acknowledge financial support from AgResearch, and funding from MBIE contract C02X0810 (Roots for Sustainable Agriculture), and DairyNZ (contract FD617).

Finally, to all my family, friends and colleagues – thank you for your support over the last 4 years.
Table of Contents

Abstract .................................................................................................................................. ii
Acknowledgements ................................................................................................................ iv
List of Tables ............................................................................................................................. x
List of Figures ............................................................................................................................ xi
List of Plates ................................................................................................................................ xiv
List of Appendices ...................................................................................................................... xv
List of Abbreviations ............................................................................................................... xvii

Chapter 1 Introduction .............................................................................................................. 1
  1.1 Background ......................................................................................................................... 1
  1.2 Production of interspecific hybrids ................................................................................... 2
  1.3 Gaps in the knowledge ........................................................................................................ 3
  1.4 Current context .................................................................................................................... 3
  1.5 Hypotheses and objectives ................................................................................................. 4
  1.6 Terminology ....................................................................................................................... 5
  1.7 Thesis structure ................................................................................................................... 7

Chapter 2 Literature Review ...................................................................................................... 8
  2.1 White clover ........................................................................................................................ 8
    2.1.1 High value pasture species ......................................................................................... 8
    2.1.2 Limitations of white clover ....................................................................................... 9
  2.2 White clover roots ............................................................................................................. 10
  2.3 Soil fertility ....................................................................................................................... 13
  2.4 Drought .............................................................................................................................. 15
    2.4.1 Measuring the effects of drought ............................................................................... 15
  2.5 Drought and white clover ................................................................................................. 17
    2.5.1 Traits that improve resistance to drought in white clover ........................................ 18
    2.5.2 Selecting for drought resistance in white clover ....................................................... 19
  2.6 *Trifolium* interspecific hybrids ......................................................................................... 20
    2.6.1 Problems with hybridisation ....................................................................................... 21
    2.6.2 Pre- and post-fertilisation barriers ............................................................................ 22
    2.6.3 Ovule and embryo rescue ......................................................................................... 23
    2.6.4 Other factors affecting hybridisation success .......................................................... 24
    2.6.5 Confirmation of hybridity ........................................................................................... 25
    2.6.6 Genetic bridges .......................................................................................................... 26
    2.6.7 New *Trifolium* phylogeny ....................................................................................... 26
    2.6.8 Development of commercial hybrids ........................................................................ 28
  2.7 *Trifolium uniflorum* ........................................................................................................ 30
    2.7.1 Morphology .................................................................................................................. 31
    2.7.2 Pest and disease resistance ......................................................................................... 33
    2.7.3 Nodulation .................................................................................................................... 34
    2.7.4 Cyanogenesis .............................................................................................................. 34
  2.8 Interspecific hybridisation between *T. repens* and *T. uniflorum* ................................. 35
    2.8.1 Hybridisation problems ............................................................................................. 36
    2.8.2 Pre- and post-fertilisation barriers ............................................................................ 36
Chapter 3 Effect of hybridisation on key white clover traits ............................................. 39
3.1 Introduction ..................................................................................................................... 39
3.2 Materials and methods ............................................................................................... 40
  3.2.1 Experimental area and preparation ....................................................................... 40
  3.2.2 Plant material ......................................................................................................... 40
  3.2.3 Experimental design ............................................................................................... 42
  3.2.4 Establishment ......................................................................................................... 43
  3.2.5 Management ........................................................................................................... 44
  3.2.6 Measurements ......................................................................................................... 45
  3.2.7 Statistical analysis .................................................................................................. 50
3.3 Results .......................................................................................................................... 51
  3.3.1 Dry matter yield ..................................................................................................... 51
  3.3.2 Tap root measurements ......................................................................................... 56
  3.3.3 Nitrogen fixation .................................................................................................... 61
  3.3.4 Stolon morphology ................................................................................................. 62
  3.3.5 Flowering ............................................................................................................... 66
  3.3.6 Other characteristics ............................................................................................... 69
3.4 Discussion ...................................................................................................................... 77
  3.4.1 Dry matter yield ..................................................................................................... 77
  3.4.2 Stolon morphology and growth form ..................................................................... 78
  3.4.3 N fixation ............................................................................................................... 82
  3.4.4 Tap root survival .................................................................................................... 83
  3.4.5 Fungal disease and virus infection ....................................................................... 85
  3.4.6 Flowering ............................................................................................................... 85
  3.4.7 Variability ............................................................................................................... 86
3.5 Conclusions .................................................................................................................... 86

Chapter 4 Root depth distribution and associated traits.................................................. 88
4.1 Introduction .................................................................................................................... 88
4.2 Materials and methods ............................................................................................... 89
  4.2.1 Experimental setup ............................................................................................... 89
  4.2.2 Plant material ......................................................................................................... 90
  4.2.3 Experimental design ............................................................................................... 91
  4.2.4 Nutrient solution .................................................................................................... 93
  4.2.5 Plant harvests ......................................................................................................... 93
  4.2.6 Statistical analysis .................................................................................................. 95
4.3 Results .......................................................................................................................... 97
  4.3.1 Nodulation ............................................................................................................. 97
  4.3.2 Plant dry weight ..................................................................................................... 97
  4.3.3 Shoot elemental analyses ...................................................................................... 103
  4.3.4 Root system shape ................................................................................................. 105
  4.3.5 Root depth penetration ......................................................................................... 109
  4.3.6 Root length density and specific root length ......................................................... 109
  4.3.7 Differences among families within hybrid populations ...................................... 112
4.4 Discussion .................................................................................................................... 114
  4.4.1 Plant growth .......................................................................................................... 114
  4.4.2 Mineral nutrition ................................................................................................... 115
  4.4.3 Root system shape and distribution ..................................................................... 117
  4.4.4 Root morphology ................................................................................................. 118
7.2.7 Statistical analysis ................................................................. 191

7.3 Results ................................................................................. 192
  7.3.1 Soil moisture .................................................................. 192
  7.3.2 Dry matter yield ............................................................. 194
  7.3.3 Stolon morphology, leaf and chlorophyll index measurements .................................................................. 198
  7.3.4 Senescence ................................................................. 203
  7.3.5 Roots ........................................................................... 204
  7.3.6 Other measurements .................................................. 206
  7.3.7 Flowering .................................................................... 210

7.4 Discussion .......................................................................... 214
  7.4.1 Dry matter production and growth .................................. 214
  7.4.2 Senescence ................................................................... 216
  7.4.3 Roots ........................................................................... 217
  7.4.4 Flowering .................................................................... 218

7.5 Conclusions ......................................................................... 218

Chapter 8 Physiological and biochemical responses to water stress ........................................... 220

8.1 Introduction ......................................................................... 220

8.2 Materials and methods .................................................... 221
  8.2.1 Measurements ............................................................. 221
  8.2.2 Statistical analysis ....................................................... 223

8.3 Results ................................................................................. 225
  8.3.1 Gas exchange ............................................................... 225
  8.3.2 Leaf water potential ...................................................... 230
  8.3.3 Chlorophyll fluorescence .............................................. 230
  8.3.4 Phenolic compounds .................................................. 233
  8.3.5 13C discrimination ....................................................... 239
  8.3.6 Feed quality ............................................................. 242

8.4 Discussion .......................................................................... 246
  8.4.1 Physiology ................................................................. 246
  8.4.2 Protective compounds ................................................ 249
  8.4.3 Feed quality ............................................................. 250

8.5 Conclusions ......................................................................... 252

Chapter 9 General discussion ................................................................................................. 253

9.1 Background ......................................................................... 253

9.2 General effects of hybridisation ......................................... 253

9.3 Growth ............................................................................... 255

9.4 Adaptation to low soil resources ......................................... 256

9.5 Interaction of soil fertility and soil moisture ....................... 258

9.6 Root morphology and architecture ...................................... 259

9.7 Genotypic variation and selection ....................................... 261

9.8 Future work ......................................................................... 263
  9.8.1 Major points of investigation ....................................... 263
  9.8.2 Other areas of interest ................................................ 263

References .................................................................................. 264

Appendices ................................................................................. 283

Appendix A Key traits .................................................................... 283
A.1 Full description of hybrid families ................................................................. 283
A.2 Clover entry means ....................................................................................... 284
A.3 Flowering score and height – variability of clover types ............................. 286
A.4 Above-ground fragmentation ........................................................................ 287
A.5 Tap root measurements ................................................................................ 288
A.6 Nitrogen fixation – variability of clover types .............................................. 289
A.7 Changes in lateral spread .............................................................................. 291
A.8 Chlorophyll index measurements ................................................................. 292

Appendix B Nutrient solution recipes ................................................................. 293
B.1 Low ionic strength solution ......................................................................... 293
B.2 Complete nutrient solution ............................................................................ 295

Appendix C Root depth distribution and associated traits ................................. 296
C.1 Differences among families - change in root length density and specific root length 296

Appendix D Nutrient effects on growth ............................................................... 297
D.1 Dry weight back-transformed means ............................................................ 297
D.2 Phosphorus and inorganic phosphorus ......................................................... 297

Appendix E Root system structure .................................................................... 299
E.1 Dry weight data ............................................................................................. 299
E.2 Root morphology ........................................................................................... 300
E.3 Root architecture ............................................................................................ 301

Appendix F Morphological and growth responses to water stress .................. 309
F.1 Senescence score examples ......................................................................... 309
F.2 Total shoot dry weight – variability of clover types ..................................... 310
F.3 Dry matter scores .......................................................................................... 311
F.4 Correlations with changes in shoot DW ....................................................... 312
F.5 Senescence – variability of clover types ....................................................... 313

Appendix G Physiological and biochemical responses to water stress .......... 314
G.1 Water potential ............................................................................................. 314
G.2 Chlorophyll fluorescence .............................................................................. 315
G.3 Correlation between phenolics and biomass .............................................. 316
G.4 δ13C ............................................................................................................... 317
G.5 Feed quality .................................................................................................. 319
G.6 Variability of clover types and entries ......................................................... 322
## List of Tables

Table 3.1. Soil test (0-75 mm) results for the key traits experimental site............................... 40
Table 3.2. Clover entries used in the key traits experiment. .................................................... 41
Table 3.3. The range of values, and corresponding descriptions, for parameters scored........ 45
Table 3.4. $R^2$ values for dry matter scores against mean total dry weight. ......................... 52
Table 3.5. Mean dry matter scores at each sampling date....................................................... 53
Table 3.6. Variability for overall mean dry matter scores.................................................... 53
Table 3.7. Variability for stolon morphological characteristics............................................. 65
Table 3.8. Variability for peduncle measurements................................................................. 69
Table 3.9. Variability for maximum lateral spread. .............................................................. 70
Table 3.10. Variability for growth habit, stolon density, fungal disease and virus scores...... 73
Table 4.1. Day/night temperatures and maximum solar radiation for each harvest period..... 90
Table 4.2. Clover entries used in the root depth distribution experiment......................... 91
Table 4.3. Variability for shoot and root dry weight............................................................. 103
Table 4.4. Means for elements showing differences among clover populations................. 104
Table 4.5. Means for elements showing differences among clover types only................... 105
Table 4.6. Root shape parameters at each harvest.............................................................. 106
Table 4.7. Variability for root morphological parameters at 50–100 mm deep.................... 112
Table 4.8. Mean shoot DW, root DW and root:shoot ratio for families within populations.. 113
Table 4.9. Model values for $\beta_j$ and $R_j$ for families within BC$_1$ populations................. 114
Table 4.10. Mean shoot and total root dry weight for the 80-2 and 900-4 families............. 115
Table 5.1. Clover entries used in the nutrient experiment.................................................... 124
Table 5.2. Number of replicates sampled for shoot elemental analyses............................. 127
Table 5.3. Main effects for shoot dry weight, root DW and root:shoot ratio......................... 129
Table 5.4. Back-transformed means for shoot and root dry weight.................................... 131
Table 5.5. Variability for shoot dry weight, root DW and root:shoot ratio........................... 134
Table 5.6. Table of main effects for shoot elemental analyses............................................. 137
Table 5.7. Back-transformed means for S, Li and Mo concentrations................................. 143
Table 5.8. Mean log concentrations of elements (excluding P and P)................................... 144
Table 6.1. Clover entries used in the root system structure experiment.............................. 156
Table 6.2. Topological indices of clover entries in Kopu II and Crusader based groups...... 172
Table 7.1. Clover entries used in the water stress experiment............................................. 181
Table 7.2. Score system for growth parameters in the water stress experiment.................. 190
Table 7.3. Score values used to assess the flowering stages of inflorescences.................... 190
Table 7.4. Variability for stolon morphological characteristics........................................... 202
Table 8.1. Main effects for feed quality parameters in the full data set.............................. 242
Table 8.2. Clover type means for feed quality parameters in the full data set.................. 243
Table 8.3. Watering treatment means for feed quality parameters in the full data set......... 244
Table 8.4. Clover type means for %CHO and protein within treatments (full data set)..... 245
Table 8.5. Clover entry means for % Protein within treatments in the Kopu II subset....... 245
List of Figures

Figure 1.1. The crossing process through which interspecific hybrids are produced .................. 3
Figure 1.2. Examples of the terminology used to describe plant material. ............................ 6
Figure 1.3. Outline of the thesis structure. ............................................................................. 7
Figure 2.1. Description of link types. ..................................................................................... 11
Figure 2.2. Three examples of root system architecture ....................................................... 12
Figure 2.3. The “white clover complex” in the new Section Trifoliastum. ............................ 27
Figure 3.1. Layout of the key traits experiment ....................................................................... 42
Figure 3.2. Rainfall and temperatures during the key traits experiment .............................. 44
Figure 3.3. Overall mean dry matter scores ......................................................................... 52
Figure 3.4. Overall mean relative dry matter production of hybrid families ......................... 54
Figure 3.5. Spline curves fitted to mean dry matter scores over time .................................. 55
Figure 3.6. Estimated total mean plant dry weight over time ............................................. 56
Figure 3.7. Mean scores for tap root condition .................................................................... 57
Figure 3.8. Proportions of intact healthy tap roots .............................................................. 58
Figure 3.9. Mean diameter of intact tap roots ....................................................................... 60
Figure 3.10. Mean proportion of N from fixation in shoots ................................................ 61
Figure 3.11. Mean %N content of shoots .............................................................................. 62
Figure 3.12. Means for stolon morphological parameters ................................................. 63
Figure 3.13. Mean flowering scores ..................................................................................... 66
Figure 3.14. Mean scores for inflorescence height relative to the canopy ............................... 67
Figure 3.15. Mean inflorescence peduncle height and length .............................................. 67
Figure 3.16. Mean peduncle:petiole ratio and peduncle height:length ratio .......................... 68
Figure 3.17. Mean maximum lateral plant spread ............................................................... 69
Figure 3.18. Mean relative lateral spread of hybrid families ................................................. 71
Figure 3.19. Mean growth habit scores ................................................................................ 73
Figure 3.20. Mean relative growth habit of hybrid families ................................................. 74
Figure 3.21. Mean stolon density scores .............................................................................. 74
Figure 3.22. Mean relative stolon density of hybrid families ............................................... 75
Figure 3.23. Mean fungal disease scores ............................................................................. 76
Figure 3.24. Mean relative fungal disease of hybrid families ............................................. 76
Figure 3.25. Mean virus scores ......................................................................................... 77
Figure 4.1. Experimental layout of the root depth distribution experiment ......................... 92
Figure 4.2. Overall means for shoot dry weight, root dry weight and root:shoot ratio .......... 98
Figure 4.3. Means for shoot dry weight at harvests 1–4 ...................................................... 101
Figure 4.4. Means for root dry weight at harvests 1–4 ....................................................... 102
Figure 4.5. Root depth distribution (root dry weight by depth) ........................................... 107
Figure 4.6. Proportion of total root mass at 0–100 mm, 100–200 mm and 400–500 mm .... 108
Figure 4.7. Root penetration over time ............................................................................... 109
Figure 4.8. Mean RLD and SRL at 50–100 mm and 400–500 mm ...................................... 110
Figure 4.9. Proportion of mean RLD and SRL at 400–500 mm .......................................... 111
Figure 5.1. Mean temperatures and daytime solar radiation .............................................. 123
Figure 5.2. Experimental layout for the nutrient experiment .............................................. 126
Figure 5.3. Mean log shoot and root dry weight within treatments .................................... 130
Figure 5.4. Mean log shoot dry weight across treatments ................................................... 131
Figure 5.5. Mean log root:shoot ratio by clover entry and nutrient treatment ..................... 133
Figure 5.6. Mean log shoot and root DW of entries sampled for shoot elemental analyses .. 135
Figure 5.7. Mean log root:shoot ratio of entries sampled for shoot elemental analyses ..... 136
Figure 5.8. Mean log P concentration, mean P concentration and mean fraction P_i ........ 138
Figure 5.9. Mean P concentration by nutrient treatment ..................................................... 139
Figure 5.10. Mean log P use-efficiency and mean log P<sub>i</sub> use-efficiency .................................. 140
Figure 5.11. Mean log S, Li and Mo concentrations within nutrient treatments ................................. 142
Figure 5.12. Mean nodulation scores within nutrient treatments ..................................................... 145
Figure 5.13. Mean pigmentation scores within nutrient treatments .................................................. 146
Figure 6.1. Experimental layout for the root system structure experiment ....................................... 156
Figure 6.2. Example of a scanned BC1 hybrid for root morphology and architecture ........................ 157
Figure 6.3. Definitions of root architecture ..................................................................................... 158
Figure 6.4. Mean root diameter ....................................................................................................... 161
Figure 6.5. Mean number of root tips per cm of root ................................................................. 162
Figure 6.6. Mean number of root tips per unit root dry weight ..................................................... 163
Figure 6.7. Mean specific root length ........................................................................................... 164
Figure 6.8. Mean root tissue density .............................................................................................. 165
Figure 6.9. Growth in total root length at each time period ....................................................... 166
Figure 6.10. Mean average link length, surface area and diameter of internal–internal links ..... 169
Figure 6.11. Mean % of primary, secondary and tertiary lateral roots ........................................ 170
Figure 6.12. Changes in topological indices from week 0 to week 4 .............................................. 171
Figure 7.1. Daily temperature and rainfall during the water stress experiment ............................. 180
Figure 7.2. Experimental layout of the water stress experiment .................................................. 183
Figure 7.3. Irrigation, PET, and weekly rainfall during the water stress experiment ................... 185
Figure 7.4. Trendlines fitted to mean soil moisture over time ...................................................... 193
Figure 7.5. Mean total shoot dry weight for BC1, BC2 and white clover ....................................... 194
Figure 7.6. Mean total shoot dry weight for clover entries in the Kopu II subset ............................. 195
Figure 7.7. Mean shoot dry matter scores for BC1, BC2 and white clover .................................... 197
Figure 7.8. Means for stolon morphological parameters of BC1, BC2 and white clover ................ 199
Figure 7.9. Mean SPAD values for BC1, BC2 and white clover ..................................................... 203
Figure 7.10. Mean senescence scores of BC1, BC2 and white clover ........................................... 204
Figure 7.11. Mean root cross-sectional area of clover entries in the Kopu II subset ...................... 205
Figure 7.12. Mean root dry weight in cores for clover entries in the Kopu II subset ....................... 205
Figure 7.13. Mean lateral spread of BC1, BC2 and white clover .................................................... 206
Figure 7.14. Mean increase in lateral spread of BC1, BC2 and white clover ................................. 207
Figure 7.15. Mean percentage rooted width of BC1, BC2 and white clover ............................... 208
Figure 7.16. Mean growth habit, growth pattern, and stolon density scores ............................... 209
Figure 7.17. Mean stolon:leaf ratio scores in the Stressed treatment ............................................. 210
Figure 7.18. Mean number of inflorescences and totally deflexed inflorescences ......................... 211
Figure 7.19. Mean number of inflorescences by category ............................................................ 212
Figure 8.1. Mean net photosynthesis of clover entries in the Kopu II subset ................................. 225
Figure 8.2. Mean stomatal conductance of clover entries in the Kopu II subset ............................ 226
Figure 8.3. Mean internal CO<sub>2</sub> concentration of clover entries in the Kopu II subset .......... 227
Figure 8.4. Mean transpiration of clover entries in the Kopu II subset ........................................... 228
Figure 8.5. Mean physiological water use efficiency of entries in the Kopu II subset .................... 229
Figure 8.6. Relationship between shoot DW and physiological WUE (Kopu II subset) .................... 229
Figure 8.7. Overall mean water potential across time for entries in the Kopu II subset ................. 230
Figure 8.8. Overall mean midday chlorophyll fluorescence in the Kopu II subset ......................... 231
Figure 8.9. Mean pre-dawn chlorophyll fluorescence for BC1, BC2 and white clover .................. 232
Figure 8.10. Mean pre-dawn chlorophyll fluorescence yield in the Kopu II subset ....................... 232
Figure 8.11. Mean phenolic compound concentrations for BC1, BC2 and white clover ................. 233
Figure 8.12. Relationship between quercetin and shoot DW in the Stressed treatment .................... 236
Figure 8.13. Mean phenolic compound concentrations for entries in the Kopu II subset ................. 237
Figure 8.14. Mean 13C discrimination for BC1, BC2 and white clover .......................................... 240
Figure 8.15. Relationship between 13C discrimination and shoot DW for all plants ....................... 240
Figure 8.16. Mean 13C discrimination for clover entries in the Kopu II subset ................................. 241
Figure 8.17. Relationship between $^{13}$C discrimination and physiological WUE. ................. 242
Figure 9.1. Summary of the major findings of the study, and their interactions..................... 254
List of Plates

Plate 2.1. *T. uniflorum* in flower ................................................................. 32
Plate 2.2. *T. uniflorum* in sand culture ....................................................... 33
Plate 3.1. Key traits experimental site at establishment .............................. 43
Plate 3.2. The top 100 mm of tap roots with a condition score of 1 ............... 46
Plate 3.3. Application of KNO$_3$-$^{15}$N ......................................................... 47
Plate 4.1. Experimental set up of the root depth distribution experiment .......... 89
Plate 4.2. Hybrid plant from the root tube experiment at harvest 4 ............... 94
Plate 4.3. Large corraloid nodules on *T. uniflorum* ....................................... 97
Plate 6.1. Hydroponics system used in the root system structure experiment .... 154
Plate 6.2. Root growth in hydroponics in the root system structure experiment .... 155
Plate 7.1. Plant and Food Research rain shelter ............................................ 179
Plate 7.2. Water stress experiment experimental site ....................................... 184
Plate 7.3. Measurement of soil moisture content using a neutron probe .......... 186
Plate 7.4. Measurement of maximum lateral spread and maximum rooted width .... 188
Plate 7.5. Examples of the score values for growth pattern scoring ............... 189
Plate 7.6. Examples of flowering scores ..................................................... 191
Plate 9.1. Sand adhering to the roots of a *T. uniflorum* plant ................. 261
List of Appendices

Appendix 1. Full descriptions of BC1 families ................................................................. 283
Appendix 2. Full descriptions of the BC2 families .......................................................... 283
Appendix 3. Clover entry means for growth parameters .................................................. 284
Appendix 4. Clover entry means for flowering parameters .............................................. 285
Appendix 5. Relative growth parameters for hybrid families ......................................... 286
Appendix 6. Variability for flowering score and inflorescence height ............................ 286
Appendix 7. Mean scores for above-ground fragmentation ............................................ 287
Appendix 8. Mean tap root dry weight ............................................................................ 288
Appendix 9. Mean diameter and number of lateral roots larger than 1 mm in diameter ... 289
Appendix 10. Variability for the proportion of N from fixation in shoots ....................... 290
Appendix 11. Variability for %N content of shoots ....................................................... 290
Appendix 12. Change in maximum lateral spread ......................................................... 291
Appendix 13. Mean SPAD values .................................................................................. 292
Appendix 14. Overall variability for SPAD values .......................................................... 292
Appendix 15. Composition of the low ionic strength nutrient solution .......................... 293
Appendix 16. Concentration of ions in the low ionic strength nutrient solution ............. 294
Appendix 17. Composition of the Complete strength nutrient solution ........................... 295
Appendix 18. Proportion of RLD and SRL at 400–500 mm for 80-2 and 900-4 families .... 296
Appendix 21. Variability for P related data .................................................................... 298
Appendix 22. Mean root dry weight, shoot dry weight and root:shoot ratio .................... 299
Appendix 23. Mean total root length, total surface area, and number of root tips .......... 300
Appendix 24. Mean number of links, altitude, external path length and %EI links......... 301
Appendix 25. Mean length, surface area and average diameter of base links ............... 302
Appendix 26. Mean length, surface area and average diameter of external-external links ... 303
Appendix 27. Mean length, surface area and average diameter of external-internal links ... 304
Appendix 28. Mean length, surface area, and average link diameter of axis order 0 ....... 305
Appendix 29. Morphological parameters of primary lateral roots .................................... 306
Appendix 30. Morphological parameters of secondary lateral roots ............................... 307
Appendix 31. Morphological parameters of tertiary lateral roots ................................... 308
Appendix 32. Examples of senescence scores ............................................................... 309
Appendix 33. Variability for shoot dry weight of BC1, BC2 and white clover ............... 310
Appendix 34. Variability for shoot dry weight in the Kopu II subset ............................... 310
Appendix 35. Treatment effects and type x treatment interactions for shoot DM scores .... 311
Appendix 36. Correlation - internode length versus changes in shoot DW .................... 312
Appendix 37. Correlation - deflexed inflorescences versus changes in shoot DW .......... 312
Appendix 38. Correlation - inflorescences (category 3) versus changes in shoot DW ...... 313
Appendix 39. Variability for senescence scores of BC1, BC2 and white clover .......... 313
Appendix 40. Mean water potential of clover entries in the Kopu II subset over time ...... 314
Appendix 41. Mean midday fluorescence of entries in the Kopu II subset over time ....... 315
Appendix 42. Relationship between quercetin and shoot DW in the Stressed treatment... 316
Appendix 43. Correlation - kaempferol concentration versus changes in shoot DW ...... 317
Appendix 44. Mean $\delta^{13}$C within treatments, for the full data set and the Kopu II subset .. 317
Appendix 45. Means for feed quality within treatments for BC1, BC2 and white clover .. 319
Appendix 46. Main effects for feed quality parameters in the Kopu II subset ................. 320
Appendix 47. Clover entry means for feed quality parameters in the Kopu II subset ....... 320
Appendix 48. Watering treatment means for feed quality in the Kopu II subset ............. 320
Appendix 49. Clover entry means for feed quality within treatments for Kopu II subset .... 321
Appendix 50. Variability, within treatments, for parameters in the Kopu II subset. .......... 322
Appendix 51. Variability, within treatments, for parameters in both data sets. .................. 323
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Parameter</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC$_1$</td>
<td>Backcross 1 hybrid</td>
<td></td>
</tr>
<tr>
<td>BC$_2$</td>
<td>Backcross 2 hybrid</td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>Base link</td>
<td></td>
</tr>
<tr>
<td>$\beta_j$</td>
<td>Model value for root mass at 0-100 mm</td>
<td>g</td>
</tr>
<tr>
<td>Ci</td>
<td>Internal CO$_2$ concentration</td>
<td>$\mu$mol CO$_2$ mol$^{-1}$</td>
</tr>
<tr>
<td>cv</td>
<td>Cultivar</td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
<td></td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
<td>mg or g</td>
</tr>
<tr>
<td>E</td>
<td>Transpiration</td>
<td>mmol H$_2$O m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>EE</td>
<td>External-external link</td>
<td></td>
</tr>
<tr>
<td>EI</td>
<td>External-internal link</td>
<td></td>
</tr>
<tr>
<td>FEL</td>
<td>Fully expanded leaf</td>
<td></td>
</tr>
<tr>
<td>F$_1$</td>
<td>First filial generation</td>
<td></td>
</tr>
<tr>
<td>F$_v$/F$_m$</td>
<td>Maximum quantum yield of photosystem II</td>
<td>Dimensionless</td>
</tr>
<tr>
<td>g</td>
<td>Stomatal conductance</td>
<td>mol H$_2$O m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>II</td>
<td>Internal-internal link</td>
<td></td>
</tr>
<tr>
<td>Physiological WUE</td>
<td>Physiological water use efficiency</td>
<td>mmol CO$_2$ mol$^{-1}$ H$_2$O</td>
</tr>
<tr>
<td>P$_i$</td>
<td>Inorganic phosphorus</td>
<td>mg kg$^{-1}$</td>
</tr>
<tr>
<td>P$_n$</td>
<td>Net photosynthesis</td>
<td>$\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>PS II</td>
<td>Photosystem II</td>
<td></td>
</tr>
<tr>
<td>R$_j$</td>
<td>Model value for decrease in root mass</td>
<td>Dimensionless</td>
</tr>
<tr>
<td></td>
<td>with depth</td>
<td></td>
</tr>
<tr>
<td>Symbol</td>
<td>Term</td>
<td>Units</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>RLD</td>
<td>Root length density</td>
<td>km root m(^{-3}) soil</td>
</tr>
<tr>
<td>SLA</td>
<td>Specific leaf area</td>
<td>mm(^2) mg(^{-1}) DW</td>
</tr>
<tr>
<td>SLM</td>
<td>Specific leaf mass</td>
<td>mg DW mm(^{-2})</td>
</tr>
<tr>
<td>SPAD</td>
<td>Chlorophyll index</td>
<td>SPAD units</td>
</tr>
<tr>
<td>SRL</td>
<td>Specific root length</td>
<td>m root g(^{-1}) root DW</td>
</tr>
<tr>
<td>TI</td>
<td>Topological index</td>
<td>Dimensionless</td>
</tr>
<tr>
<td>(\Psi)</td>
<td>Leaf water potential</td>
<td>MPa</td>
</tr>
<tr>
<td>(\Delta)</td>
<td>(^{13})C discrimination</td>
<td>(%)</td>
</tr>
<tr>
<td>(\delta^{13})C</td>
<td>(^{13})C/(^{12})C isotopic composition</td>
<td>(%)</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

1.1 Background

The genus *Trifolium* contains approximately 250 species, representing a wide range of geographical distribution, habitats and morphological features. Species diversity is concentrated in the Mediterranean, western North America, and the east African highlands (Zohary and Heller, 1984; Ellison *et al.*, 2006). Habitats range from coastal to mountainous environments, meadows, woodlands and semi-desert areas. Morphologically, species are stoloniferous and non-stoloniferous, prostrate and erect. In addition there are both annual and perennial species.

White clover (*Trifolium repens* L.) is a major pasture legume worldwide, and a dominant component of New Zealand agricultural systems. It possesses desirable characteristics for pastoral systems such as a stoloniferous growth habit, high nutritive value, and the ability to fix atmospheric nitrogen (N) (Williams *et al.*, 2006a; Caradus *et al.*, 1996). However, it has a relatively small root system, limited persistence and a requirement for moist growing conditions. This restricts the use of this species in arid regions and makes it susceptible to drought in temperate areas of the world, such as New Zealand (Knowles *et al.*, 2003).

White clover breeding programmes in New Zealand over the last 70 years have involved breeding work within the white clover gene pool to recombine desirable characteristics and harness the natural variability of the species to gain improvements in morphology and agronomic performance. This has predominantly concentrated on shoot characteristics and knowledge of root traits is relatively low.

Other *Trifolium* species possess characteristics that would potentially be useful in white clover, such as rhizomes and/or thick roots, resistance to certain pests and diseases, increased flowering, persistence and drought resistance (Abberton, 2007). Combining such characteristics with the agronomically and economically important traits of white clover could be achieved through interspecific hybridisation (Abberton, 2007; Widdup *et al.*, 2003).

Interspecific hybrids are produced by crossing two parental species. Recurrent backcrossing to the parent of interest results in plants that are predominantly one parent, with some genetic material from the second (Williams and Hussain, 2008; Williams *et al.*, 2008). The introduction of genes or traits from one species into another is called introgression (Williams
et al., 2008; 2006b). In this way it is possible to maintain the desirable characteristics of white clover, while introducing new characteristics from related species. The ability to introduce new traits, or the expression of traits outside the existing variation of white clover, has potential for large scale improvements and increased agricultural performance. This PhD project investigated an interspecific hybrid between white clover and *T. uniflorum* L., a wild species from the Mediterranean region of Europe characterised by small, thick, waxy leaves and thick, strong, deep roots.

1.2 Production of interspecific hybrids

F₁ (first filial generation) interspecific hybrids are produced by cross pollination of two plants, in this case from two different parental species, each contributing 50% to the genetic makeup of the F₁ (Figure 1.1). In most *Trifolium* interspecific combinations, the F₁ hybrids are raised by embryo culture, due to post-fertilisation barriers to hybridisation, so there is no seed available for the F₁ crosses. Backcrossing of the F₁ to either of the parental species produces the BC₁ (backcross 1) generation, in which 75% of the genes, on average, come from the backcross parent and 25% from the second parental species. Recurrent use of one parent results in a series of backcross generations (BC₂, BC₃, BC₄ etc.) in which the genetic contribution of the second parent is progressively reduced. Intercrossing within backcross generations creates a series of F generations (BC₁F₂, BC₁F₃, BC₁F₄ etc.). This kind of crossing scheme was used by Williams and Hussain (2008) for *T. repens* x *T. ambiguum* M Bieb. hybrids.

The phenotype of the hybrids is dependent on which parts of the genome from each parent have been combined and how these interact, e.g. genes controlling cyanogenesis, perenniality, stolon production etc. For example, *T. repens* has roots at its nodes, while *T. nigrescens* Viv. does not. Marshall *et al.* (1995) found F₁ hybrids between the two did not have nodal roots, but some of the BC₁ generation did, suggesting this is a recessive trait.
1.3 Gaps in the knowledge

There is little data on the morphological and physiological characteristics of *T. repens* x *T. uniflorum* hybrids. Previous workers did not produce large numbers of plants and most effort concentrated on the process of producing the hybrids rather than extensive morphological and physiological studies. There is virtually no mention, in the literature, of hybrid performance in the field and no studies on agronomic traits or performance in conditions where an advantage over white clover would be desirable. Apart from qualitative descriptions there are also no reports on the *T. repens* x *T. uniflorum* hybrid root system. In addition, the performance of these hybrids in terms of key white clover traits is not known. This information is necessary to further determine the usefulness of *T. repens* x *T. uniflorum* hybrids, identify important characteristics, and assist with developing breeding programmes to produce improved germplasm for agricultural use.

1.4 Current context

AgResearch has a large *Trifolium* hybridisation programme, which has included larger scale hybridisation of *T. repens* x *T. uniflorum* than that carried out previously. Higher numbers of hybrids have been produced, including new F1 crosses and new backcrosses. The F1 hybrids were characterised as being of low agronomic value, closely resembling the *T. uniflorum*
parent. However, the BC₁ generation proved to be quite different and their phenotype is basically white clover-like above ground, but with large, thick roots (W. Williams, pers. comm.). The nature of the leaves of *T. uniflorum* and its natural habitat (dry, Mediterranean) suggest that drought resistant characteristics or mechanisms are likely to exist. The introduction of less desirable characteristics (from an agricultural perspective) is also likely to occur in at least some individuals. For example, the BC₁ and BC₂ hybrids can exhibit poor seed-set, some of which is probably due to inheritance of low floret numbers from *T. uniflorum* (W. Williams, pers. comm.).

Cytological studies have confirmed the chromosome pairing relationships reported previously by Pandey et al. (1987), with the presence of multivalents indicating that crossing-over of genes occurs between white clover and *T. uniflorum* chromosomes (Hussain et al., 2012). Variation is likely in the backcross generations, depending on which parts of the *T. uniflorum* genome have been passed on, and also due to crossing over of genes between the two species. A range of morphological features are therefore possible, and segregating populations with distinctive characteristics could develop. In addition, recombination of the two genomes of white clover in interspecific hybrids will alter the variability of the white clover component, and changes to white clover characteristics could also be seen (W. Williams, pers. comm.). The use of a range of white clover cultivars as backcross parents will further increase the range of variation in the hybrids.

### 1.5 Hypotheses and objectives

The underlying hypothesis of this study was that hybrids with *T. uniflorum* have different morphological and physiological characteristics than their white clover parents. *T. uniflorum* characteristics are expected to have been transferred into white clover, resulting in hybrids that are more *T. uniflorum*-like compared with white clover. The extent to which such traits have been introduced, and the effects or potential effects of such changes (both positive and negative) will be studied. It is specifically hypothesised that the hybrids will be more drought resistant than white clover, due to *T. uniflorum* characteristics that may confer resistance to moisture stress, and that the root systems of the hybrids will contribute to improved agronomic performance.

Overall, the aim was to describe important morphological and physiological characteristics in *T. repens x T. uniflorum* hybrids and compare them to the white clover parents and, where possible, *T. uniflorum*. Due to the general scarcity of information on *T. repens x T. uniflorum* hybrids, this study focussed on characteristics of *T. uniflorum* that would improve white
clover, particularly those related to drought resistance and persistence. It also included key traits that need to be retained in the hybrid material, to maintain - or improve upon - the high value contribution of white clover to agricultural systems.

Five objectives were identified, to:

1. Quantify key white clover traits to determine whether these are maintained or improved in the hybrids;
2. Describe the root depth distribution of the hybrids and compare this to the white clover and *T. uniflorum* parents;
3. Quantify and compare the growth of hybrids and their white clover parents under varying nutrient regimes;
4. Describe the root morphology and architecture of the hybrids and compare these to the white clover and *T. uniflorum* parents; and
5. Quantify the physiological and morphological responses of hybrids to drought and compare them to their white clover parents.

### 1.6 Terminology

Throughout this thesis the terminology used for plant material is as follows (examples are presented in Figure 1.2). “Entry” is a plant breeding term synonymous with the term “line” used by some authors. Entries are comprised of *T. uniflorum* accessions, hybrid families, and white and red clover cultivars. In some instances entries are combined into groups with a common background - these are referred to as “types”. Types may be species, hybrid generations or cultivars. Where appropriate, hybrid families with common parents are referred to as “populations”.

The general names of hybrids (e.g. *T. repens* x *T. uniflorum*) as well as the descriptions of individual crosses are set out as *Female parent* x *Male parent*, so that each generation reads as follows:

\[ F_1 = T. repens \times T. uniflorum, \]  

each \( F_1 \) cross is described by its own unique code, e.g. 80-2, which is subsequently used in the naming of \( BC_1 \) crosses. Full details of the \( F_1 \) parentage are presented in Appendices.

\[ BC_1 = \text{White clover} \times F_1, \]  

for example Kopu II x 80-2.

\[ BC_2 = \text{White clover} \times (\text{White clover} \times F_1), \]  

for example Kopu II x (Kopu II x 80-2).
Any instances where the crossing regime departs from this convention are noted in the Appendices, where families are fully described. Suffixes at the end of parental names or crosses refer to the specific genotypes used, e.g. Kopu II-2 denotes an individual Kopu II plant.

<table>
<thead>
<tr>
<th>“Entry”</th>
<th>“Type”</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>= Cultivars or accessions</td>
</tr>
<tr>
<td>2</td>
<td>e.g. Species</td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>= Families</td>
</tr>
<tr>
<td>5</td>
<td>e.g. Hybrid generation</td>
</tr>
<tr>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>= Families</td>
</tr>
<tr>
<td>9</td>
<td>e.g. Hybrid generation</td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.2. Examples of the terminology used throughout the thesis to describe plant material, including the grouping of entries into types. An illustration of populations, formed by related families, is shown by shading.**
1.7 Thesis structure

The outline of the thesis is shown in Figure 1.3. Based on the objectives outlined in the previous section, six experimental chapters are presented to address the benefits and limitations of white clover, as related to the effects of interspecific hybridisation with *T. uniflorum*. For conciseness, the main results are presented in each chapter, and supplementary material of interest is shown in the Appendices.

![Thesis structure diagram](image)

Figure 1.3. Outline of the thesis structure, showing the relationship of each experimental chapter to the broad objectives of this project.
2.1 White clover

2.1.1 High value pasture species

White clover is a highly heterozygous and outcrossing species, which leads to genetic variation through recombination of genes (Williams, 1987a; Williams, 1987b). This variation can be harnessed through breeding to create cultivars adapted to particular environments and agronomic situations, and also enables the plant to adapt naturally to – and thus survive in – a range of conditions. Considerable variation exists within white clover for numerous characteristics (Caradus, 1994b).

The shoot morphology of white clover varies mainly in leaf lamina size and stolon density. Small leaf types tend to have high stolon densities, which give tolerance to grazing, and are therefore used in set stocked grazing systems (Brock and Hay, 1996). Large leaf types generally have low stolon densities and are utilised under rotational grazing. The stoloniferous growth habit is an important characteristic of white clover, allowing it to spread through the sward and colonise new ground. It also provides a means of vegetative reproduction, enabling the plant to persist following the death of the tap root.

Nitrogen fixation, through the symbiotic relationship with *Rhizobium trifolii*, is one of the most important characteristics of white clover. Caradus *et al.* (1996) quoted an estimated average N fixation value in New Zealand of 1.57 million tonnes of N per year, valued at that time at NZ$ 1.49 billion, from a total financial impact of NZ$ 3.1 billion for white clover. The transfer of fixed N from white clover to grasses in grazed swards has been measured using the $^{15}$N dilution method (Ledgard, 1991), with above- and below-ground transfer estimated at 60 and 70 kg N ha$^{-1}$ year$^{-1}$ respectively, accounting for 50% of grass N.

Nitrogen fixation is closely linked with plant growth and thus is influenced by edaphic factors such as soil temperature and moisture (which also have direct effects on fixation) (Crush, 1987). However, the influence of plant growth on fixation can be overridden by the availability of soil mineral N, which is utilised in preference to fixed N. Other factors which directly affect N fixation include soil pH and nutrient status, and grazing or defoliation of the shoots. Fixation rates can, therefore, be highly variable. Crush (1987) lists rates of 17-380 kg
N ha⁻¹ year⁻¹ fixed from studies in grazed pastures, and over 600 kg N ha⁻¹ year⁻¹ in mown plots.

White clover also provides high quality feed. Sheep liveweight gains are higher when grazing white clover compared with perennial ryegrass (*Lolium perenne* L.), due to higher feed intakes and efficiency (gain per unit intake = nutritional value) (Ulyatt, 1981). Castle *et al.* (1983) found that increasing the proportion of white clover silage versus perennial ryegrass silage increased the intake and milk yield of dairy cows. No difference has been found in the digestibility of the two species (Ulyatt, 1981), but white clover is more easily broken down by chewing and rumination, leading to increased intakes in some situations (Caradus *et al.*, 1996).

Compared with perennial ryegrass, white clover has higher levels of crude protein (CP) and readily fermented carbohydrates; and lower lipids, water soluble carbohydrates (WSC), lignin, cellulose and fibre (Caradus *et al.*, 1996). Higher CP can be undesirable as excess N must be metabolised to waste, utilising energy that could otherwise be used for meat, wool or milk production (Pacheco and Waghorn, 2008; Kolver, 2003). As reviewed by Williams (1987b), some studies indicate there is genetic variation for protein concentrations in white clover, while others have found no differences among cultivars. In addition, increased WSC levels in forage grasses are proposed to increase N utilisation and animal production (Edwards *et al.*, 2007), and both high and low WSC levels can have negative effects on ensilation of feed (Kruse *et al.*, 2008). The ratio of CP:WSC also has important effects on rumen microbial activity and animal production (Hoover and Stokes, 1991; Fulkerson *et al.*, 1998).

### 2.1.2 Limitations of white clover

White clover is adapted to a moist growing environment and production is relatively poor in dry conditions. Knowles *et al.* (2003) reported the effects of a summer/autumn drought on clover presence in five regions throughout New Zealand. They found that 23% of long-term mean rainfall in Marlborough reduced white clover presence by 95%, while 80% of long-term mean rainfall reduced white clover by just 8% in the Wairarapa. Recovery following drought was lowest where white clover losses were most severe.

The relatively limited root system of white clover affects its ability to access water and nutrients. Thomas (1984) attributed the poor performance of white clover under drought to the impact of its small root system on its ability to compete with ryegrass. Root system characteristics, combined with poor survival under drought, contribute to poor persistence in white clover.
Compared with other legumes, white clover is small-seeded and slow to establish, leading to poor establishment in competition with forage grasses. It is also susceptible to a range of pests, diseases and viruses (Skipp and Gaynor, 1987; Latch and Skipp, 1987; Gaynor and Skipp, 1987), which can also affect its long term persistence in the sward.

2.2 White clover roots

Young white clover plants develop a tap root through the secondary thickening of the seedling seminal root. Nodal roots then develop as the primary shoot axis elongates and branches to form a network of stolons. Between 12-18 months after establishment, tap root death occurs due to a combination of factors, such as natural senescence, pathogen and insect attack, and carbon allocation to nodal roots (Westbrooks and Tesar, 1955; Brock et al., 2000). This is followed by loss of the primary shoot, leading to fragmentation of the plant into smaller clonal units, dependent on nodal root systems. Subsequently, any stolon losses not balanced by renewal lead to decreases in plant size and production, and loss of the plant itself may ultimately occur. Clonal plants are most vulnerable to biotic and edaphic stresses following fragmentation during spring, when they are smallest (Woodfield and Caradus, 1996; Brock et al., 1988).

White clover nodal roots are relatively small and shallow, and form only in the presence of adequate soil moisture (Thomas, 1987b; Thomas, 2003). There are two nodal root primordia at each node but usually only one develops into a nodal root system (Thomas, 1987b). These are usually fibrous roots but tap root-like nodal roots may also form. Caradus (1977) found that the root morphology of white clover varied between two extremes – from predominantly “tap rooted” plants with few fibrous roots, to those that were mainly fibrous with no nodal tap roots. Diameter of these larger nodal roots, and their proportion of the total, has been shown to be positively correlated with large leaf size (Caradus, 1977; Caradus and Woodfield, 1998). Furthermore, such roots are described in the literature as “vertically penetrating” (Caradus and Woodfield, 1998; Caradus, 1977). Generally, white clover roots are thicker, shorter and less branched than grasses, with fewer, shorter root hairs (Dunlop and Hart, 1987). The root characteristics of grasses give them a competitive advantage over white clover in acquisition of nutrients, particularly immobile nutrients such as phosphorus (P) (Mouat and Walker, 1959; Jackman and Mouat, 1972).

Smaller diameter roots have a higher absorption of water and nutrients per unit root mass than thicker roots (Eissenstat, 1992; Jungk, 1996), due to a higher surface area to volume ratio, and root length density is also important, with higher uptake rates occurring with increasing root
length per unit volume of soil (Dunbabin et al., 2003a). Root length density can be increased by increasing numbers of individual (e.g. nodal) roots and also by increased branching of roots. Crush et al. (2008) found that a white clover genotype selected for relatively long, fine roots had greater P uptake per unit root mass than one selected for short, thick roots. Roots of the long, fine-rooted genotype were also more highly branched.

Root architecture describes the positioning of roots in the soil, and there are several systems by which it can be described. The developmental system identifies individual roots according to the order in which they develop. While this is useful for studying the growth of root systems, topology is considered to provide a more functional description of branching patterns (Fitter et al., 1988; Fitter, 1996). The main architectural analysis applied in this study is the topological system described by Fitter (1987), and is commonly used in root research. This firstly divides the root system into segments called “links”, which are subdivided into four classes (Figure 2.1). The link which joins the base of the shoot is called the base link (BL). Links between two branching points are called internal links (denoted here as II), while links ending in a meristem (or root tip) are called external links. External links which join another external link at the base are external–external links (EE) and those which join an internal link are external–internal links (EI).

Figure 2.1. Link types as described by Fitter (1987). BL = base link; II = internal links (links between two branching points); EE = external–external links (links ending in a meristem, which join other external links); EI = external–internal links (links ending in a meristem, which join an internal link). Adapted from Crush et al. (2005a).
There are a number of parameters which can be used to describe the branching pattern (Figure 2.2). For each link, a continuous path can be traced back to the base link - the number of links in this path (including the starting link and BL) is the “path length”. The “external path length” of the entire root system is the sum of the path lengths of all external links in the system. “Altitude” is the longest path length in the root system, and the “magnitude” of the root system is the total number of external links. Link parameters provide information on the morphology and connectivity of the individual root segments, but altitude and magnitude can be used to derive the topological index (TI), which describes the topology of the root system as a whole (Fitter, 1993). This varies between two extremes (Figure 2.2). In roots with a strictly herringbone topology, branching occurs only on the main axis (i.e. there is a main root and primary laterals only), while dichotomous systems have extensive branching on the lateral roots (Fitter, 1991). The TI is the slope of the regression of log altitude on log magnitude (Fitter, 1993). Values close to 1.0 represent a herringbone system, while lower values are more dichotomous. Higher proportions of EI links, out of all external links, are also indicative of a more herringbone-like root system (Fitter, 1986).

![Figure 2.2. Three examples of root system architecture, with a magnitude of 8 (total number of external links or links ending in a meristem). Topological extremes for this magnitude are shown in B (herringbone) and C (dichotomous). Numbers indicate the path length (number of links back to the origin) of each link. The altitude (longest individual path length) of each root system is 5 (A), 8 (B) and 4 (C); and the external path length (sum of the path lengths of all external links) is 33 (A), 43 (B) and 32 (C). From Fitter (1987).](image)

The morphology and architecture of root systems can be highly plastic, changing in response to pulses or deficits of water and nutrients (Lynch, 1995; Fransen et al., 1999; Linkohr et al.,
There are few studies on the root architecture of white clover, but Crush et al. (2005a) found that white clover root systems are essentially herringbone-like, and also suggested that there were differences among the genotypes studied that would affect the degree of soil exploration by roots. Inbreeding has been found to have little effect on the herringbone architecture of white clover roots (Nichols et al., 2007), indicating that root architecture is strongly fixed genetically in this species.

Studies on the root depth distribution of forage grasses have found that up to 80% of root mass occurs in the top 100-150 mm of the soil (Crush et al., 2007; 2005b). In lower soil layers the opportunity for interception of water and nutrients must therefore be greatly reduced. Root depth distribution of white clover has not been extensively studied, but relatively shallow rooting is also characteristic. Caradus (1981) found an average of 50-70% of total root mass occurred in the top 150 mm of the soil in glass-fronted cabinets. There was evidence for variation among white clover entries, with some having more roots at depth and less at the surface compared with others. In sand tubes, Nichols et al. (2007) found a similar distribution for nodal roots of the white clover cultivar Crau and a sequence of inbred generations, where 66% of the root mass was in the top 100 mm. Ninety per cent of white clovers absorption of nutrients occurs in the top 80 mm (Boggie and Knight, 1960).

Control of root to shoot dry weight ratios were reviewed by Wilson (1988). There are two main theories regarding the control mechanisms. Firstly, that the ratio of root versus shoot biomass is set and maintained at all times. In contrast, the optimisation theory asserts that root to shoot ratio can vary, with more biomass being allocated where resources are limiting. For example, when nutrients or water are limiting, more biomass is allocated to roots, and under shading more biomass is allocated to shoots. There is evidence for both theories in white clover in relation to nutrient and water stress (Blaikie and Mason, 1990; Hill et al., 2006; Davidson, 1969), suggesting more work is necessary to understand carbon allocation in this species.

### 2.3 Soil fertility

A requirement for high soil fertility currently limits the environments which are suitable for white clover, and necessitates high inputs of mineral fertiliser in high production pastures. In particular, low soil P levels are a major limitation to the growth of white clover. Grasses are highly competitive against white clover for P acquisition, due to their finer, higher density roots per unit of soil volume. Fine roots are able to absorb more P than coarser roots due to a greater root surface area:soil volume ratio (Jungk, 1996; Eissenstat, 1992), and increased
rooting density allows roots to better access immobile nutrients such as P (Dunbabin et al., 2003a). Mouat and Walker (1959), found that P uptake rates in white clover growing in monoculture were 3-9 times higher than white clover growing with ryegrass, depending on P and N supply. Considerable amounts of additional superphosphate are required to grow white clover with grasses compared to that needed for white clover on its own (Jackman and Mouat, 1970).

There are numerous published studies on the P physiology of white clover. Although data have suggested it should be possible to breed more P-efficient white clover cultivars, attempts to transfer selected genotypes from controlled environments to the field have not been successful (Caradus and Dunn, 2000; Caradus, 1994a). Many studies have focused on the fractionation of P into organic and inorganic (P_i) pools (Hart and Jessop, 1983; Caradus et al., 1998). P_i in plant cells is found in the cytoplasm and the vacuole, with the latter pool being the largest (Vogel, 1987; Bieleski, 1973). At high soil P supplies, uptake of P_i increases and it is sequestered in the vacuoles, where it is largely unavailable until soil P becomes limiting. It is then transferred to the cytoplasm where it can be used for growth, which stops once the vacuolar supply of P_i is depleted (Bieleski, 1973). The rate at which P_i is transferred from the vacuole to the cytoplasm under deficient conditions may still limit plant growth. Bieleski (1973) reports that P_i exchange half-times can be more than three days, which may exceed short term requirements of white clover (Hart and Jessop, 1982). White clover adapted to low P soils has been shown to accumulate higher levels of P_i than populations from high P soils (Caradus and Snaydon, 1987), but other studies show no such effect (Caradus et al., 1998). The impact of this higher accumulation of P_i on plant growth was not reported by Caradus and Snaydon (1987), although Caradus et al. (1998) subsequently stated that the material in question was of no agronomic merit.

Hart and Jessop (1983) compared the growth and P nutrition of white clover with lotus (Lotus pedunculatus Cav.) over a range of external P supplies, as lotus grows well in low P soils. Growth of lotus was higher than white clover at both high and low P supply, and was continuing to increase at the highest P levels. P_i concentrations were lower than those of white clover, and remained relatively constant over all external P levels. In contrast, growth of white clover reached a maximum at the highest P levels, and P_i concentrations were increasing. High amounts of unavailable P_i sequestered in the vacuoles of white clover may therefore contribute to a less efficient use of absorbed P.

Hart and Colville (1988) suggested that the P_i fraction of total P may be a useful indicator of differences in P responses among white clover genotypes. Caradus et al. (1998) found low
broad sense heritability for this trait, but it was not part of the breeding programme for the material studied, and they suggested that selecting for extreme genotypes should be considered in breeding populations.

2.4 Drought

Water is one of the most important resources for the growth and survival of terrestrial plants. Drought is therefore a major constraint to plant production worldwide. In New Zealand, dryland environments are classified as those in which summer evapotranspiration is greater than summer rainfall in most years (Brown and Green, 2003). Such environments occur on the east coast from Hawkes Bay to Otago, with inland pockets in Central Otago and South Canterbury. The cumulative difference between potential evapotranspiration (PET) and rainfall is the potential soil moisture deficit (PSMD). Climate predictions indicate that eastern areas of New Zealand are likely to become drier, with an increase in PSMD of 20-30% (Salinger, 2003).

2.4.1 Measuring the effects of drought

One of the earliest plant responses to drought is the closure of stomata to limit water loss through transpiration (Chaves et al., 2003), which ultimately reduces the intake of CO$_2$ and thus photosynthesis. Many studies indicate that, in drying soil, chemical signals between the roots and shoots play a major role in stomatal closure (Davies et al., 1994; Chaves et al., 2002). The impact of water stress on gas exchange can be monitored using an infrared gas analyser, which measures net photosynthesis, stomatal conductance, transpiration and internal CO$_2$ concentration (Erice et al., 2011; Gilbert et al., 2011). Due to the gradients in the concentrations of CO$_2$ and water vapour between the inside ($C_i$ and $W_i$) and outside ($C_a$ and $W_a$) of the leaf, many water molecules are lost for every CO$_2$ molecule that is gained (Schulze, 1986; Salisbury and Ross, 1992). This relationship means that stomatal closure affects water loss before it affects photosynthesis (Chaves et al., 2003). Despite changes in stomatal aperture, there can still be some water loss under water stress, reducing turgor and therefore cell expansion and growth. The sensitivity of cell growth to water stress also means that the first impact of drought is a decrease in growth, in particular a decrease in organ size (Salisbury and Ross, 1992; Tardieu et al., 2011).

Most plants have increased water use efficiency (WUE) during mild drought due to the non-linear relationship between stomatal conductance and carbon assimilation (Chaves et al., 2003). Physiological WUE at the leaf level can be calculated using measurements of net photosynthesis and transpiration (Condon et al., 2002; Hall et al., 1994). However, due to
requirements for uniform light conditions during photosynthesis measurements, only limited numbers of plants can be measured in the field. In contrast, the $^{13}\text{C}/^{12}\text{C}$ isotopic composition ($\delta^{13}\text{C}$) of plants can be used to calculate $^{13}\text{C}$ discrimination ($\Delta$) as a measure of WUE in a large number of plants, and also provides an integrated measurement over time. The isotopic composition is measured relative to a standard – usually CO$_2$ from a fossil belemnite from the Pee Dee Formation (PDB) – using mass spectrometry (Farquhar et al., 1989). $\Delta$ is then calculated from the $\delta^{13}\text{C}$ values of the product (plant) and the source (air) (where $\delta_{\text{source}}$ is assumed to be -8‰ (Hall et al., 1994)) using:

$$\Delta = \frac{\delta_{\text{source}} - \delta_{\text{product}}}{1 + \delta_{\text{source}}/1000}$$  

(Farquhar et al., 1982)

Plants discriminate against $^{13}\text{C}$ during photosynthesis, and in C$_3$ plants this discrimination is approximately 20‰ ($20 \times 10^{-3}$ or 0.02 per mil) (Farquhar et al., 1989). The relationship between WUE and $\Delta$ suggests that plants with a higher WUE will discriminate less against $^{13}\text{C}$ (Farquhar et al., 1982; Farquhar and Richards, 1984), and $\Delta$ may then be used as a tool to select for high WUE.

The impact of water stress on photosynthesis can also be determined by measuring chlorophyll $a$ fluorescence. Light energy intercepted by plants is either used in photochemistry, dissipated as heat, or emitted as chlorophyll fluorescence (Baker, 2008; Maxwell and Johnson, 2000). These three processes compete, so that an increase in any one of them results in a decrease in the others. In this way, chlorophyll fluorescence can be used as an indicator of the state of photochemistry in photosystem II (PS II). Essentially, light is absorbed by chlorophyll, then special chlorophyll molecules (P680) in the reaction centres of PS II pass electrons to a plastoquinone acceptor molecule (Raven et al., 1992; Krause and Weis, 1991). From there they are passed through an electron transport chain to PS I. There are multiple chlorophyll fluorescence parameters, but a simple and widely used measure is the maximum quantum yield of PS II efficiency – $F_v/F_m$ – in dark-adapted leaves (Krause and Weis, 1991; Baker and Rosenqvist, 2004). $F_v/F_m$ is known to decrease under environmental stress (Maxwell and Johnson, 2000; Krause and Weis, 1991). $F_m$ is maximal fluorescence, which occurs when reaction centres are “closed” (i.e. contain electrons); and $F_v$, variable fluorescence, is the difference between $F_m$ and minimal fluorescence $F_o$, which occurs when reaction centres are “open” (i.e. contain no electrons).

The effect of drought on plant water status can be assessed by measuring leaf water potential ($\Psi$), which decreases under water stress (Lee et al., 2009; Maricle and Adler, 2011). In white
clover, decreases in Ψ are associated with decreases in relative water content, photosynthesis and stomatal conductance (Lee et al., 2009; Grieu et al., 1995). Various studies record differences among species, and genotypes within species, in the response of Ψ to changes in soil moisture (Maricle and Adler, 2011; Santos et al., 2009; Iannucci et al., 2002; Silim et al., 2009). In conjunction with photosynthesis, stomatal conductance and osmotic adjustment, these differences have been used to distinguish among drought tolerant and drought sensitive species or genotypes.

The flavonol quercetin is a potential marker for stress tolerance in plants. Accumulation of quercetin glycosides in white clover is increased by exposure to UV-B radiation (Hofmann et al., 2003; 2000), and these higher levels are associated with increased tolerance of UV-B. Hofmann et al. (2003) also found that the accumulation of quercetin was further increased by the addition of drought stress. White clover ecotypes adapted to a range of stresses had higher constitutive levels of total flavonols, including quercetin, and these increased more under UV-B than they did for white clover cultivars and breeding populations. Hofmann and Jahufer (2011) also found higher levels of quercetin in stress-resistant white clover ecotypes and populations, which were associated with lower levels of biomass productivity. However, in a full-sib population generated by crossing an elite white clover cultivar with a stress-resistant ecotype, Ballizany et al. (2012a; 2012b) found that smaller decreases in biomass production under water stress were associated with larger increases in quercetin accumulation. Measurement of quercetin levels using HPLC may therefore provide information on the relative stress tolerance of different clover populations.

2.5 Drought and white clover

Moisture stress decreases the growth of white clover, as reported by Knowles et al. (2003). Barbour et al. (1996) recorded decreases in leaf dry matter production as moisture stress increased, with larger leaved (initially more productive) cultivars being more sensitive. Belaygue et al. (1996) found that drought stress decreased the total leaf area of white clover through changes in stolon number, leaf number and size of individual leaves. The contribution of each component depended on the severity of the drought. Stolon number accounted for 66% of the leaf area reduction under mild drought, individual leaf area accounted for 40% under intermediate drought, and leaf appearance rate accounted for 66% of the reduction under severe drought. Brock and Kim (1994) also recorded decreased white clover dry matter production during drought, but concluded that the greatest effect of water stress was on the survival of plants. Johns and Lazenby (1973a), Turner (1990a) and Brock and Kim (1994) all reported wilting and death of leaves in white clover under drought.
White clover has poor control of water loss under dry conditions (Johns, 1978; Aparicio-Tejo et al., 1980; Hart, 1987), which may be due to failure to close the stomata or low cuticular resistance. Hart (1987) suggested this was the cause of low WUE in dry conditions, as observed by Johns and Lazenby (1973a, 1973b). In moisture limiting conditions, water use of white clover and forage grasses was similar but the clover produced significantly less herbage. Aparicio-Tejo et al. (1980) concluded that subterranean clover (T. subterraneum L.) was better adapted to water stress than white clover, due to larger decreases in transpiration, higher cuticular resistance, and better recovery of N-fixation after drought.

High and rapid leaf senescence in white clover (Johns and Lazenby, 1973a; Brock and Kim, 1994) may be a direct consequence of water loss, or a response mechanism to limit dehydration. Turner (1990b) concluded that white clover adjusts osmotically to maintain stolons at the expense of leaf biomass. Similarly, high levels of white clover leaf death observed by Brock and Kim (1994) were attributed to the maintenance of plant survival.

As mentioned previously, nodal roots of white clover require moist conditions to form. In addition, humidity requirements for nodal root initiation are higher for water stressed (over 93% relative humidity) versus well watered (over 83% relative humidity) stolons (Stevenson and Laidlaw, 1985). Decreased nodal root formation under drought would reduce total root mass for access to available soil water, and affect persistence of clonal plants during fragmentation.

Despite possessing a relatively shallow root system, water extraction by white clover has been recorded to 900 mm (Evans, 1977). Burch and Johns (1978) and Karsten and MacAdam (2001) found that grasses (tall fescue and perennial ryegrass) extracted more water in deeper soil than white clover. However, there have also been studies showing that white clover can extract water to the same depth as, or even deeper than, ryegrass (Grieu et al., 2001; Hogh-Jensen and Schjoerring, 1997).

### 2.5.1 Traits that improve resistance to drought in white clover

White clover genotypes selected from New Zealand dryland environments have higher survival rates in dry conditions than other selections (Woodfield and Caradus, 1987; van den Bosch et al., 1993). van den Bosch et al. (1993) also found that dryland selections had higher forage yields in a drought-prone environment. Woodfield and Caradus (1987) reported that dryland white clover populations appeared to be “more tap rooted” than those from moist hill country, and some populations had a larger “tap root diameter” (diameter of the largest root) and higher numbers of “tap roots” (roots >1 mm basal diameter) for their leaf size. Selections
which survived a summer drought also had higher proportions of total root weight as “tap root” weight (roots >1 mm basal diameter) and higher proportions of total plant weight as root weight than Tahora, a cultivar bred for moist hill country conditions. Dryland ecotypes and genotypes which survived the drought generally had the root morphology of the cultivar Huia, an old cultivar derived from New Zealand ecotypes, with reduced leaf size as an adaptation to moisture stress (Woodfield and Caradus, 1987).

MacFarlane et al. (1990) concluded that tolerance of intensive grazing through maintenance of high stolon density was an important factor in the persistence of cultivars in dry hill country, and proposed that large nodal roots may contribute to this. Brock and Kim (1994) also suggested that grazing, particularly grazing management, is a key factor in drought survival of white clover. Removal of pasture cover exposed stolons to direct radiation and high temperatures at the soil surface, leading to lower survival for exposed versus shaded stolons. The use of set stocking, rather than rotational grazing, was considered a better management option in dry conditions, although stocking rates are also likely to be important.

The potential influence of shoot morphology on leaf production and survival was also discussed by Brock and Kim (1994). Unlike MacFarlane et al. (1990), these authors concluded that the effects of drought were severe regardless of morphology, despite finding that leaf production and survival were affected differently in cultivars with different morphologies. Faster recovery was recorded for a smaller-leaved cultivar compared with those with larger leaves and thick stolons (Brock and Kim, 1994).

2.5.2 Selecting for drought resistance in white clover

Blaikie and Mason (1990) observed a high correlation between root and shoot growth in white clover. A decrease in white clover shoot growth under water stress was redressed after a period of time by reallocation of growth from the roots, and the authors suggested that high root growth is necessary for high shoot yields. Selection for high root:shoot ratio – i.e. a high proportion of root biomass – and increased proportions of “tap root” has been studied as a means of increasing drought resistance. Woodfield and Caradus (1987) suggested that there is scope to select for these traits independently, using shoot or total yield as a co-variate. Some dryland populations exhibited high proportions of root and “tap root” (roots >1 mm basal diameter) in conjunction with high shoot yields.

Response to selection for root characteristics is high (Woodfield and Caradus, 1990; Caradus and Woodfield, 1998). Selection for seedling tap root diameter has increased this trait by 2.4% per breeding cycle (Caradus and Woodfield, 1998). Some characteristics may be more
easily targeted than others. Woodfield and Caradus (1990) found that selecting for “tap root diameter” (diameter of the largest root), and the “tap root” (roots >1 mm basal diameter) proportion of total root weight, was more successful than selecting for number of “tap roots” (>1 mm basal diameter) and the root proportion of total plant weight.

Selection for root characteristics can improve yield and persistence of white clover under drought (Caradus and Woodfield, 1998). Increased root weight ratios improved growth, spread and survival in seasonally dry hill country, while the combination of medium leaf size and large “tap root diameter” (basal diameter of the largest root) increased yield in both the irrigated (+70%) and dry (+35%) treatments of a rain shelter experiment, relative to the control white clover cultivar (Huia) (Caradus and Woodfield, 1998). However, van den Bosch et al. (1993) found that selection for root morphology alone was not as effective as selecting genotypes from dryland environments. Selecting for root morphology within these dryland selections did improve the yield and persistence of some genotypes at a dryland site.

Barbour et al. (1996) observed no differences in WUE among ten white clover cultivars, concluding that little genetic variability exists within the species for photosynthetic capacity and control of water loss. However, they suggested that genotypic variation within cultivars may be more important.

*T. ambiguum* x *T. repens* interspecific hybrids have root characteristics that may improve drought tolerance, compared with white clover (Widdup et al., 2003). Marshall et al. (2001) also reported that *T. ambiguum* x *T. repens* hybrids had higher relative water contents, higher leaf water potentials and lower decreases in dry matter yield under water stress compared with white clover. In that study, the backcross 1 (BC₁) generation was able to maintain growth during drought.

### 2.6 *Trifolium* interspecific hybrids

The *Trifolium* genus includes a few species that are known, or suggested, to be natural hybrids. Allotetraploid *T. repens* has long been accepted to have arisen by interspecific hybridisation of two ancestral species. *T. nigrescens*, *T. occidentale* Coombe, *T. isthmocarpum* Brot., and *T. uniflorum* have all been proposed as possible parents, based on the success of their hybridisation with white clover and chromosome pairing in the hybrids (Gibson and Beinhart, 1969; Brewbaker and Keim, 1953; Chen and Gibson, 1972a; Kazimierski and Kazimierska, 1973; Evans, 1962a). In particular, many studies have promoted *T. nigrescens* as one of the two parents, and this has commonly been accepted. More recently Badr et al. (2002) suggested that *T. uniflorum* and *T. nigrescens* are the likely
parents, with introgression of genes also occurring from *T. occidentale* and *T. isthmocarpum*. However, based on a new phylogenetic analysis, Ellison *et al.* (2006) concluded that *T. occidentale* and *T. pallescens* Schreb. are the putative parents of white clover.

Early work on white clover interspecific hybrids included *T. isthmocarpum* (Kazimierski and Kazimierska, 1973), *T. nigrescens* (Hovin, 1962b; Keim, 1953), *T. occidentale* (Chen and Gibson, 1970b; Chou and Gibson, 1968), *T. ambiguum* (Williams, 1978) and *T. uniflorum* (Pandey, 1957). Hybrids have also been made between *T. repens* and *T. hybridum* L. (Przywara *et al.*, 1989). Non white clover hybrids include *T. alexandrinum* L. and *T. resupinatum* L. (Kaushal *et al.*, 2005), *T. alexandrinum* and *T. apertum* Bobrov (Malaviya *et al.*, 2004), and *T. alexandrinum* and *T. constantinopolitanum* Ser. (Roy *et al.*, 2004). The genetic resource present in wild relatives of white clover, and other *Trifolium* species of agricultural interest, is reviewed by Williams and Nichols (2011).

Most *Trifolium* species do not hybridise naturally. Ellison *et al.* (2006) found evidence for only 5–6 cases of historical hybridisation out of 218 species studied. However, Williams *et al.* (2008) concluded that *T. nigrescens* and *T. occidentale* are not biologically isolated, and have a close ancestry despite a wide current geographic separation, suggesting they could hybridise naturally if the two species occurred together in the wild. It was suggested that such hybrids may not have been recognised due to their resemblance to the *T. nigrescens* parent.

### 2.6.1 Problems with hybridisation

*Trifolium* hybrids can be produced by hand pollination but are often difficult to achieve. This was particularly so in the early work as understanding of hybridisation increased, and improved methods were developed. Low seed production and germination, followed by low survival and fertility in the resulting hybrids, were common problems.

Gibson *et al.* (1971) carried out 2160 pollinations between *T. uniflorum* and *T. repens*, *T. occidentale* and the three subspecies of *T. nigrescens* – producing only one hybrid plant. Kazimierski and Kazimierska (1973) pollinated 77 flowers in a *T. repens* x *T. isthmocarpum* cross and obtained only two seeds, with just one seedling surviving. The reciprocal cross (*T. isthmocarpum* x *T. repens*) produced no seed from 110 pollinations. Attempts to hybridise *T. ambiguum* x *T. repens* were unsuccessful (Williams and White, 1976) until embryo culture was used (Williams, 1978).

Chlorophyll deficiencies are also reported. Hovin (1962a) produced 35 *T. nigrescens* x *T. repens* hybrid seedlings which developed with albino cotyledons and/or albino or pale leaves.
or sectors of leaves. All these plants died within two weeks. The reciprocal cross (*T. repens* x *T. nigrescens*) produced approximately 130 healthy seedlings, which survived to flowering. Pandey (1957) produced only two seedlings from a *T. uniflorum* x *T. repens* cross, one of which was albino and died. The other was ¾ albino, but a healthy plant resulted from the green sector.

Variable fertility of F₁ and backcross hybrids initially provided a barrier to further development of interspecific hybrids. Even after Williams (1978) had produced the first *T. ambiguum* x *T. repens* hybrid through embryo culture, it was highly sterile, with pollen fertility ranging from 0.96–3.59% depending on the stage of the flowering season. Further work produced partially fertile F₁ hybrids from which almost sterile F₂ progeny were obtained, resulting in some F₃ progeny and backcrosses to *T. repens* with low pollen fertility (Williams and Verry, 1981). Ferguson *et al.* (1990) successfully produced a *T. ambiguum* x *T. occidentale* F₁ hybrid but it did not flower, and the first fertile hybrids of this combination were not reported until Williams *et al.* (2006b). Despite improved success of hybridisation, the fertility of hybrids continues to be an issue. Williams *et al.*’s (2006b) new *T. pallescens* x *T. occidentale* hybrid was only partially fertile, and Hussain *et al.* (1997b) obtained no seed from 2950 reciprocal backcrosses with a 3x *T. repens* x *T. nigrescens* F₁ hybrid.

### 2.6.2 Pre- and post-fertilisation barriers

The limited success of hybridisation in *Trifolium* is due to the presence of pre- and post-fertilisation barriers. These fall into four categories: failure of the pollen to germinate on the stigma; slow or abnormal growth of the pollen tube; failure of fertilisation; and abortion of the embryo (Chen and Gibson, 1972a). However, the results of many studies indicate that post-fertilisation barriers are the most important of these. Chen and Gibson (1972a) found low germination of pollen and slow or abnormal pollen tube growth in crosses of *T. repens* with several other species. Although fertilisation of ovules was variable, and lower than in intraspecific controls, it was still achieved in all interspecific combinations. Many workers have recorded fertilisation and pod enlargement, with no production of viable seed (Williams and White, 1976; White and Williams, 1976). White and Williams (1976) also found that the percentage of fertilised ovules in *T. semipilosum* Fresen. x *T. repens* was similar to intraspecific crosses in *T. semipilosum*, suggesting a post-fertilisation barrier.

Observations of embryo development have shown that hybrids are initially comparable to intraspecific controls, but the growth of hybrid embryos then slows and they begin to degenerate (Williams and White, 1976; White and Williams, 1976; Kazimierski *et al.*, 1972;
Chen and Gibson, 1971). This phenomenon has been recorded in a range of interspecific crosses – e.g. *T. repens* x *T. uniflorum*, *T. hybridum* x *T. michelianum* Savi, *T. ambiguum* x *T. repens*, and *T. semipilosum* x *T. repens*. Failure of the endosperm has also been observed, and is thought to lead to degeneration of the embryo through disruption of nutrient transfer and, ultimately, starvation (Chen and Gibson, 1971; White and Williams, 1976; Williams, 1987a). Several causes for endosperm failure have been suggested for a range of species, including competition with the maternal tissue for nutrients, alterations to nutrient supply, genomic imbalances in the endosperm, and negative interactions between the endosperm and maternal tissues (Williams, 1987a; Chen and Gibson, 1971). The endosperm balance number (EBN), or effective ploidy, affects the genomic balance within the endosperm, with normal endosperm development occurring at a ratio of 2:1 (maternal:paternal derived EBN’s) (Williams, 1987a; Williams et al., 2008).

### 2.6.3 Ovule and embryo rescue

Ovule culture and embryo rescue techniques have been developed to overcome post-fertilisation barriers to hybridisation, and are widely used (Ferguson et al., 1990; Williams et al., 2006b; Meredith et al., 1995). These techniques have greatly improved the success of known hybrid combinations and have also been used to produce new hybrids (Williams et al., 2006b). The first successful attempts at embryo rescue in *Trifolium* were reported by Keim (1953) with interspecific hybrids of *T. ambiguum* x *T. hybridum*, *T. repens* x *T. nigrescens*, and *T. nigrescens* x *T. repens*.

In general, the ovule or embryos are excised from the pods – usually those which have enlarged. Embryos may be transplanted to a nurse endosperm (Williams, 1987a; Przywara et al., 1989) from an intraspecific cross before being transferred to artificial growth media. But in most cases the embryos or ovules are transplanted directly into a culture medium. The optimal point for embryo rescue appears to be the heart-shaped to torpedo stages of development, as described in *T. alexandrinum* x *T. apertum*, *T. repens* x *T. uniflorum*, and *T. alexandrinum* x *T. constantinopolitanum* hybrids (Malaviya et al., 2004; Roy et al., 2004; Pandey et al., 1987). Ovule culture can be used to extend embryo development to the stage where they can be excised and successfully cultured. The length of time after pollination at which these optimal developmental stages occur differs according to the interspecific combination. Various plant tissue culture media have been used depending on the species involved and the stage of development of the embryos (e.g. Kaushal et al., 2005; Yamada and Fukuoka, 1985; Przywara et al., 1989; Williams, 1987a). Embryos and plantlets are usually
transplanted through a series of media, differing in composition according to the requirements of various growth stages.

### 2.6.4 Other factors affecting hybridisation success

#### 2.6.4.1 Ploidy level

The ploidy level of the parental material is important in the success of interspecific crosses, with some species crossing with more success at higher ploidy levels. Many workers have studied plants in which the chromosomes were doubled through the use of colchicine (Evans, 1955; Gibson and Beinhart, 1969; Chou and Gibson, 1968). Chromosome doubling can also be achieved by treating pollinated plants or flower heads with nitrous oxide (Taylor *et al.*, 1980). Taylor *et al.* (1976) reported that this method was easier and more successful than reported studies at the time using colchicine. However, colchicine has been the most commonly used method. Earlier work exposed seeds and seedlings directly to aqueous colchicine (Evans, 1955), while more recent *in vitro* methods combine shoot proliferation media with colchicine to target axillary meristems (Hussain *et al.*, 1997a; Anderson *et al.*, 1991a).

In crosses with *T. nigrescens*, Chou and Gibson (1968) achieved a seed set of zero with diploid *T. occidentale*, and 0.48 with tetraploid *T. occidentale*. Gibson and Beinhart (1969) also found crosses with *T. nigrescens* and *T. repens* to *T. occidentale* were more successful using induced tetraploid *T. occidentale*. Anderson *et al.* (1991b) doubled the chromosomes of the 4x *T. ambiguum x T. repens* F₁ hybrid of Williams and Verry (1981) to produce an octoploid with improved fertility. Hussain and Williams (1997) used this octoploid to produce a range of backcross generations, one of which could serve as a genetic bridge between the two parental species. A series of backcross and intercross generations was then developed (Williams and Hussain, 2008). Hussain *et al.* (1997b) also increased the fertility of a highly sterile 3x *T. repens x T. nigrescens* F₁ hybrid through chromosome doubling, with pollen stainability (an indication of pollen fertility) increasing from an average of 9.9% in 3x plants to 89.2% in the 6x plants.

#### 2.6.4.2 Direction of crosses

The choice of maternal and paternal parents is also important – that is, which of the two species in the cross is the maternal parent and which species is the paternal parent. Pandey *et al.* (1987) found that *T. uniflorum x T. repens* F₁ hybrids had higher seed development than *T. repens x T. uniflorum* crosses but were ultimately less viable. Similarly, Williams *et al.* (2006a) found an increased frequency of abnormal seedlings, and lower fertility and vigour in
adult plants, when white clover was backcrossed as the male parent to the 8x *T. ambiguum* x *T. repens* F₁, compared with the reciprocal backcross with white clover as the maternal plant.

Williams *et al.* (2008) suggested that gene flow only occurs in the direction of *T. occidentale* to *T. nigrescens*, as F₁ hybrids have only been successfully produced using *T. nigrescens* as the female parent. As mentioned in Section 2.6.1.2, Hovin (1962a) had much greater success crossing *T. repens* x *T. nigrescens* than with the reciprocal cross. They also concluded that this was a one-way barrier that could not be overcome by embryo rescue in the reciprocal cross.

### 2.6.4.3 Genotypic variation

Genotypic variation can also influence the success of hybridisation. White and Williams (1976) observed variation (frequency and size) in the pod enlargement of *T. semipilosum* x *T. repens* hybrids according to parental genotype, and suggested that selection of parents could lead to improved embryo development. Williams (1987a) also attributed observed variation in endosperm and embryo development of *Trifolium* hybrids to differences in compatibility of different genotypes. Hovin (1962a) found that Italian accessions of *T. nigrescens* were five times more fertile than Turkish accessions, when crossed with white clover, and produced more vigorous hybrids that had greater survival to flowering.

### 2.6.5 Confirmation of hybridity

Confirmation of hybridity can be made morphologically, cytologically and by using molecular markers. Possession of morphological characteristics that are intermediate to the parents (e.g. plant size, leaf size, size and arrangement of floral parts, stoloniferous habit) is seen as evidence of hybridity (Williams, 1978; Gibson and Beinhart, 1969). The presence of dominant leaf markings (Pandey *et al.*, 1987; Williams, 1978; Hussain *et al.*, 2012) and isozyme bands (Williams, 1980; Williams, 1978; Pandey *et al.*, 1987) from the male parent also confirm hybridity.

Somatic chromosome counts are made in root tip squashes, as described in Hussain *et al.* (1997b) and Meredith *et al.* (1995), and compared with those expected given the chromosome numbers of the parental species. For example, *T. repens* (2n = 4x = 32) x *T. nigrescens* (2n = 2x = 16) hybrids should have a chromosome count of 24 (16 from *T. repens* and 8 from *T. nigrescens*). Chromosome associations during meiosis are also studied in pollen mother cells (Hussain *et al.*, 1997b; Anderson *et al.*, 1991b). The presence of a high number of multivalents (pairing of three or four chromosomes) is seen as evidence of hybridisation, with at least one chromosome from one species pairing with one or more from the other species.
(Hussain and Williams, 1997; Williams et al., 1982). Bivalents may be autosyndetic (pairing between two chromosomes from the same species) or allosyndetic (one chromosome from each species) (Williams et al., 1982).

Fluorescence in situ hybridization (FISH) with labelled known DNA sequences, or genomic in situ hybridisation (GISH) with labelled total genomic DNA, can also be used to study hybridity and pairing – e.g Meredith et al. (1995) for T. ambiguum x T. repens, and Williams et al. (2008) for T. nigrescens x T. occidentale.

2.6.6 Genetic bridges

In addition to hybrids between two species, multi-species hybrids have also been produced. These include (T. ambiguum x T. repens) x T. uniflorum (Williams et al., 2006b); (T. repens x T. uniflorum) x T. occidentale, [(T. repens x T. uniflorum) x T. occidentale] x T. ambiguum, and (T. repens x T. isthmocarpum) x T. repens with T. nigrescens and T. occidentale (Ferguson et al., 1990). As well as introducing genes from two species into a third, this is also a method of overcoming compatibility barriers between species by using another as a bridge between them. Genetic bridges have also been produced within interspecific combinations, through the manipulation of chromosome numbers, to facilitate crossing between parental species that are otherwise difficult to cross (Hussain and Williams, 1997).

2.6.7 New Trifolium phylogeny

Ellison et al. (2006) used molecular phylogenetics, through analysis of both nuclear and chloroplast DNA, to redefine Trifolium species relationships and proposed a new classification of the genus. The new Section Trifoliastrum contains a group of species that has been named “the white clover complex”. The revised version of this group, found in Williams et al. (2006b), consists of white clover plus eight species which are closely related, and are therefore potential sources of genetic material through interspecific hybridisation (Figure 2.3). These species represent a wide range of geographic locations, habitats and morphology. Notably, the putative parents of white clover suggested by Ellison et al. (2006), T. occidentale and T. pallescens, come from widely different habitats – coastal and alpine, respectively (Williams et al., 2008; Zohary and Heller, 1984).
Figure 2.3. The “white clover complex” proposed by Williams et al. (2006b) as part of the new Section *Trifoliastrum*.

Based on this new classification, Williams et al. (2006b) carried out a series of hybridisations within the white clover complex. This produced eight new fertile interspecific hybrid combinations, including the first ones involving *T. pallescens* and *T. thalii* Vill.:

- *T. ambiguum* (2x) x *T. occidentale* (2x)
- *T. ambiguum* (6x) x *T. occidentale* (2x)
- *T. ambiguum* x *T. pallescens*
- *T. ambiguum* x *T. nigrescens ssp. nigrescens*
- *T. ambiguum* x *T. nigrescens ssp. meneghinianum*
- *T. ambiguum* x *T. thalii*
- *T. pallescens* x *T. occidentale*
- (*T. ambiguum* x *T. repens*) x *T. uniflorum*

A more detailed description of hybrids between *T. ambiguum* (Caucasian clover) and *T. occidentale* is reported by Williams et al. (2011), and crossing within the white clover complex is reviewed by Williams et al. (2010).
2.6.8 Development of commercial hybrids

For many years, studies on interspecific hybridisation of white clover, and *Trifolium* species in general, focused on successfully producing confirmed hybrids, production of new interspecific combinations, understanding and overcoming barriers to hybridisation, and developing new methods to improve success (Sections 2.6.1–2.6.5). As knowledge about hybridisation and understanding of genetic interactions and their influence has grown, more progress has been made (Abberton, 2007). However, the difficulty in producing hybrids and limited success in improving key traits for an agronomic environment has limited the development of commercial interspecific hybrids. Two combinations have been the focus for development of commercial material – *T. repens* with *T. nigrescens*, and *T. repens* with *T. ambiguum*.

2.6.8.1 *T. ambiguum* x *T. repens*

*T. ambiguum* (Caucasian clover) has thick roots, underground rhizomes, is persistent in the field and possesses resistance to some pests and diseases of white clover. Fertile *T. ambiguum* x *T. repens* hybrids have been produced by research groups at AgResearch in New Zealand and the Institute of Grassland and Environmental Research (IGER), now the Institute of Biological, Environmental and Rural Sciences (IBERS) at Aberystwyth University, in the United Kingdom.

Marshall *et al.* (2003b) found that with white clover as the recurrent parent, dry matter production of BC1 and BC2 generations was less than that of white clover in the first year of growth, but similar in the second and third years. Widdup *et al.* (2003) reported lower mean growth at multiple sampling times for 63 6x BC1 hybrids compared with white clover, but found that the best performing hybrid entry had improved growth relative to white clover in the second year of the experiment. *T. ambiguum* x *T. repens* hybrids have also been shown to partition a greater proportion of biomass to root material than white clover (Widdup *et al.*, 2003). Despite the effects on reduced herbage production, this feature, along with thicker and deeper roots, could have benefits in assisting with drought resistance. Improved drought resistance in *T. ambiguum* x *T. repens* backcross hybrids, compared with white clover, has been reported by Marshall *et al.* (2001) (see Section 2.5.3).

Both Marshall *et al.* (2004) and Abberton *et al.* (2002) reported that the forage quality of white clover was maintained in *T. ambiguum* x *T. repens* hybrids. Backcross material had similar dry matter digestibility, higher WSC, and lower CP and %N relative to white clover. The latter three features were suggested to represent potential for better fermentation in silage.
and better utilisation of N in the rumen compared with white clover (leading to decreased N losses in urine and gaseous emissions).

Abberton et al. (1998) and Widdup et al. (2003) reported that T. ambiguum x T. repens backcross generations had a similar morphology to white clover. Hybrids from the New Zealand breeding programme have not yet been reported to form rhizomes (Widdup et al., 2003) but Abberton et al. (1998) found an average of 3% of total plant dry weight (DW) as rhizomes in the BC3 generation (range 0.5–6%). Compared with an average of 55% in rhizomes for Caucasian clover this is still relatively small, and Meredith et al. (1995) also found rhizomes in only 10% of BC2 hybrids. However, the rhizomatous trait is only fully expressed in well established plants and Abberton et al. (2003) classified 14% of 18 month old BC3 plants as having a high proportion of rhizomes. Forty two percent had no rhizomes and there was considerable variation within and among families. A genetic marker for the rhizomatous trait was identified.

Abberton et al. (2000) inoculated T. ambiguum x T. repens hybrids and the parental species with strains of Rhizobium known to be effective on both parents. They concluded that there is no inhibition of N fixation in the hybrids, with BC2 plants being similar to white clover in dry matter production, nodulation patterns, nodule growth and N fixation per plant. The effectiveness of the various rhizobia was not studied, but Lowther et al. (2002) found that growth of white clover and the hybrids was higher with a mixture of strains of rhizobia from white clover in the field than with a New Zealand inoculant strain, and lower again with an inoculant strain for Caucasian clover. The F1 plants grew equally well with rhizobia from either parent. Contrary to Abberton et al. (2000), growth of BC2 plants was significantly lower than white clover, although there was considerable variability. Lower growth could simply reflect intermediate characteristics between the two parents, which may be expected in interspecific hybrids. However, there was a significant correlation between plant weight and ethylene production, the latter of which was used as a measure of N fixation rates and accounted for 60% of the variation in plant fresh weight.

2.6.8.2 T. repens x T. nigrescens

T. nigrescens is an annual species with prolific flowering and resistance to clover cyst nematode. Hybridisation with white clover is targeted at increased seed production and nematode resistance. Hussain et al. (1997a) reported that clover cyst nematode resistance in T. repens x T. nigrescens hybrids was at least as good as that of T. nigrescens.
Studies by Marshall et al. (1995; 1998) showed that *T. repens x T. nigrescens* backcross hybrids have an increased number of inflorescences compared with white clover. However, Marshall et al. (2002a) observed that the number of seeds per floret and inflorescence were lower, meaning total seed yields were no greater than those of white clover. Variability in the later BC₃ generation included individual plants that did produce more seeds, providing potential for selection for increased seed yields.

Marshall et al. (2002b; 2005; 2003a) concluded that increased inflorescence production had not compromised the agronomic traits of the *T. repens x T. nigrescens* hybrids, unlike Hussain et al. (1997a) who observed that hybrids had poorer growth and perenniality than white clover, although no measurements were reported. Dry matter yields of backcrosses were generally similar to white clover, and any decreases were suggested to be due to a smaller leaf size in the white clover parent. Dry matter yields of companion grasses growing with the hybrids were also similar to those growing with white clover. Differences in forage quality between the hybrids and white clover were small. Unlike the Caucasian clover hybrids, levels of WSC were lower than white clover in the *T. repens x T. nigrescens* hybrids, and CP was greater, but these differences were not always significant. There were few differences in the forage quality of the companion grasses, and these were also small.

The *T. repens x T. nigrescens* hybrids retained the stoloniferous habit of white clover, and later generations in particular produced nodal roots (*T. nigrescens* does not), maintaining the persistence of white clover (Marshall et al., 1995; 1998). Nitrogen fixation appeared to be unaffected by hybridisation, being similar in the hybrids and the white clover parent (Abberton et al., 1999).

### 2.7 *Trifolium uniflorum*

*T. uniflorum* L. (2n = 32) is a perennial, wild species from the Mediterranean region, found in Greece, Turkey, southern France, southern Italy, and Libya (Zohary and Heller, 1984). In a literature and field survey of *Trifolium* species, Fotiadis et al. (2010) identified *T. uniflorum* in 9 out of 13 phytogeographical areas in Greece, from sea level to 2400 m altitude. It is reported as being tolerant of dry environments (Tela Botanica, 2012), and occurs in coastal to inland habitats, including halophilous coastal communities (Brullo et al., 2000; Tela Botanica, 2012). There is very little published data on the eco-geography of *T. uniflorum*, but Mt Parnitha in Greece, where germplasm has been collected, has been described as having a “...long dry season and high temperatures during summer, poor and shallow soils” (Forest Service of Parnitha, 2012). *T. uniflorum* has also been considered for use in the dryland
wheatbelt of southern Australia, where rising water tables, salinisation and acid soils are also issues (Cocks, 2001; Dear et al., 2003; Li et al., 2008). These evaluations ranked *T. uniflorum* highly for persistence but poorly for productivity among the species investigated, in both general and acid soil nurseries (Li et al., 2008). It is of no real economic importance, but the United States Department of Agriculture, Agricultural Research Service (2007), reports it is used for ornamental purposes.

Like white clover, it is self incompatible (Pandey, 1957), although Gibson and Chen (1971) found some self compatibility in three seedlines. Pandey (1957) determined the chromosome number of *T. uniflorum* to be 32, which was confirmed by Gibson and Chen (1971) who also found predominantly bivalent and quadrivalent chromosome pairing. It has four satellite chromosomes (Gibson and Chen, 1971; Gibson et al., 1971) which, along with the chromosome pairing arrangements, is interpreted as evidence of an autotetraploid origin (Gibson and Chen, 1971).

*T. uniflorum* appears to be highly variable. Badr et al. (2002) calculated genetic diversity in a range of *Trifolium* species and accessions, and reported that *T. uniflorum* had the highest diversity and differentiation among accessions. Vierhapper (1919) divided the species into seven varieties, based mainly on floral morphology: *cryptoscias*, *Sternbergianum*, *Buxbaumii*, *varians*, *Savianum*, *macrodan*, and *breviflorum*. Hossain (1961) listed four synonyms – *T. uniflorum* var. *sternbergianum*, *T. uniflorum* var. *breviflorum*, *T. uniflorum* var. *macrodan*, and *T. uniflorum* var. *varians* – and also described the species as variable, including for leaflet size and shape, peduncle length, pedicel length and breadth, and floral characteristics. Greuter (1972) also listed the co-existence of *T. uniflorum* with a mountain ecotype on the Greek island of Crete (*T. uniflorum* var. *breviflorum* Boiss.).

Some authors treat *T. savianum* Guss. as a synonym or subspecies of *T. uniflorum*, while others consider it to be a distinct species. Brullo et al. (2000) proposed that *T. savianum* is an endemic Sicilian species separated from *T. uniflorum* by geographic isolation, with the two exhibiting differing morphological characteristics and ecological associations. They speculated that *T. savianum* could have adopted a mountainous habitat prior to the ice-age, isolating it from low land connections which may have allowed genetic exchange in the eastern populations of *T. uniflorum* during glaciations.

### 2.7.1 Morphology

Detailed morphological descriptions of *T. uniflorum* are given by Zohary and Heller (1984) and Brullo et al. (2000). Its name derives from the production of groups of 1–3 large florets,
in contrast to the inflorescences of other *Trifolium* species (Plate 2.1). Various authors have particularly noted its short internodes, thick and deep roots, a woody tap root (see Plate 2.2), and a relatively large seed (Pandey and Petterson, 1978; Chen and Gibson, 1971; Pandey *et al*., 1987). These characteristics were suggested to have potential to improve white clover through interspecific hybridisation.

Gibson *et al.* (1971) suggested that the larger seed size could improve the seedling vigour of white clover. The stronger, deeper roots were also suggested to improve drought resistance, pest resistance, nutrient interception and soil conservation (Pandey *et al*., 1987; Pandey and Petterson, 1978). Vierhapper (1919) noted that low nutrients or exposure to drought caused a decrease in the size of above ground plant parts (e.g. leaves, flowers and petiole length) of *T. uniflorum*.

**Plate 2.1.** *T. uniflorum* in flower.
Plate 2.2. T. uniflorum after 24 weeks of growth in sand culture in a glasshouse, showing thick roots, short internodes and small leaf size.

2.7.2 Pest and disease resistance

Dymock and Hunt (1989) reported that grass grub (Costelytra zealandica (White)) fed equally on white clover and T. uniflorum in terms of percentage of root DW consumed. However, there was a difference in consumption of different sized roots. For both species, roots over 2 mm in diameter were eaten less than those under 2 mm, but particularly so for T. uniflorum (-4.0% of root DW versus +38.1% for white clover). The authors speculated that the roots of T. uniflorum in this class were thicker than those of white clover, and that T. uniflorum may not have enough fine roots to support grass grub in the field. Dymock et al. (1989) subsequently found that grass grub larval growth was reduced on T. uniflorum compared with other Trifolium species. Growth of several seedlines was comparable to, or lower than, Lotus pedunculatus, a species known to be resistant to grass grub. The authors speculated that pest resistance in T. uniflorum could be due to nutritional quality of the roots, absence of feeding stimulants, or the production of feeding deterrents. Sutherland (1979) also suggested that the woody nature of T. uniflorum roots could provide a mechanical method of tolerance. Dymock and Hunt (1989) suggested the resistance of T. uniflorum to grass grub could be utilised in white clover by hybridisation.
Pederson and Windham (1989) studied the resistance of eight *Trifolium* species to southern root-knot nematode (*Meloidogyne incognita*). Four species, including *T. uniflorum*, had lower mean gall indexes than white clover. One accession of *T. uniflorum* had the lowest mean gall index but only two plants out of 10 were classified as resistant, so the species was not considered to be as resistant as *T. nigrescens* and Caucasian clover. This accession also had the smallest proportion of the root system affected by galls. A second *T. uniflorum* accession had no resistant plants among the 10 that were examined.

Gibson *et al.* (1971) also suggested that *T. uniflorum* may have some virus tolerance, listing only one virus (clover yellow mosaic virus) as causing symptoms, out of six tested. These authors also reported a lower effect of sooty blotch and powdery mildew on *T. uniflorum* compared with *T. occidentale*.

### 2.7.3 Nodulation

In laboratory studies, *T. uniflorum* has been found to form either partially effective or effective nodules with three *Rhizobium leguminosarum* bv *trifolii* strains from *T. subterraneum* and one from *T. medium* L. (both annual species) (Yates *et al.*, 2003; Howieson *et al.*, 2005). Those from *T. subterraneum* are commercial Australian rhizobia strains. Rhizobia from white clover were not tested on *T. uniflorum*, but the two strains tested from *T. uniflorum* were ineffective on white clover. One strain of *Rhizobium* from *T. uniflorum* was effective or partially effective with ten *Trifolium* species (Howieson *et al.*, 2005). Of two other strains, one was effective and one ineffective, with *T. fragiferum* L. (perennial) (Yates *et al.*, 2003; Howieson *et al.*, 2005).

All successful relationships involving *T. uniflorum* were with European clovers and *Rhizobium* strains isolated from European species. They included both perennial and annual clovers. *T. uniflorum* rhizobia were unsuccessful with the African, North American and South American clover species tested and, similarly, rhizobia isolated from clovers from these regions were unsuccessful with *T. uniflorum* (Yates *et al.*, 2003; Howieson *et al.*, 2005).

### 2.7.4 Cyanogenesis

It is unclear whether *T. uniflorum* is cyanogenic. Gibson *et al.* (1971) used the presence of cyanoglucoside to distinguish between *T. uniflorum*, *T. occidentale* and their F1 hybrids. They found that *T. occidentale* contained cyanoglucoside (presence or absence of the hydrolysing enzyme linamarase was not stated) but *T. uniflorum* did not. However, Gibson *et al.* (1972) later found two out of ten *T. uniflorum* plants tested did contain cyanoglucoside, although only trace amounts were detected. None of the *T. uniflorum* plants tested contained the
hydrolysing enzyme. The authors questioned the significance of the weak response of *T. uniflorum* and recommended that further tests be carried out using more accessions and greater sample sizes. Given the variability in morphological features observed in *T. uniflorum* by other authors, it is possible that variability may also exist for cyanogenesis. Within white clover there are certainly cyanogenic and acyanogenic genotypes, plus variation in the level of hydrocyanic acid produced (Crush and Caradus, 1995). The variation in cyanogenesis with latitude found by Daday (1954) (i.e. decreasing frequency of genes for cyanogenesis between Mediterranean and northern European populations of white clover) may also suggest that high levels may be expected in a Mediterranean species such as *T. uniflorum*, at least in some populations.

2.8 Interspecific hybridisation between *T. repens* and *T. uniflorum*

Studies by Chen and Gibson (1970a, 1970b) found homology between the chromosomes of *T. repens*, *T. occidentale*, and *T. nigrescens*, suggesting closely related genomes. Chen and Gibson (1972b) subsequently found that *T. uniflorum* may share a similar genome to these three species.

As mentioned in Section 2.6, Badr *et al.* (2002) concluded that *T. uniflorum* is one of the ancestors of white clover, and Evans (1962a) also suggested this was possible given the success in hybridising the two species. However, the results of Ellison *et al.*’s (2006) molecular-based phylogeny of the *Trifolium* genus found otherwise. Still, the new Section *Trifoliastrum* produced by Ellison *et al.* (2006) does show that *T. uniflorum* is closely related to white clover (see Figure 2.3). Hybridisation of white clover with *T. uniflorum* could therefore be expected to be relatively successful compared with other more distantly related species, such as *T. ambiguum*.

Interspecific hybrids between white clover and *T. uniflorum* were first produced by Pandey (1957), and the F₁ generation was successfully backcrossed to both parents. The F₁ was also self-compatible, in contrast to both parents which are self-incompatible. The author interpreted this as indicating that the S gene of the two species, which governs self incompatibility, occurs at different loci either on the same homologous chromosome or on non-homologous chromosomes. Hussain *et al.* (2012) also reported self-compatibility in *T. repens x T. uniflorum* F₁ hybrids. Overall, relatively little information is available on white clover and *T. uniflorum* hybrids, but studies have also been published by Evans (1962a, 1962b), Gibson *et al.* (1971), Gibson and Chen (1973), Chen and Gibson (1971; 1972a, 1972b), Pandey and Petterson (1978), and Pandey *et al.* (1987).
T. uniflorum has also been hybridised with 4x T. occidentale, and the F₁ crossed with white clover (Gibson et al., 1971; Gibson and Chen, 1975; Williams et al., 2010). In addition, T. uniflorum x T. occidentale has been backcrossed to T. uniflorum (Gibson and Chen, 1975), and T. repens x T. occidentale hybrids have also been crossed with T. uniflorum (Gibson et al., 1971). Crossing white clover and T. uniflorum with T. occidentale was used to overcome compatibility barriers between the two species. The multi-species hybrids were also seen as a means of introducing genes to T. repens from two species at once.

2.8.1 Hybridisation problems

Hybridisation of white clover and T. uniflorum has had the same problems as reported for other interspecific hybrids. For the first T. uniflorum x T. repens crosses produced by Pandey (1957), seed production was 30–50% that of intraspecific crosses, but germination (2 out of 30 seeds) and seedling survival (one of the two seedlings) were low. The two germinated seedlings also exhibited chlorophyll deficiencies. Pandey et al. (1987) subsequently found that the reciprocal cross (T. repens x T. uniflorum) was more successful. Although this combination produced no seed, the use of embryo rescue ultimately resulted in plants that were more viable than those from T. uniflorum x T. repens crosses. In contrast, T. uniflorum x T. repens F₁ hybrids had low germination, poor seedling survival and a high proportion of chlorotic or albino seedlings.

Evans (1962a) initially gained no seed from both T. uniflorum x T. repens and T. repens x T. uniflorum crosses, but observed development of embryos after hybridisation of compatible genotypes. With embryo rescue she then successfully produced several seedlings from each cross direction. Gibson et al. (1971) also initially failed to produce hybrids from T. repens x T. uniflorum crosses, although pod enlargement and production of non-viable seed suggested that some embryo development was occurring. Further attempts, utilising genotypes which exhibited pod enlargement, were successful.

2.8.2 Pre- and post-fertilisation barriers

The findings of Evans (1962a), Gibson et al. (1971) and Pandey (1987) indicate the presence of barriers to hybridisation between these two species. These have been studied in more depth by Evans (1962b) and Chen and Gibson (1971; 1972a). As with interspecific crosses in general, post-fertilisation barriers appear to be more important than pre-fertilisation barriers. Evans (1962b) reported that T. uniflorum had the longest pistil and style out of the 10 species studied, and also the highest mean pollen tube growth rate. This growth rate was lower in T.
repens x T. uniflorum crosses, but still relatively high up to 24 hours after pollination, unlike some other interspecific combinations where abnormal pollen tube growth was observed.

Conversely, Chen and Gibson (1972a) observed that T. repens x T. uniflorum hybrids had the slowest pollen tube growth, with more abnormalities, compared with other interspecific crosses, although few of these cross combinations were the same as those studied by Evans (1962b). Pollen germination of T. repens x T. uniflorum was lower than in white clover intraspecific crosses, and fertilisation was also slower than in both intraspecific and interspecific crosses of white clover. Chen and Gibson (1971) examined the seed development of T. repens x T. uniflorum crosses and also observed both delayed fertilisation and a decrease in the frequency of ovule fertilisation. Abnormal growth of the hybrid endosperm appeared four days after pollination, followed by abnormal growth of the embryo. The authors speculated that failure of the embryos was due to starvation following the disintegration of the hybrid endosperm. Embryo rescue has improved the success of interspecific hybridisation between the two species (Evans, 1962a; Pandey and Petterson, 1978; Pandey et al., 1987).

2.8.3 Morphology of the hybrids

Morphological descriptions of the hybrids are generally intermediate to the two parental species. Pandey (1957) described the T. uniflorum x T. repens F\textsubscript{1} as vigorous and intermediate to the parents. Gibson \textit{et al.} (1971) also described their T. repens x T. uniflorum F\textsubscript{1} hybrids as vigorous, with intermediate stipule shape and internode length. Example images of floral form were intermediate to the parents, and the hybrids were stoloniferous perennials. F\textsubscript{1}, F\textsubscript{2} and BC\textsubscript{1} hybrids produced by Pandey \textit{et al.} (1987) were generally intermediate, but variability was observed within the cross combinations, reflecting heterozygosity of the parents. Backcrosses to white clover more closely resembled white clover in vegetative characteristics, while those to T. uniflorum showed more T. uniflorum-like floral characteristics. Some hybrids were nodulated, but the authors do not mention whether plants were inoculated. If not, this indicates they are likely to be compatible with the same rhizobia as white clover, which was likely to be present in background populations. Hybrids in the field were reported to have low vigour but no further data has been presented on this.

The F\textsubscript{1} hybrids produced by Hussain \textit{et al.} (2012) had a smaller root and shoot DW than BC\textsubscript{1} and white clover plants, but had the highest ratio of thick roots (>2 mm) to total root mass. The lower and upper ranges of shoot and root DW of BC\textsubscript{1} hybrids were below those for white clover, but the best-performing BC\textsubscript{1} plants were reported to be similar to the best-performing
white clover plants. Furthermore, BC\textsubscript{1} hybrids had more thick roots than white clover. There was significant variation among the hybrids for these morphological traits.

Pandey \textit{et al.} (1987) also reported that some hybrid plants showed transgressive segregation (genetic variation outside the range of either parent (Grant, 1975; Rieseberg \textit{et al.}, 1999)), notably the formation of tap root-like nodal roots. The authors describe most vigorous hybrids as having stronger and deeper root systems than white clover, with most roots from the central crown. Pandey and Petterson (1978) had previously reported similar transgressive segregation in a white clover hybrid with \textit{T. uniflorum}, with a central tap root plus tap roots at the nodes. A second hybrid plant had a root system more like that of white clover. Transgressive segregation is widely reported in interspecific hybrids, across a range of genera (e.g. Nasrallah \textit{et al.} (2000) (\textit{Arabidopsis}); Rosenthal \textit{et al.} (2002) and Gross \textit{et al.} (2004) (\textit{Helianthus}); Kirk \textit{et al.} (2011) (\textit{Jacobaea})). In addition, relatively high proportions of transgressive traits are reported. For example, Rosenthal \textit{et al.} (2002) found that 20-39% of traits were transgressive in a study of three hybrid sunflower species. In \textit{Helianthus anomalus}, Schwarzbach \textit{et al.} (2001) separated traits into morphological and ecophysiological characteristics, and found transgressive segregation in 41.5% of the former and 24% of the latter. Given the common occurrence of transgressive segregation, it is likely that transgressive traits will also be found in \textit{T. repens x T. uniflorum} interspecific hybrids.
Chapter 3
Effect of hybridisation on key white clover traits

3.1 Introduction

The value of white clover to pastoral agriculture lies in its productivity, nutritive value, and the ability to fix atmospheric N (Caradus et al., 1996). In addition, its perenniality and stoloniferous nature are important characteristics governing its ability to spread and persist in the sward. However, loss of the white clover tap root is a pivotal stage in the decline of the white clover component of pastures (Woodfield and Caradus, 1996; Westbrooks and Tesar, 1955; Brock et al., 2000). Persistence and performance are also poor under drought and in dryland environments (Knowles et al., 2003; Brock et al., 2003). Morphological characteristics such as leaf size, stolon diameter, stolon density, and internode length contribute to the performance of white clover and are often the basis of plant breeding selections (Caradus and Woodfield, 1997; Williams, 1987b). While the limitations of white clover may be addressed through interspecific hybridisation, it is important that key white clover traits are not adversely affected in the process. As well as improved traits, it is likely that negative characteristics will also be present in some hybrid individuals or populations.

The effect of hybridisation with T. uniflorum on key characteristics of white clover has not been quantified. It can be expected that traits in the hybrids will be intermediate to the parents, becoming more like white clover with successive backcross generations. The limited information in the literature does report intermediate morphological traits in hybrids between T. repens and T. uniflorum (Pandey, 1957; Pandey et al., 1987; Gibson et al., 1971), but little or no data is presented. In particular, there is no published data on the performance of T. repens x T. uniflorum hybrids in the field. No information is published about N fixation of T. uniflorum, and how this may affect N fixation of T. repens x T. uniflorum hybrids. Hybrids grown in the glasshouse without targeted rhizobia inoculation form healthy nodules, suggesting they use the same rhizobia as white clover and are naturally inoculated with bacteria that are present in the glasshouse environment. However the relative N fixation of white clover and T. repens x T. uniflorum hybrids has not been quantified.

The objective of this study was to measure important white clover traits in a range of BC$_1$ (backcross 1) and BC$_2$ (backcross 2) hybrids under field conditions, and to compare their performance to white clover cultivars. It was hypothesised that growth and morphological characteristics of the hybrids would be intermediate to the T. uniflorum and white clover
parents, and that the BC₂ generation would be more like white clover than the BC₁ generation. In addition, it was hypothesised that N fixation of the hybrids would not differ to that of white clover. This experiment also presented the opportunity to quantify tap root survival of the hybrids compared with the white clover and *T. uniflorum* parents. Given the thick, woody nature of the *T. uniflorum* tap root reported in the literature (Pandey and Petterson, 1978; Dymock and Hunt, 1989), it was hypothesised that tap roots of *T. uniflorum* would survive longer than those of white clover. In addition, given the expected intermediate characteristics of hybrids, it was also hypothesized that tap roots of the hybrids would survive longer than those of white clover, and that this may be attributed to increased root diameter.

3.2 Materials and methods

3.2.1 Experimental area and preparation

The experiment was conducted from November 2008 to May 2010 on the AgResearch farm, Boundary Road, Lincoln (43° 37’ 38.17"S, 172° 28’ 10.2"E). The soil type was a Wakanui silt loam (Cox, 1978) (Udic Ustochrept, USDA soil taxonomy). Prior to this experiment, the paddock contained a perennial ryegrass/white clover experiment. The existing vegetation was sprayed with 2.5 l ha⁻¹ of Roundup® (glyphosate). Soil samples (0–75 mm) taken at the beginning of the experiment showed that soil fertility conditions were generally not limiting for growth (Table 3.1), and no fertiliser was added at establishment.

Table 3.1. Soil test (0-75 mm) results from December 2008 for the key traits experimental site at AgResearch, Boundary Rd, Lincoln. me = milli equivalents. Numbers in parentheses are results in MAF Quick Test units. ‡target values for New Zealand sheep and beef farms (sedimentary soils) (Fert Research, 2009)

<table>
<thead>
<tr>
<th>pH</th>
<th>Olsen P (mg l⁻¹)</th>
<th>SO₄-S (mg kg⁻¹)</th>
<th>K (me 100 g⁻¹)</th>
<th>Ca (me 100 g⁻¹)</th>
<th>Mg (me 100 g⁻¹)</th>
<th>Na (me 100 g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>39</td>
<td>7</td>
<td>1.18</td>
<td>9.5</td>
<td>1.13</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>(23)</td>
<td>(11)</td>
<td>(24)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

‡ pH = 5.8–6.0; Olsen P = 20–30; SO₄-S = 10–12; K = 5–8 MAF units; Mg = 8–10 MAF units

3.2.2 Plant material

Plant material was grouped into five clover types – *T. uniflorum*, BC₁, BC₂, white clover and red clover (*T. pratense* L.). There were 27 clover entries, including 10 BC₁ and six BC₂ families, two *T. uniflorum* accessions, eight white clover cultivars representing a range of morphologies, and one red clover cultivar (Sensation) as a standard control. BC₁ and BC₂ entries were selected from top performing entries, based on dry matter (DM) scores, from an
existing field experiment at AgResearch, Lincoln. Clover entries are shown in Table 3.2. A full description of hybrid families is presented in Appendices 1 and 2. Plants were grown from seed to monitor tap root life, so F₁ (first filial) generations were not included due to the absence of seed for this material.

Table 3.2. *T. uniflorum* accessions, BC₁ and BC₂ families, and white clover and red clover cultivars used in the key traits experiment. Entry numbers correspond to the experimental design (Figure 3.1). OP = open pollinated; cv = cultivar.

<table>
<thead>
<tr>
<th>Entry number</th>
<th>Clover type</th>
<th>Description</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>T. uniflorum</em></td>
<td>AZ4382⁷ OP</td>
<td>Greek origin</td>
</tr>
<tr>
<td>2</td>
<td><em>T. uniflorum</em></td>
<td>AZ4383⁷ OP</td>
<td>Turkish origin</td>
</tr>
<tr>
<td>3</td>
<td>BC₁</td>
<td>Aran x 900-3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>BC₁</td>
<td>Barblanca x 82-3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>BC₁</td>
<td>Crusader x 80-2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>BC₁</td>
<td>Crusader x 900-4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>BC₁</td>
<td>Crusader x 902-11</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>BC₁</td>
<td>Kopu II x 900-4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>BC₁</td>
<td>Kopu II x 80-2</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>BC₁</td>
<td>Sustain x 82-3</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>BC₁</td>
<td>Tribute x 900-4</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>BC₁</td>
<td>Trophy x 902-6</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>BC₂</td>
<td>Crusader x (Crusader x 900-5)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>BC₂</td>
<td>Kopu II x (Kopu II x 902-1)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>BC₂</td>
<td>902-1-OP-4 x Trophy</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>BC₂</td>
<td>(Crusader x 902-1) OP</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>BC₂</td>
<td>Durana x (Crusader x 902-1)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>BC₂</td>
<td>Durana x (Kopu II x 902-4)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>White clover</td>
<td>cv. Crusader</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>White clover</td>
<td>cv. Grasslands Kopu II</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>White clover</td>
<td>cv. Grasslands Sustain</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>White clover</td>
<td>cv. Grasslands Tahora</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>White clover</td>
<td>cv. Grasslands Tribute</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>White clover</td>
<td>cv. Trophy</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>White clover</td>
<td>cv. Aran</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>White clover</td>
<td>cv. Barblanca</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Red clover</td>
<td>cv. Grasslands Sensation</td>
<td></td>
</tr>
</tbody>
</table>

⁷ Accession number, Margot Forde Germplasm Centre (Palmerston North, New Zealand).
Seedlings were established in a glasshouse at AgResearch, Lincoln in September 2008. Seed scarified with sandpaper was germinated on damp filter paper in Petri dishes, then transplanted to 40 x 40 x 120 mm root trainers containing a sand/peat potting mix. Seedlings were not inoculated with rhizobia due to its availability in pasture soils.

### 3.2.3 Experimental design

A total area of 25 m x 27 m was marked out in the middle of the paddock. This covered its full width, but was positioned to avoid high stock traffic areas such as gateways and shelter belts. A split plot design was used, with five replicates (blocks) and four sub-plots within each replicate, for a total of 540 experimental plants (Figure 3.1). The four sub-plots allowed for harvesting of plants over time to study tap root life span (see Section 3.2.6.2). Each sub-plot contained 30 plants in a 5 x 6 randomised design at 1 m spacings. All 27 entries were replicated in every sub-plot, with the three extra spaces in the design filled by spare plants to eliminate gap effects (see ^ in Figure 3.1), for an overall total of 600 plants. Where insufficient seed of experimental entries germinated, spare plants from other entries were used as replacements (see # in Figure 3.1).

<table>
<thead>
<tr>
<th>Pilot plants</th>
<th>Block 1</th>
<th>Block 2</th>
<th>Block 3</th>
<th>Block 4</th>
<th>Block 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>16</td>
<td>21</td>
<td>14</td>
<td>9</td>
<td>23</td>
</tr>
<tr>
<td>14</td>
<td>25</td>
<td>11</td>
<td>26</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>26</td>
<td>22</td>
<td>2</td>
<td>6</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>26</td>
<td>23</td>
<td>17</td>
<td>24</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>24</td>
<td>27</td>
<td>19</td>
<td>4</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>23</td>
<td>18^</td>
<td>13</td>
<td>1</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>20</td>
<td>23</td>
<td>16</td>
<td>22</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>2</td>
<td>14</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>1</td>
<td>7</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>19</td>
<td>24^</td>
<td>12</td>
<td>9</td>
<td>26</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>27</td>
<td>10</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>11</td>
<td>24</td>
<td>21</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>1</td>
<td>7</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>5</td>
<td>23</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>23^</td>
<td>7</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>1</td>
<td>17</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>21</td>
<td>18</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>14^</td>
<td>10</td>
<td>19^</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>23</td>
<td>21</td>
<td>13</td>
<td>3</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>19</td>
<td>14</td>
<td>16</td>
<td>8</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>25</td>
<td>17</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>12</td>
<td>9</td>
<td>6</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>10</td>
<td>16</td>
<td>19</td>
<td>5</td>
</tr>
</tbody>
</table>

**Figure 3.1. Layout of the key traits experiment. Numbers 1–27 represent entries as outlined in Table 3.2; ^ indicates plants filling gaps in the design; # indicates replacements for missing plants.**
A row of pilot plants was established along one edge of the experimental area to provide material for monitoring the onset of tap root death. A mixture of clover types was used for this, including white clover cultivars, BC1 hybrids, BC2 hybrids and *T. uniflorum* accessions. These plants were treated and maintained in the same manner as the experimental material.

### 3.2.4 Establishment

Tall fescue (*Festuca arundinacea* (Schreb.) syn. *Schedonorus arundinaceus* (Schreb.) Dumort.), cultivar Advance with MaxP endophyte (75% endophyte, 91% germination), was direct-drilled into the entire paddock at 20 kg ha⁻¹ on 10 November 2008, using a Duncan Linkage Renovator drill with Baker tips. Grass was used to provide the competition expected in normal grazing situations, and enabled the use of ¹⁵N techniques to study fixation of atmospheric N by the clovers. Tall fescue was chosen due to its expected complementarity with the hybrid clover material.

The young clover plants were transplanted to the field eight weeks after they were planted in the root trainers (12–18 November 2008) to minimise disturbance of the tap root. Planting took place 2–8 days after the tall fescue was drilled, prior to emergence of grass seedlings (Plate 3.1).

**Plate 3.1.** Key traits experimental site (AgResearch, Lincoln) at establishment (24 November 2008).
3.2.5 Management

The entire area was irrigated after drilling, and again after the clovers were planted, to aid establishment. After this no further irrigation was applied. Three passes, of 1.5 hours each, were made across the paddock with a 2 inch hand shift pipe irrigation system. Total monthly rainfall and mean monthly temperatures during the course of the experiment are presented in Figure 3.2, along with the mean monthly rainfall from 2000–2009 for comparison. The mean annual rainfall for 2000–2009 was 604 mm. Rainfall and temperature data were recorded at the Broadfield meteorological station, 300 m from the site (43° 37′ 28.43"S, 172° 28′ 13.5"E).

![Figure 3.2 Total monthly rainfall (solid bars) and mean monthly temperature (line) over the period of the experiment (November 2008 to May 2010). Mean monthly rainfall calculated from 2000–2009 is shown by hatched bars. Data collected from the Broadfield meteorological station.](image)

The experiment was grazed by sheep at 5–12 week intervals, depending on season, at approximately 2000–2500 kg ha⁻¹ total DM. Grazing dates were 21 January, 20 March, 4 June, 26 August, 24 September, 29 October, and 24 November 2009, and 5 February 2010. Mobs of 150-200 ewes and hoggets were moved into the whole paddock for 24–48 hours, to provide hard grazing (approximately 800–1000 kg ha⁻¹ post-grazing total DM).

After each of the first two grazings, the paddock was mown to control large weeds which had established, such as black nightshade (Solanum nigrum L.), wireweed (Polygonum aviculare L.) and broad-leaved dock (Rumex obtusifolius L.). Hand weeding of volunteer white clovers
from buried seed was also carried out in the first four months after establishment, and again in January/February 2010.

### 3.2.6 Measurements

Parameters which were visually scored during the experiment are shown in Table 3.3, with ranges and descriptions of score values.

#### Table 3.3. The range of values, and corresponding descriptions, for parameters scored.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score value</th>
<th>Score range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter yield</td>
<td>1 – 9</td>
<td>Smallest – largest</td>
</tr>
<tr>
<td>Above-ground fragmentation of stolons</td>
<td>1 – 4</td>
<td>Intact – obvious fragmentation</td>
</tr>
<tr>
<td>Tap root condition</td>
<td>1 – 6</td>
<td>Intact and healthy – no trace</td>
</tr>
<tr>
<td>Inflorescence height</td>
<td>1 – 6</td>
<td>Within canopy – well above canopy</td>
</tr>
<tr>
<td>Growth habit</td>
<td>1 – 4</td>
<td>Prostrate – erect</td>
</tr>
<tr>
<td>Fungal disease</td>
<td>0 – 5</td>
<td>None – severe</td>
</tr>
<tr>
<td>Stolon density</td>
<td>1 – 5</td>
<td>Low – high</td>
</tr>
<tr>
<td>Flowering density</td>
<td>0 – 5</td>
<td>None – high density</td>
</tr>
<tr>
<td>Virus</td>
<td>0 – 4</td>
<td>None – severe</td>
</tr>
</tbody>
</table>

#### 3.2.6.1 Dry matter yield

Dry matter yield of all plants was assessed visually prior to each grazing on: 16 January, 19 March, 2 June, 20 August, 23 September, 27 October and 20 November 2009, and 18 January and 20 May 2010. Assessments were made by scoring on a scale of 1 (smallest) to 9 (largest), taking into account lateral spread, density and height. Three plants of each score value were then cut to grazing height, dissected from the companion grass, and dried overnight (approximately 15 hours) (Crush et al., 2010b) at 80ºC to calibrate the visual DM scores with DM yield.

#### 3.2.6.2 Tap root measurements

Tap root life span was monitored by destructive harvesting of plants, beginning after the expected period of time at which tap root death occurs in white clover (12–18 months old) (Westbrooks and Tesar, 1955). This was confirmed by harvesting pilot plants from each of the clover types and identifying the onset of tap root loss. Age of the tap roots was measured from the time of seedling germination. Three harvests were conducted on 12–21 October 2009 (13 months old), 25–26 January 2010 (16 months old) and 22–30 April/24–28 May 2010 (19–20
months old). The third harvest combined plants from the harvest 3 and 4 sub-plots, due to low numbers of surviving tap roots.

All plants were first scored for above-ground fragmentation of the stolons on a scale from 1 (intact) to 4 (obvious fragmentation into clonal plants) (Table 3.3). Turves of one spade square (180 mm x 180 mm) and 150–200 mm deep were then dug out around the centre of each plant. These were broken apart, and the original centre of the plant identified. Where the tap root was intact, or appeared to be intact, it was collected and stored at 5°C for further analysis. The condition of the tap root was scored on a scale from 1 (intact and healthy) to 6 (not present).

Collected roots were washed to remove the soil and confirm the tap root condition score. At the first harvest (13 months old), measurements of tap root diameter were then made at the base (where it attaches to the crown of the plant), 10 mm below the base, 20 mm below the base, and at the beginning of the primary root, for plants with a tap root score of 1 (intact and healthy). The beginning of the primary root was considered to be the point immediately distal to the basal thickening at the top of the tap root.

The diameter of lateral roots greater than 1 mm in diameter, arising from the top 20 mm of the tap root, were also measured. The top 100 mm of the tap roots (including lateral roots) were then dried for approximately 15 hours (Crush et al., 2010b) at 80°C and weighed (Plate 3.2).

Plate 3.2. The top 100 mm of tap roots with a condition score of 1 (intact and healthy) at 13 months old (harvest 1) from block 3.
3.2.6.3 Nitrogen fixation

A $^{15}$N isotope method was used to measure the proportion of N from N fixation (Ledgard and Peoples, 1988) on plants in the harvest 4 sub-plots (five replicates). KNO$_3$-$^{15}$N was applied on 6 October 2009, at 1 kg N ha$^{-1}$, to 400 mm diameter rings (0.1256 m$^2$) centred over each plant. The isotope was applied in 120 ml of water with a small watering can (Plate 3.3). 250 ml of water was then applied to each ring to wash the solution further into the soil and rinse off isotope adhering to the foliage. The total volume of water applied was equivalent to 3 mm of irrigation.

Plate 3.3. Application of KNO$_3$-$^{15}$N to circular plots over each clover plant in harvest 4 sub-plots on 6 October 2009.

Herbage (grass and clover) was sampled for $^{15}$N analysis on 28 October 2009, 23 November 2009 and 19 January 2010, and DM yield scored for the whole experiment prior to each sampling. The rings were re-centred over each plant, ensuring that the area sampled was that to which the isotope was applied. Electric shears were used to cut the herbage within each ring to stolon level. Each sample was then dissected into tall fescue and clover, oven dried for approximately 15 hours (Crush et al., 2010b) at 80°C, and ground.
Some clover plants scored zero for DM yield, and others were too small for $^{15}$N analysis. The trimmed fescue from each block was bulked to provide a representative companion grass reference sample, by taking 0.2 g from each well-mixed ground sample. Fescue from rings where the clover component was not analysed were not included in bulk samples.

All the samples were analysed by EA-CF-IRMS (Elemental Analyser – Continuous Flow Isotope Ratio Mass Spectrophotometry) (PDZ Europa Ltd., United Kingdom) for atom %$^{15}$N and %N. Analyses were performed by Analytical Services, Faculty of Agriculture and Life Sciences, Lincoln University. The proportion of N derived from fixation was calculated for each clover plant using the following equation based on Ledgard et al. (1985):

\[
\% \text{ clover N fixed} = \frac{\text{grass atm\%} - \text{clover atm\%}}{\text{grass atm\%} - 0.3663} \times 100
\]

where grass atm\% is the atom\% $^{15}$N content of the bulked fescue sample for the corresponding block; and 0.3663 is the standard value used for clover that fixes all of its N, based on the atom\% $^{15}$N of the atmosphere (Rennie and Rennie, 1983).

### 3.2.6.4 Stolon morphology

Stolon morphological measurements were made on 10-18 March, 10-14 August and 26-27 November 2009 on plants in the harvest 4 sub-plots (five replicates). Red clover plants were not included as they do not form true stolons comparable to white clover. For each plant, two well-developed stolons were measured to account for within-plant variability. These were randomly selected from stolon tips which had extended to the margins of each plant, i.e. the longest primary stolons. Stolons were collected and measured block by block.

Stolon tips were excised, sealed in zip lock bags, and stored at 5ºC until measured. The 1st fully expanded leaf (FEL) (relative to the stolon tip) can be used as the basis for measuring stolon characteristics, however the petioles of these young leaves may not yet be fully extended. Therefore, the 2nd FEL was used for measurements in this experiment. Digital callipers were used to measure the length of the internode proximal to the 2nd FEL, as well as stolon diameter (width and height) at the midpoint of this internode. The petiole of the 2nd FEL was then excised and the trifoliolate leaf lamina removed. The length of the petiole was measured with a ruler, and lamina area determined using a leaf area meter (ADT Bioscientific Ltd., Hoddesdon, England). Laminae were then dried for 15 hours (Crush et al., 2010b) at 80ºC and weighed to obtain leaf lamina dry weights (DW). These were subsequently used to calculate specific leaf area (SLA, mm$^2$ mg$^{-1}$).
3.2.6.5 Other measurements

Observations suggest that hybridisation of white clover with *T. uniflorum* can affect flowering characteristics, which could impact the seed production of potential cultivars (K. Widdup, pers. comm.). Therefore, on 13–15 January 2010, measurements of some of these characteristics were made on flowering plants in the harvest 3 and 4 sub-plots (10 replicates). It was not possible to accurately measure the peduncle height of *T. uniflorum in situ*, due to its short length, and therefore this clover type was not included. Plants that were highly fragmented were not measured.

Three inflorescences were chosen at random from each plant, and height of the distal end of the peduncle above the ground was measured. The peduncle and supporting leaf were then excised, and peduncle and petiole lengths were measured. From this, the peduncle:petiole length ratio was calculated, to determine the position of the inflorescences relative to the leaf laminae. The peduncle height:length ratio was also calculated, to estimate the angle of the peduncle relative to the ground. In addition, the height of the inflorescences in relation to the canopy was scored for each plant (Table 3.3). Relative numbers of inflorescences were also scored on 16 January 2009 by assessing their density on all plants (Table 3.3).

Lateral plant spread was monitored by measuring the maximum width of plants in the harvest 3 and 4 sub-plots (10 replicates) on 27 May, 8 July, 17 August and 20 November 2009. Chlorophyll content was measured in harvest 4 sub-plots (5 replicates) on 19 March and 19 November 2009 using a SPAD-502 chlorophyll meter (Konica Minolta Sensing Inc., Osaka, Japan), which measures the optical density of the leaf at two wavelengths. Two central leaflets were measured and averaged for each plant.

In late May 2009, growth habit, stolon density and fungal disease were scored in harvest 3 and 4 sub-plots (10 replicates). Growth habit scores were based on the orientation of the foliage in relation to the ground, and stolon density was based on the density of developed stolons. The fungal diseases present were primarily a combination of rust (*Uromyces trifolii*), pepper spot (*Leptosphaerulina trifolii*) and sooty blotch (*Mycosphaerella killianii*) (Latch and Skipp, 1987). Widespread virus infection was observed in the experiment on 20 November 2009. Virus infection was therefore scored on all plants (excluding those from harvest 1 sub-plots, which had been harvested) by noting the severity of interveinal chlorosis and associated distortion of the leaves (Latch and Skipp, 1987). Details of all score values are given in Table 3.3.
3.2.7 Statistical analysis

The trends in DM scores over time were analysed using a generalised additive modelling (GAM) approach, which produced a series of flexible, clover type-specific spline curves. As the trends appeared similar for all clover types, another GAM approach was used to compare types based on new parallel curves, assuming common trendlines. Overall means were estimated from these parallel trendlines. Where GAM did not detect differences between trendlines, those clover types were compared at each sampling date using analysis of variance (ANOVA) and Tukey’s method, which is appropriate for multiple comparisons and unbalanced data (Milliken and Johnson, 2009). All plants, including those filling gaps and replacing missing entries, were included in the DM analysis.

Nitrogen fixation, flowering, growth habit, density, fungal disease, tap root diameter, tap root DW, and diameter of lateral roots (log-transformed to satisfy normality assumptions) were also analysed using ANOVA and Tukey’s method, to account for multiple comparisons and unbalanced data. The tap root diameter analyses presented exclude red clover, due to its larger size compared with the other clover types. However, a separate ANOVA was conducted to compare red clover diameters with those of the other clovers, and any significant differences are mentioned in the text, along with the red clover means. Back-transformed means are presented for lateral root diameters, with the estimated standard error of the mean (SEM) (back-transformed mean x log SEM). Above-ground fragmentation data was analysed using either the one sample or two sample Wilcoxon test as appropriate, and tap root scores were analysed using the two sample Wilcoxon test (Conover, 1980), as these data were not normally distributed. The Wilcoxon test does not require any normality assumptions. It compares median values, but mean data is presented. The proportion of intact tap roots was analysed with Fisher’s exact test, which is appropriate for comparing ratios of counted values (which are binomially distributed).

Virus scores were converted to either 0 or 1 (absence or presence), due to small numbers of observations for some score values. Data were then analysed either using a binary logistic regression approach or Fisher’s exact test depending on whether comparisons involved zero or non-zero mean values. Stolon morphology and plant spread were analysed using linear mixed modelling (LMM) to take into account the correlation between measurements made on the same plants over time. A generalised linear modelling (GLM) approach was used to analyse the number of lateral roots arising from the top 20 mm of the tap root, in order to account for the expected negative binomial distribution found in count data.
All analyses were carried out using Minitab version 15 (Minitab Inc.), apart from GAM (R version 2.8.1 (R Core Team, 2012)), LMM (SAS version 9.1 (SAS Institute Inc.)) and GLM (R version 2.8.1 (R Core Team, 2012)). The analyses took into account block and clover type effects, plus their interactions where appropriate. Differences in variability among clover types for key parameters were analysed using a test for equal variances in Minitab version 15 (Minitab Inc.), which compares two variances using the F-test or Levene’s test, depending on the normality distribution of the data. Standard deviations are presented to indicate the relative size of the variance for each clover type, while significant differences \( P<0.05 \) between clover types are indicated with lettering.

As well as the general effects of hybridisation (i.e. BC\(_1\) v BC\(_2\) v white clover), there was also interest in determining the performance of individual hybrid families for major characteristics. Therefore, DM yield, stolon density, fungal disease, growth habit and plant spread data were also analysed for hybrid family differences. Only hybrid families whose white clover parental cultivars were part of the experiment were used. This included all BC\(_1\) families, plus the Crusader BC\(_2\) and Kopu II BC\(_2\) families. The performance of each individual plant was determined, relative to the overall mean of its parental cultivar. Unbalanced ANOVA were then performed in Genstat version 11.1 (VSN International Ltd.), using replicates as block effects, to determine whether there were overall differences among entries. For DM yield data, the parental means were calculated over all sampling dates, and the ANOVA was performed on all sampling dates combined.

In graphs and tables, means with the same letter were not significantly different at the 5% level, using the means separation methods stated above for the respective traits. Trends nearing statistical significance \( P=0.05–0.099 \) are noted in the text.

For DM production, lateral spread, fungal disease, stolon density, growth habit, virus infection and flowering measurements, the means for each entry are shown in Appendix 3 and Appendix 4. Where measurements were made at multiple times during the experiment, overall means are given. The performance of hybrid families relative to their respective white clover parental cultivar is shown in Appendix 5.

### 3.3 Results

#### 3.3.1 Dry matter yield

When plotted against mean plant DW, \( R^2 \) values for the polynomial curves indicated that the scoring system gave an accurate representation of clover DM yield (Table 3.4).
Table 3.4. $R^2$ values for polynomial curves fitted to dry matter scores against mean total dry weight of three representative plants of each score value.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$R^2$</td>
<td>0.9849</td>
<td>0.9846</td>
<td>0.9837</td>
<td>0.953</td>
<td>0.9406</td>
<td>0.9591</td>
<td>0.8865</td>
<td>0.9279</td>
</tr>
</tbody>
</table>

Comparison of the estimated overall mean DM scores shows that *T. uniflorum* was smaller ($P<0.001$) than all other types, while white clover was larger ($P<0.001$) (Figure 3.3). Dry matter production of BC$_1$, BC$_2$ and red clover did not differ to each other overall, but showed some differences when compared in January and June 2009 (Table 3.5). The overall difference between *T. uniflorum* and the other clover types was consistent for all individual sampling dates, but some of the clover type differences compared with white clover disappeared later in the experiment (Table 3.5). In particular, DM yield scores of the BC$_1$ and BC$_2$ generations did not differ to white clover at the end of the experiment in May 2010.

![Figure 3.3](image)

Figure 3.3. Overall mean dry matter scores ($\pm$SEM) of the five clover types (see Table 3.3 for details of the scoring system), estimated from fitted trendlines. Means with the same letter show no significant differences at the 5% level.
Table 3.5. Mean dry matter scores (±SEM) of the five clover types at each sampling date. Means with the same letter within sampling dates show no significant differences at the 5% level.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>T. uniflorum</td>
<td>1.65\textsuperscript{a}</td>
<td>1.38\textsuperscript{a}</td>
<td>2.28\textsuperscript{a}</td>
<td>1.53\textsuperscript{a}</td>
<td>1.63\textsuperscript{a}</td>
<td>1.33\textsuperscript{a}</td>
<td>0.97\textsuperscript{a}</td>
<td>0.30\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>±0.202</td>
<td>±0.209</td>
<td>±0.213</td>
<td>±0.238</td>
<td>±0.255</td>
<td>±0.322</td>
<td>±0.359</td>
<td>±0.334</td>
<td>±0.576</td>
</tr>
<tr>
<td>BC\textsubscript{1}</td>
<td>5.08\textsuperscript{b}</td>
<td>4.92\textsuperscript{b}</td>
<td>4.56\textsuperscript{b}</td>
<td>4.39\textsuperscript{b}</td>
<td>4.12\textsuperscript{b}</td>
<td>4.37\textsuperscript{b}</td>
<td>4.08\textsuperscript{b}</td>
<td>3.67\textsuperscript{b}</td>
<td>2.33\textsuperscript{bc}</td>
</tr>
<tr>
<td></td>
<td>±0.088</td>
<td>±0.098</td>
<td>±0.108</td>
<td>±0.118</td>
<td>±0.155</td>
<td>±0.163</td>
<td>±0.148</td>
<td>±0.253</td>
<td></td>
</tr>
<tr>
<td>BC\textsubscript{2}</td>
<td>4.87\textsuperscript{b}</td>
<td>4.64\textsuperscript{b}</td>
<td>4.15\textsuperscript{b}</td>
<td>4.27\textsuperscript{b}</td>
<td>4.35\textsuperscript{b}</td>
<td>4.78\textsuperscript{b}</td>
<td>4.39\textsuperscript{b}</td>
<td>3.68\textsuperscript{b}</td>
<td>2.20\textsuperscript{bc}</td>
</tr>
<tr>
<td></td>
<td>±0.132</td>
<td>±0.124</td>
<td>±0.113</td>
<td>±0.136</td>
<td>±0.153</td>
<td>±0.188</td>
<td>±0.204</td>
<td>±0.170</td>
<td>±0.351</td>
</tr>
<tr>
<td>White clover</td>
<td>5.90\textsuperscript{c}</td>
<td>5.74\textsuperscript{c}</td>
<td>5.07\textsuperscript{d}</td>
<td>5.50\textsuperscript{c}</td>
<td>5.18\textsuperscript{c}</td>
<td>5.63\textsuperscript{c}</td>
<td>5.49\textsuperscript{c}</td>
<td>4.81\textsuperscript{c}</td>
<td>3.01\textsuperscript{c}</td>
</tr>
<tr>
<td></td>
<td>±0.089</td>
<td>±0.094</td>
<td>±0.107</td>
<td>±0.114</td>
<td>±0.144</td>
<td>±0.161</td>
<td>±0.150</td>
<td>±0.287</td>
<td></td>
</tr>
<tr>
<td>Red clover</td>
<td>8.20\textsuperscript{e}</td>
<td>4.55\textsuperscript{b}</td>
<td>3.70\textsuperscript{b}</td>
<td>3.65\textsuperscript{b}</td>
<td>3.90\textsuperscript{b}</td>
<td>4.00\textsuperscript{b}</td>
<td>4.27\textsuperscript{bc}</td>
<td>3.53\textsuperscript{bc}</td>
<td>0.60\textsuperscript{ab}</td>
</tr>
<tr>
<td></td>
<td>±0.213</td>
<td>±0.312</td>
<td>±0.263</td>
<td>±0.254</td>
<td>±0.307</td>
<td>±0.378</td>
<td>±0.483</td>
<td>±0.786</td>
<td>±0.600</td>
</tr>
</tbody>
</table>

Red clover DM scores were more variable than those of the other four clover types ($P<0.001$), and $T. uniflorum$ was less variable ($P<0.001$) (Table 3.6). Of the remaining clover types, the variability of BC\textsubscript{1} did not differ to BC\textsubscript{2} and white clover, but BC\textsubscript{2} was more variable than white clover ($P=0.031$).

Table 3.6. Standard deviations for overall mean dry matter scores of the five clover types, derived from the original data. Clover types with the same letter show no significant differences in variability at the 5% level.

<table>
<thead>
<tr>
<th>Clover type</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. uniflorum</td>
<td>1.02\textsuperscript{a}</td>
</tr>
<tr>
<td>BC\textsubscript{1}</td>
<td>1.70\textsuperscript{bc}</td>
</tr>
<tr>
<td>BC\textsubscript{2}</td>
<td>1.75\textsuperscript{c}</td>
</tr>
<tr>
<td>White clover</td>
<td>1.65\textsuperscript{b}</td>
</tr>
<tr>
<td>Red clover</td>
<td>2.26\textsuperscript{d}</td>
</tr>
</tbody>
</table>
Relative dry matter

The relative performance of hybrid families was determined by comparing the DM score of each family with that of their respective white clover parental cultivar. Although the relative performance of all families was below their parental cultivars, there were significant differences among families ($P<0.001$) (Figure 3.4). The Kopu II BC$_1$ families had the highest relative DM, while Crusader BC$_1$ (entry 7) and Barblanca BC$_1$ had the lowest.

![Graph showing DM scores of hybrid families](image)

**Figure 3.4.** Overall mean relative dry matter production of hybrid families, compared with their white clover parental cultivar means ($\pm$SEM). Means with the same letter show no significant differences at the 5% level. The dashed line represents the parental mean (100%).

The DM scores of all clover types decreased over time (Figure 3.5), and in most cases the rate of decrease was higher later in the experiment. White clover and BC$_2$ had a similar pattern, with an increase in DM yield through winter and early spring 2009. Red clover also increased DM production through winter and spring but showed much greater decreases in DM before and after these times than white clover and BC$_2$ (Figure 3.5).

The DM scores of *T. uniflorum* also increased in winter but this began earlier and reached a peak in June, compared with October/November for BC$_2$, white clover and red clover (Figure 3.5). Unlike the other four clover types, which had their highest scores at the start of the experiment (January 2009), June represented the peak of DM production for *T. uniflorum*. In contrast to the other types, BC$_1$ had a continuous decrease in DM, with no increase in winter/spring (Figure 3.5).
However, when converted to total mean plant DW using calibration cuts at each sampling date, the growth patterns of BC1, BC2, white clover and red clover were similar over time (Figure 3.6).

**Figure 3.6. Estimated total mean plant dry weight of the five clover types over time. Values are derived from polynomial curves fitted to plots of dry matter scores versus mean plant dry weight of three representative plants of each score value.**

### 3.3.2 Tap root measurements

#### 3.3.2.1 Tap root condition

The tap root scores of BC1, BC2, and white clover increased (i.e. became more fragmented) from the first sampling date, while those of *T. uniflorum* remained constant until the final harvest (Figure 3.7). The condition scores of *T. uniflorum* were lower than those of BC1, BC2 and white clover on all occasions (i.e. less deterioration). This was significant for 16 (January 2010) and 19–20 (April/May 2010) month old plants (*P*<0.05), with a similar trend at 13 months when compared with white clover (*P*=0.069). Deterioration of the tap root was lower for BC1 at 13 (*P*=0.017) and 19-20 months old (*P*<0.001) compared with white clover, and at the final harvest (19–20 month old plants) (*P*<0.009) when compared with BC2. The BC2 generation and white clover did not differ at any time, but showed a trend for less deterioration for BC2 at 13 months old (*P*=0.088).
3.3.2 Tap root survival

Tap root survival of the *T. uniflorum* parent and BC1 was higher than that of white clover and BC2 (Figure 3.8). At 13 months old, 50% of *T. uniflorum* tap roots were still intact compared with just 11% for white clover (*P*=0.012) and 13% for BC2 (*P*=0.029). Survival of BC1 tap roots (31%) was not different to *T. uniflorum* at that time, but was higher (*P*=0.032) than white clover (11%).

No healthy intact tap roots of white clover or BC2 were found after the first sampling date (13 months old), but some *T. uniflorum* and BC1 tap roots were still present (Figure 3.8). At 16 months old, 60% of *T. uniflorum* tap roots and 10% of BC1 tap roots were still intact. 30% of *T. uniflorum* tap roots were still intact at 19–20 months old, and 2% of BC1 tap roots. Tap root survival of *T. uniflorum* was higher than BC1 at both 16 (*P*=0.003) and 19–20 months old (*P*<0.001). At 16 months old, there were trends towards higher survival for BC1 compared to white clover.
with BC₂ ($P=0.074$) and white clover ($P=0.061$). Survival of red clover tap roots did not differ to BC₁ at any time, and was different to *T. uniflorum* only at 16 months old ($P=0.044$).

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Clover type**

Figure 3.8. Proportions of intact healthy tap roots (condition scores of 1) for the five clover types in 13 (A), 16 (B) and 19–20 (C) month old plants. Values with the same letter within each sampling date show no significant differences at the 5% level.
### 3.3.2.3 Tap root diameter

In most instances, tap root diameter decreased in the order of: \( T. \text{uniflorum} > \text{BC}_1 > \text{BC}_2 > \) white clover, apart from the very base of the tap root at the crown of the plant, where the diameter of \( \text{BC}_1 \) was the largest (Figure 3.9). Differences between the \( T. \text{uniflorum} \) and white clover parents were not significant near the top of the tap root but white clover was 43\% smaller \((P=0.001)\) at 20 mm (Figure 3.9C) and 46\% smaller \((P=0.001)\) at the beginning of the primary root (below the basal thickening at the top of the tap root) (Figure 3.9D). This indicates that its tap root tapers more rapidly than \( T. \text{uniflorum} \). The intermediate diameters of the two hybrids were also approximately 30\% smaller \((P<0.05)\) than \( T. \text{uniflorum} \) at 20 mm (Figure 3.9C) and 22–25\% smaller \((P<0.05)\) at the beginning of the primary root (Figure 3.9D). Tap root diameter of \( \text{BC}_2 \) did not differ to white clover at any point, but that of \( \text{BC}_1 \) was 37\% greater \((P=0.026)\) than that of white clover at the base of primary tap root (Figure 3.9D).

Tap root diameter of red clover was 18.9 mm at the base, 12.4 mm at 10 mm, 9.3 mm at 20 mm, and 11.3 mm at the beginning of the primary tap root. Although these measurements were thicker than those for \( T. \text{uniflorum} \), when included in the analysis the differences were only significant \((P=0.035)\) at the base of the tap root (+52\%) (data not shown). Red clover tap root diameter was also greater than the diameters of \( \text{BC}_2 \) \((P=0.018)\) and white clover \((P=0.008)\) at the base of the tap root; greater than \( \text{BC}_1 \) \((P=0.033)\) and white clover \((P=0.011)\) at 20 mm; and greater than \( \text{BC}_1 \) \((P=0.022)\), \( \text{BC}_2 \) \((P=0.035)\) and white clover \((P=0.001)\) at the beginning of the primary tap root (data not shown).
Figure 3.9. Mean diameter of intact tap roots (tap root condition scores of 1) (±SEM) of *T. uniflorum*, BC1, BC2 and white clover in 13 month old plants (October 2009). Measurements were made at the base (A), 10 mm from the base (B), 20 mm from the base (C), and at the start of the primary root (D). Means with the same letters show no significant differences at the 5% level.
3.3.3 Nitrogen fixation

The proportion of N in the shoots derived from N fixation did not differ among the clover types in spring (October and November 2009) (Figure 3.10), but in summer (January 2010) it was lower ($P=0.027$) in BC1 (91%) than in white clover (95%). In January 2010, the proportion of N from fixation in *T. uniflorum* (73%) was also lower ($P<0.05$) than in the other clover types (>90%). At that time the sample size of *T. uniflorum* was only two plants, and the standard error was relatively large compared with the hybrids and white clover.

![Figure 3.10. Mean proportion of N from fixation in shoots (±SEM) for the five clover types, sampled in October 2009, November 2009 and January 2010. Means with the same letter within sampling dates show no significant differences at the 5% level.](image)

Despite these differences in the proportion of N from fixation, the only difference among clover types in the %N content of the shoots (Figure 3.11) at any sampling time was for October 2009 when %N of *T. uniflorum* (3.61%) was lower ($P=0.04$) than that of white clover (4.12%). At that time, there was also a trend for a lower %N content for *T. uniflorum* than for BC2 (4.09%) ($P=0.078$).
3.3.4 Stolon morphology

3.3.4.1 *T. uniflorum*

The stolon morphology of *T. uniflorum* differed considerably to the other three clover types measured (Figure 3.12). Petiole length, leaf lamina area and SLA of *T. uniflorum* were all smaller than those of BC₁, BC₂ and white clover. For example, petiole length was approximately 65% shorter in March ($P<0.001$), 35–39% shorter in August ($P<0.006$), and 45–58% shorter in November ($P<0.018$) (Figure 3.12D), compared with the other clover types. *T. uniflorum* leaf lamina area was also much smaller than the hybrids and white clover, by 90–93% in March ($P<0.001$), 72–77% in August ($P<0.003$) and 80–86% in November ($P<0.001$) (Figure 3.12E).

Internode length of *T. uniflorum* was also shorter than BC₂ and white clover, by 95% in March ($P<0.001$), 58–64% in August ($P<0.018$), and 81–85% in November ($P<0.023$) (Figure 3.12A). Compared with BC₁, internode length of *T. uniflorum* was 94% shorter in March ($P<0.001$) and 60% shorter in August ($P=0.01$). Stolon height:width ratios of *T. uniflorum* were smaller than BC₁ and white clover ($P<0.06$) (Figure 3.12C), and also smaller than those of BC₂ in March ($P<0.001$) and August ($P=0.012$). All clover types had ratios less than 1, meaning the stolons were wider than they were high, but this transverse flattening was greater in *T. uniflorum*. 

Figure 3.11. Mean %N content (±SEM) of shoots for the five clover types sampled in October 2009, November 2009 and January 2010. Means with the same letter within sampling dates show no significant differences at the 5% level.
3.3.4.2 BC2 compared with white clover and BC1

Stolon morphological characteristics of the BC2 generation did not differ to those of white clover (Figure 3.12), except for leaf lamina area in November, which was 17% higher in white clover ($P=0.029$). The BC2 generation also had few differences in stolon morphology compared with BC1 (Figure 3.12).

3.3.4.3 BC1 and white clover

There were a greater number of differences in stolon morphology between BC1 and white clover than between BC2 and white clover (Figure 3.12), all in March and November. White clover leaf lamina area was 39% higher than BC1 in March ($P=0.002$) and 69% higher in November ($P<0.001$) (Figure 3.12E), while SLA was 9% higher than BC1 in March ($P=0.011$) and 7% higher in November ($P=0.003$) (Figure 3.12F). The internode length of white clover was 34% higher than that of the BC1 generation in March ($P=0.005$) and 82% higher in November ($P<0.001$) (Figure 3.12A), and petiole length was also 30% higher than BC1 in November ($P=0.001$) (Figure 3.12D).
3.3.4.4 Variability of T. uniflorum

*T. uniflorum* was less variable than the other clover types for internode length (*P*<0.002) and leaf lamina area (*P*<0.001), at all sampling dates (Table 3.7). Petiole length of *T. uniflorum* also tended to be less variable than BC1 (*P*=0.03), BC2 (*P*=0.079) and white clover (*P*=0.01) in March 2009, but did not differ to the other clover types in August and November. The stolon height:width ratio of *T. uniflorum* tended to be more variable than BC1 and BC2 (*P*=0.035), and white clover (*P*=0.077) in March, and was also more variable than the other clover types in August (*P*<0.001). Stolon diameter of *T. uniflorum* tended to be less variable than BC2 in August (*P*=0.04) and November (*P*=0.09), and was less variable than white clover in November (*P*=0.046). In March, variability for SLA tended to be higher for *T. uniflorum* than for BC1 (*P*=0.036), BC2 (*P*=0.063) and white clover (*P*=0.069). In contrast, in August, SLA of *T. uniflorum* tended to be less variable than BC1 (*P*=0.003), BC2 (*P*=0.065) and white clover (*P*=0.037).

3.3.4.5 Variability of BC2 compared with white clover and BC1

As with the means, BC2 and white clover had no differences in variability for any stolon morphological parameters (although the stolon diameter of BC2 tended to more variable (*P*=0.053) in August), but some differences occurred between BC2 and BC1 (Table 3.7). Compared with BC1, the BC2 generation had a higher variability for leaf lamina area (*P*<0.009) and stolon diameter (*P*=0.002), and internode length was also more variable in March (*P*<0.001) and November (*P*=0.001). Specific leaf area of BC2 tended to be less variable than BC1 in August (*P*=0.015) and November (*P*=0.095).

3.3.4.6 Variability of BC1 and white clover

The variability for leaf lamina area in white clover was higher than in BC1 in all months (*P*<0.008), as was that for internode length in March and November (*P*<0.001), and for stolon diameter in March (*P*=0.002) and November (*P*<0.001) (Table 3.7). In contrast, the BC1 generation was more variable than white clover in August (*P*=0.019) and November (*P*=0.03) for SLA. The variability of petiole length and stolon height:width did not differ among BC1, BC2 or white clover.
Table 3.7. Standard deviations for stolon morphological characteristics of *T. uniflorum*, BC1, BC2 and white clover, measured in March, August and November 2009. Clover types with the same letter, within sampling dates, show no significant differences in variability at the 5% level.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Clover type</th>
<th>March 2009</th>
<th>August 2009</th>
<th>November 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internode length (mm)</td>
<td><em>T. uniflorum</em></td>
<td>0.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.56&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;1&lt;/sub&gt;</td>
<td>7.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;2&lt;/sub&gt;</td>
<td>12.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>10.86&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.53&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stolon diameter (mm)</td>
<td><em>T. uniflorum</em></td>
<td>0.36&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.20&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.35&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.42&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.38&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stolon height:width</td>
<td><em>T. uniflorum</em></td>
<td>0.068&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.096&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.061&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.045&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.042&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.061&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.047&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.047&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.055&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>0.051&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.046&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.054&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Petiole length (mm)</td>
<td><em>T. uniflorum</em></td>
<td>19.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;1&lt;/sub&gt;</td>
<td>30.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;2&lt;/sub&gt;</td>
<td>27.80&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>15.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>33.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leaf lamina area (mm²)</td>
<td><em>T. uniflorum</em></td>
<td>38.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;1&lt;/sub&gt;</td>
<td>396.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>193.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>175.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;2&lt;/sub&gt;</td>
<td>589.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>269.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>250.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>585.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>263.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>271.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Specific leaf area (mm² mg⁻¹)</td>
<td><em>T. uniflorum</em></td>
<td>5.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.86&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;1&lt;/sub&gt;</td>
<td>4.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.95&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BC&lt;sub&gt;2&lt;/sub&gt;</td>
<td>4.22&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.84&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.24&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>4.32&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
3.3.5 Flowering

When assessed early in the experiment, the mean flowering density score of BC1 was lower ($P<0.003$) than all other clover types, and that of white clover was also lower ($P=0.001$) than BC2 (Figure 3.13). There was also a trend for a lower flowering score ($P=0.066$) for white clover compared with red clover. The flowering scores of *T. uniflorum* were more variable than BC1 ($P=0.009$), BC2 ($P=0.007$) and white clover ($P=0.003$) (Appendix 6).

![Flowering scores chart](chart.jpg)

**Figure 3.13.** Mean flowering scores (±SEM) of the five clover types in January 2009 (see Table 3.3 for details of the scoring system). Means with the same letter show no significant differences at the 5% level.

The position of white clover inflorescences in relation to the canopy was higher than that of BC1 ($P<0.001$) and BC2 ($P<0.001$) (Figure 3.14), and in BC2 it was higher than in BC1 ($P<0.001$). The variability did not differ among the three clover types (Appendix 6).
There were significant effects of hybridisation on the peduncle characteristics measured. Peduncle height and peduncle length differed among all three clover types ($P<0.001$) and were smallest in BC$_1$ and biggest in white clover (Figure 3.15). Compared with white clover, the peduncle height of BC$_1$ was 60% shorter and that of BC$_2$ was 38% shorter, while peduncle length was 54% shorter in BC$_1$ and 33% shorter in BC$_2$.

**Figure 3.14.** Mean scores (±SEM) for inflorescence height relative to the canopy, for *T. uniflorum*, BC$_1$, BC$_2$ and white clover in January 2010 (see Table 3.3 for details of the scoring system). Means with the same letter show no significant differences at the 5% level.

**Figure 3.15.** Mean (±SEM) peduncle height and peduncle length for BC$_1$, BC$_2$ and white clover in January 2010. Means with the same letter within parameters show no significant differences at the 5% level.
The peduncle:petiole ratio also differed among the clover types ($P<0.0001$). This was highest in white clover and smallest in BC$_1$ (Figure 3.16). The peduncles were 26% longer than the subtending petiole in the BC$_1$ generation and 79% longer in the BC$_2$ generation, whereas the peduncles of white clover were more than twice the length of the subtending petiole. Clover types also differed for the peduncle height:length ratio ($P<0.001$). Compared with white clover, this ratio was 13% lower in BC$_1$ ($P<0.0001$) and 9% lower in BC$_2$ ($P=0.015$) (Figure 3.16), indicating that the angle of the peduncle was lower for the hybrids. Although the angle became more erect with subsequent backcrossing, the difference between the BC$_1$ and BC$_2$ generations was not significant.

![Figure 3.16. Mean (±SEM) peduncle:petiole ratio and peduncle height:length ratio for BC$_1$, BC$_2$ and white clover in January 2010. Means with the same letter within parameters show no significant differences at the 5% level.](image)

Variability of all clover types differed for peduncle height ($P<0.033$), with white clover being the most variable and BC$_1$ the least variable (Table 3.8). The BC$_1$ generation was also less variable than white clover for peduncle length ($P=0.001$), and less variable than both white clover ($P<0.001$) and BC$_2$ ($P=0.048$) for peduncle:petiole ratio. However, the BC$_1$ generation was more variable than both the BC$_2$ generation ($P=0.001$) and white clover ($P=0.004$) for peduncle height:length ratio.
Table 3.8. Standard deviations for peduncle height, peduncle length, peduncle:petiole ratio and peduncle height:length ratio of BC1, BC2 and white clover in January 2010. Clover types with the same letter, within parameters, show no significant differences in variability at the 5% level.

<table>
<thead>
<tr>
<th>Clover type</th>
<th>Peduncle height</th>
<th>Peduncle length</th>
<th>Peduncle:petiole</th>
<th>Peduncle height:length</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC1</td>
<td>22.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.561&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.259&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC2</td>
<td>28.98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.62&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.659&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.195&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>White clover</td>
<td>34.42&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.744&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.210&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

### 3.3.6 Other characteristics

#### 3.3.6.1 Lateral spread

The lateral spread of *T. uniflorum* was smaller (*P*<0.001) than both the hybrids and white clover at all sampling dates (Figure 3.17). *T. uniflorum* was 73–77% smaller than BC1, 71–77% smaller than BC2, and 75–83% smaller than white clover. Lateral spread of red clover was also smaller than white clover by 57% in May (*P*=0.019), 37% in July (*P*<0.001), 35% in August (*P*<0.001) and 32% in November (*P*<0.001) (Figure 3.17). There were no differences in the lateral spread of BC1, BC2 and white clover in May, July and August, but white clover had a wider (*P*<0.001) spread than the hybrids at the last measurement in November (Figure 3.17).

![Figure 3.17. Mean maximum lateral plant spread (±SEM) of the five clover types in May, July, August and November 2009. Means with the same letter within sampling dates show no significant differences at the 5% level.](image-url)
The BC₁ and BC₂ generations showed no difference in the variability of lateral spread, but all other clover type comparisons were significant (\(P<0.002\)) (Table 3.9). Variability was lowest in *T. uniflorum*, with the largest variation occurring in white clover.

**Table 3.9. Standard deviations for maximum lateral spread of the five clover types, calculated from combined data from all sampling dates. Clover types with the same letter show no significant differences in variability at the 5% level.**

<table>
<thead>
<tr>
<th>Clover type</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. uniflorum</em></td>
<td>5.57\textsuperscript{a}</td>
</tr>
<tr>
<td>BC₁</td>
<td>15.08\textsuperscript{c}</td>
</tr>
<tr>
<td>BC₂</td>
<td>15.45\textsuperscript{c}</td>
</tr>
<tr>
<td>White clover</td>
<td>19.34\textsuperscript{d}</td>
</tr>
<tr>
<td>Red clover</td>
<td>8.55\textsuperscript{b}</td>
</tr>
</tbody>
</table>

**Relative lateral spread**

The overall mean spread of individual clover entries covered a similar range of values for BC₁, BC₂ and white clover (Appendix 3). However, the lateral spread of hybrid families relative to their parental cultivars differed significantly (\(P<0.05\)) (Figure 3.18). In general, the spread of some hybrid families was just over half that of their white clover parent, while others had similar or slightly wider spread than their white clover parent. Relative performance ranged from 51–108% in May, 51–99% in July, 55–106% in August, and 61–139% in November.

Kopu II BC₁ and BC₂ entries usually had the highest relative lateral spread, and Crusader BC₁ and BC₂ entries often had the lowest relative lateral spread (Figure 3.18). Tribute BC₁ was also one of the lowest performing entries in August and November (Figure 3.18C and D).
Figure 3.18. Mean relative lateral spread (±SEM) of hybrid families, compared with their white clover parental cultivar means in May (A) and July (B) 2009. The dashed line represents the parental mean (100%).
Figure 3.18 continued. Mean relative lateral spread (±SEM) of hybrid families, compared with their white clover parental cultivar means in August (C) and November (D) 2009. The dashed line represents the parental mean (100%).

### 3.3.6.2 Growth habit, stolon density, fungal disease, and virus infection

**Growth habit**

*T. uniflorum* was more prostrate (i.e. lower mean growth habit score) than all the other clover types (*P*<0.001), and BC₁ was also more prostrate than white clover (*P*=0.034) (Figure 3.19). Variability for growth habit scores was lowest for BC₁, particularly compared with red clover (*P*=0.029) and BC₂ (*P*=0.048) which had the highest standard deviations (Table 3.10).
Figure 3.19. Mean growth habit scores (±SEM) (prostrate to erect) of the five clover types in May 2009 (see Table 3.3 for details of the scoring system). Means with the same letter show no significant differences at the 5% level.

Table 3.10. Standard deviations for growth habit, stolon density and fungal disease scores in May 2009, and virus scores in November 2009, for the five clover types. Clover types with the same letter show no significant differences in variability at the 5% level.

<table>
<thead>
<tr>
<th>Clover type</th>
<th>Growth habit</th>
<th>Stolon density</th>
<th>Fungal disease</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. uniflorum</td>
<td>0.74&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.42&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.46&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>White clover</td>
<td>0.72&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.73&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Red clover</td>
<td>0.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Relative growth habit
Among individual families, some hybrids had maintained the more erect growth habit of white clover (Appendix 3), particularly entry 8 (Kopu II BC<sub>1</sub>) and entry 11 (Tribute BC<sub>1</sub>). As with growth and spread, there were differences among families in their performance relative to the parental cultivars ($P<0.001$) (Figure 3.20). The Tribute family was the only BC<sub>1</sub> to increase mean growth habit score relative to its parent (114%), while the Trophy BC<sub>1</sub> family was the most prostrate. Trophy BC<sub>1</sub> was 30% lower (i.e. more prostrate) than the Trophy cultivar.
Figure 3.20. Mean relative growth habit (erectness) (±SEM) of hybrid families, compared with their white clover parental cultivar means. The dashed line represents the parental mean (100%).

Stolon density
Stolon density was highest for *T. uniflorum* and lowest for BC₂ (*P*=0.027), otherwise there were no differences among clover types (Figure 3.21). The variability of clover types did not differ (Table 3.10).

Figure 3.21. Mean stolon density scores (±SEM) of the five clover types in May 2009 (see Table 3.3 for details of the scoring system). Means with the same letter show no significant differences at the 5% level.
Relative stolon density

There was a wide range in the relative performance of hybrid families for stolon density ($P=0.098$) (Figure 3.22). The family with the highest relative stolon density was Tribute BC$_1$, with a 43% increase in mean density score compared with the Tribute cultivar. Although the Barblanca cultivar (entry 26) had the highest mean stolon density score out of all the white clover parents (Appendix 3), Barblanca BC$_1$ had the lowest relative density (79%).

![Figure 3.22. Mean relative stolon density (±SEM) of hybrid families, compared with their white clover parental cultivar means. The dashed line represents the parental mean (100%).](image)

Fungal disease

Mean fungal disease scores of *T. uniflorum* and red clover were lower than those of white clover and the hybrids ($P<0.001$), while BC$_1$ had higher scores than BC$_2$ ($P=0.026$), white clover ($P=0.018$) and red clover ($P<0.001$) (Figure 3.23). Variability for fungal disease scores was also lower for *T. uniflorum* ($P<0.024$) and red clover ($P=0.001$) than for the other clover types (Table 3.10), with *T. uniflorum* tending to be more variable than red clover ($P=0.066$).
Figure 3.23. Mean fungal disease scores (±SEM) of the five clover types in May 2009 (see Table 3.3 for details of the scoring system). Means with the same letter show no significant differences at the 5% level.

Relative fungal disease
The mean fungal disease scores of most BC₁ families were within the same range as the BC₂ families and white clover cultivars (Appendix 3), but the relative fungal disease of hybrid families ranged from 13% lower (less disease) to 92% higher (more disease) than the parental cultivars ($P<0.001$) (Figure 3.24).

Figure 3.24. Mean relative fungal disease (±SEM) of hybrid families, compared with their white clover parental cultivar means. The dashed line represents the parental mean (100%).
As with stolon density, the most affected hybrid family was Barblanca BC\textsubscript{1} (192%), although the Barblanca cultivar (entry 26) again had the lowest mean fungal disease score out of the white clover parents (Appendix 3). The Sustain BC\textsubscript{1}, Tribute BC\textsubscript{1} and Trophy BC\textsubscript{1} families had the lowest relative fungal disease.

**Virus infection**

No virus infection was observed for *T. uniflorum* and red clover, and the mean virus score of *T. uniflorum* was lower than that of white clover (*P*=0.046) (Figure 3.25). Means comparisons with white clover gave different results for *T. uniflorum* and red clover, due to different sample sizes. There were no differences in mean virus score among BC\textsubscript{1}, BC\textsubscript{2} and white clover, but the variability of white clover was higher than that of BC\textsubscript{1} (*P*=0.028) (Table 3.10). White clover, BC\textsubscript{1} and BC\textsubscript{2} were also more variable than *T. uniflorum* and red clover (*P*<0.001).

![Figure 3.25. Mean virus scores (±SEM) of the five clover types in November 2009 (see Table 3.3 for details of the scoring system). Means with the same letter show no significant differences at the 5% level.](image)

**3.4 Discussion**

**3.4.1 Dry matter yield**

Dry matter production of the hybrids was expected to be intermediate to the two parents and this hypothesis was proven for the BC\textsubscript{1} generation, but a second generation of backcrossing did not further increase DM production as expected. This was apparent both across the broad clover types (BC\textsubscript{1} and BC\textsubscript{2}) (Figure 3.3) and also for families for which both backcross
generations were present (Crusader and Kopu II hybrids) (Figure 3.4). In contrast, DM production of white clover backcross hybrids with *T. ambiguum* and *T. nigrescens* has generally been found to be similar to that of white clover in the field (Marshall *et al.*, 2003b; Marshall *et al.*, 2005; Marshall *et al.*, 2002b). However, in glasshouse experiments, total shoot DW (stolon + petiole + leaf) of both BC1 and BC2 *T. repens* x *T. nigrescens* hybrids was lower than that of white clover (Marshall *et al.*, 1998).

The difference in DM yield between the hybrids and white clover in the current experiment was not as large as expected, being on average equivalent to one size class in the scoring protocol used. This gap could be closed through selection and crossing within generations. Variation among BC1 hybrid families shows that while some could be improved through selection and crossing, other families may be more difficult to improve as they show larger decreases compared with their white clover parental cultivars (Figure 3.4). The hybrid plants in the current experiment represent the first cycle of crossing for this particular material, and no selections have been made for agronomic performance. The variation shown in this experiment suggests that it should be possible to improve DM production through selection of superior performing plants and families. Hybrids between white clover and *T. ambiguum* have shown improved performance for percentage clover content over small-leaved white clover cultivars after several selection cycles (Widdup and Barrett, 2011). In the same experiment, unselected *T. repens* x *T. uniflorum* BC1 entries showed no advantage over white clover cultivars, although white clover was often not significantly different to the hybrids, particularly after two years of growth. Dry matter production of white clover in the current experiment was significantly higher than that of the hybrids at all but the final measurement date, when plants were highly fragmented and dependent on nodal roots (19 months old). Due to tap root harvesting these plants could not be followed on through winter and spring to determine whether there was still an advantage for DM production of white clover over the hybrids, or vice versa, once clonal plants were stabilised. Under sheep grazing with companion grasses, Brock *et al.* (2000) found that it took 2.5 years from sowing to reach a stable white clover clonal population. A range of plant sizes, of varying orders of branching, are present as the plant population undergoes fragmentation.

### 3.4.2 Stolon morphology and growth form

The underlying reason for reduced growth in the hybrids is likely to be related to the presence of intermediate characteristics between the two parental species. Less productive white clover types tend to be prostrate, with smaller leaves, short internodes and high stolon density (Caradus and Williams, 1989; Caradus *et al.*, 1997). Of these characteristics, growth habit,
leaf lamina area and internode length were all hypothesised to be reduced by hybridisation with *T. uniflorum*. Stolon morphology of the BC₁ generation was intermediate to the parents for many characteristics, as predicted (Figure 3.12). While several important characteristics were changed as expected by hybridisation (e.g. shorter internodes and smaller leaf lamina area), others were not. Transversely flattened stolons in the *T. uniflorum* parent were not inherited by the hybrids nor, generally, were their significantly shorter petioles. The reduced leaf lamina area and shorter internodes of the BC₁ generation could contribute to its lower DM production. Lower SLA compared with white clover suggests that productivity is lower in BC₁ types, as less lamina area is produced per unit of investment in dry weight. In addition, SLA is positively correlated with potential relative growth rate (Poorter and De Jong, 1999). Specific leaf area has also been correlated with habitat productivity (Poorter and De Jong, 1999; Poorter and Remkes, 1990). The lower SLA of *T. uniflorum* in the current experiment may, therefore, reflect the productivity of its natural habitat, the influence of which may also be seen in the SLA of the BC₁ generation. This suggests the habitat of *T. uniflorum* is nutrient limited. There is little published information on this, although poor soils have been reported for some locations where seed has been collected (Forest Service of Parnitha, 2012). The inverse measure to SLA is specific leaf mass (SLM), which can be used as a proxy for lamina thickness or density (Garnier and Laurent, 1994; Poorter *et al.*, 2009). As SLA decreases, SLM and therefore lamina thickness or density, increases. While thicker or denser leaves could convey some moisture stress tolerance, they also represent a cost to the plant which could reduce carbon investment in other areas, such as individual leaf lamina area or number.

Unlike DM production, stolon morphology of the second generation of back crosses was similar to that of white clover (Figure 3.12). This could suggest that morphological characteristics may not contribute to the observed reduction in DM production for BC₂ hybrids, but different mechanisms may be responsible in the BC₁ and BC₂ generations due to the varying contribution of genes from the two parents.

Generally, white clover leaf size is inversely correlated with stolon density, with small-leaved cultivars having a higher density (Brock and Hay, 1996). The cultivar Sustain was bred to break this correlation, by increasing stolon density in a medium-large leaved white clover (Caradus *et al.*, 1997). However, Widdup and Barrett (2011) suggested that the stolon density of more recent cultivars has been increased across all leaf size classes. Compared with lower density white clover types, high stolon density improves persistence through maintenance of growing points, and increases productivity under frequent grazing by compensating for decreased leaf size with higher stolon numbers (Brock and Hay, 1996). In the current
experiment, the decreases in leaf lamina area and internode length in the BC₁ generation should theoretically result in a higher stolon density, assuming branching frequency is unchanged. However, qualitative measurements of stolon density showed no difference between the hybrid generations and white clover (Figure 3.21), although individual BC₁ families again showed variability relative to their white clover parents (Figure 3.22). In particular, the increased stolon density score of the Tribute BC₁ hybrid family, compared with the Tribute cultivar, indicates possible transgressive segregation (phenotypes outside the range of the parents) for this trait. Other studies have shown no differences in growing point number or stolon density between a large-leaved white clover cultivar and T. repens hybrids with T. nigrescens, or between a medium-leaved white clover cultivar and T. repens hybrids with T. ambiguum, after 3 years in the field (Marshall et al., 2002b; 2003b). In both instances, earlier measurements did find differences in the number of growing points after two years of growth, with those in white clover being higher than T. ambiguum hybrids and lower than T. nigrescens hybrids. Assessment of T. repens x T. uniflorum plants of a range of ages would therefore be valuable, to determine the potential effect of stolon density on persistence of tap rooted plants under short term stresses, versus the longer term productivity and vegetative persistence of fragmented plants. Another study found that, after 3 years, the differences in stolon density between T. repens x T. nigrescens hybrids and white clover were associated with the leaf size of the white clover cultivars (Marshall et al., 2005). Therefore, as with white clover cultivars, it is important to assess stolon density of interspecific hybrids in association with leaf size.

The current study confirmed previous observations (K. Widdup pers. comm.) that the growth habit of T. repens x T. uniflorum BC₁ hybrids is, on average, more prostrate than white clover (Figure 3.19). This is consistent with the negative white clover relationships between leaf size, growth habit and internode length, and may also have contributed to the lower DM production of the hybrids. Elgersma and Fengrui (1997) also suggested that height and leaf area distribution of white clover cultivars could be responsible for yield differences, based on their ability to intercept light. Plants with a prostrate growth habit would intercept less light than those with a more erect habit, especially in competition with companion grasses. However, there was variability in growth habit among BC₁ families in the current study, with some maintaining the erect growth habit of their white clover parents (Kopu II BC₁ families) while others were very prostrate (e.g. Trophy BC₁) (Figure 3.20). A second generation of backcrossing produced a growth habit that was, on average, more like the white clover parent, but still not significantly different to the BC₁ generation. Growth habit was measured on only one occasion and more frequent measurements would provide confirmation of the
performance of individual families. While the growth habit of white clover is known to be highly plastic – for example, it is more prostrate under grazing and drought (Thomas, 1984; Caradus et al., 1993) – the results of this experiment still reflect the genotypic differences between white clover and the hybrid generations.

Hybridisation generally did not affect the lateral spread of the BC1 and BC2 generations (Figure 3.17). In contrast, Marshall et al. (1998) found that the spread of T. repens x T. nigrescens BC1 and BC2 hybrids, measured by area, was significantly smaller than that of the white clover parent. As with other characteristics, the performance of T. repens x T. uniflorum hybrid families varied for this trait – some were very compact while others maintained the spread of their parental cultivar (Figure 3.18). Maintenance of lateral spread may have contributed to the maintenance of DM production in individual families (e.g. Kopu II BC1 and Sustain BC1), while others may have been influenced by stolon density. For example, Tribute BC1 was very compact but still performed relatively well at maintaining DM production. This may have been achieved through the observed increase in stolon density. Lower DM production of other families may have been influenced by decreases in both spread and stolon density (e.g. Barblanca BC1).

For characteristics where BC1 and white clover differed (internode length, lamina area, SLA), the presence of differences in March (representing late summer growth) and November (spring), but an absence in August (winter), suggests seasonal differences between these clover types in the growth of morphological units (Figure 3.12). Lateral spread and petiole length of white clover also became significantly greater than BC1 in November (Figures 3.17 and 3.12D respectively). White clover appeared to be better able to expand internode length and lamina area during peak growing conditions, which may enable it to more fully reach its growth potential. The less expansive growth of the BC1 generation in spring could also represent an adaptation, from the T. uniflorum parent, for subsequent dry summer conditions.

The relative performance of the BC1, BC2 and white clover types in the current experiment may vary under different conditions, such as grazing regime, soil fertility and temperature. For example, the general morphological type of the BC1 generation (smaller lamina area, shorter internodes and a more prostrate growth habit) may be more tolerant of management regimes which utilise frequent, close grazing. Individual hybrid families, or segregating populations, which are particularly suited to specific conditions could also be developed through recurrent cycles of selection and crossing.
3.4.3 N fixation

The proportion of N from fixation was very high in the current experiment, with levels in white clover and the hybrids of 86-95% from mid-spring to mid-summer, compared with 35-80% for Waikato dairy pastures (Crush et al., 2006). However, the late spring levels of 86-89% were not dissimilar to the late spring-early summer levels of 80% measured in the Waikato. The generally high levels of N fixation indicate that mineral N supply was low. Application of fertiliser was not necessary at establishment, and by the following spring, sampling for N fixation had begun. Mineral N was therefore not added, as this would have reduced the need for clover to fix N. Clover plants were beginning to fragment at this time, so other fertilisers were not applied in order to reduce competition from the tall fescue.

However, in this situation, where plants were highly dependent on fixed N, the N fixation of the hybrids generally did not differ to white clover (Figure 3.10). This supports the hypothesis that hybridisation has not affected the ability of hybrid clovers to fix N. Further studies at lower levels of N fixation, which would occur with application of mineral N, would be valuable to confirm this. In those months with good sample sizes, the N fixation of the *T. uniflorum* parent was also the same as the hybrids and white clover (Figure 3.10). This indicates compatibility of *T. uniflorum* with white clover rhizobia present in New Zealand soils. Plants in the experiment were not inoculated with rhizobia at any time. Lower N fixation by the BC1 generation in summer, compared with white clover, may reflect a faster response to summer dry conditions, inherited from the *T. uniflorum* parent. However, this was only measured once during summer, and further measurements throughout the whole season, plus the months preceding and following it, would be necessary to identify any inherent differences among clover types in seasonal fixation. Although the mean fixation by *T. uniflorum* was much lower than of that the hybrids and white clover in summer (January 2010), this result should be treated with caution as only two samples from *T. uniflorum* could be measured at that time. Despite these lower levels of N fixation, %N content of the *T. uniflorum* parent and BC1 did not differ to white clover at this time (Figure 3.11). In fact, the %N content of BC1, BC2 and white clover did not differ at any time.

The %N content of all clover types was below optimal levels of at least 4.5% for white clover growth (McNaught, 1970) throughout the measurement period, and decreased over time. This probably reflects the lack of mineral N, with plants being unable to supply all their N needs from fixation. However, the critical N level of *T. uniflorum* is unknown, as is the effect of this on the critical N level of white clover hybrids. Therefore, it cannot be determined whether the %N content of *T. uniflorum* and *T. repens* x *T. uniflorum* hybrids was actually inadequate for
growth. However, results in Chapter 4 suggest the *T. uniflorum* parent and BC₁ hybrids are able to grow well compared with white clover at very low %N levels.

### 3.4.4 Tap root survival

Results confirmed the hypothesis that tap root survival of *T. uniflorum* is higher than that of white clover (Figures 3.7 and 3.8). Survival of BC₁ tap roots was also greater than that of white clover, but this trait was lost in the second backcross generation (Figures 3.7 and 3.8). Tap root death in white clover generally occurs 12–18 months after establishment (Westbrooks and Tesar, 1955; Brock and Tilbrook, 2000), but has been recorded as early as six months and as late as two years after sowing (Brock *et al.*, 2000). In the current study, all white clover tap roots had disintegrated by 16 months old, but *T. uniflorum* and BC₁ tap roots were still present at the end of the experiment (19-20 months). The proportion of *T. uniflorum* tap roots that were still intact at that time (30%) suggests they would have survived for at least several months more.

Although the proportion of surviving BC₁ tap roots at the end of the experiment was small, survival was much higher in 13 and 16 month old plants (Figure 3.8). Most importantly, there were differences between BC₁ and white clover at these times. As mentioned previously, the hybrid plants in the current experiment are from early crosses in the hybridisation program, and no selections have been made for rooting characteristics. If the existing difference between BC₁ hybrids and white clover could be improved further by increasing the tap root survival at 19 months old and later, valuable impacts on persistence and production could be achieved. It appears there is scope for such selection, through crossing individuals with increased tap root survival at 13–16 months old to increase the frequency of the genes responsible. In addition, although the proportion of intact, healthy tap roots of BC₁ did not differ significantly to BC₂ and white clover at 19 months old (Figure 3.8), the tap root condition scores show that fragmentation of BC₁ tap roots was still lower at this time (Figure 3.7). In white clover, response to selection for root characteristics is high (Woodfield and Caradus, 1990; Caradus and Woodfield, 1998). For example, Caradus and Woodfield (1998) found that the diameter of the seedling tap root increased by 2.4% per breeding cycle. To establish a breeding population, higher replication than that used in the current experiment would be necessary to obtain a sufficient number of plants with the target characteristics.

Numerous studies have found correlations between leaf size and root diameter in white clover (Brock and Tilbrook, 2000; Caradus and Woodfield, 1986). In particular, Brock and Tilbrook (2000) found that leaf size was positively associated with tap root diameter, and that loss of
the tap root was faster in small-leaved cultivars. It appears the inverse may be the case in *T. repens* x *T. uniflorum* hybrids as, on average, leaf lamina area of BC$_1$ hybrids was smaller than that of white clover, but tap root survival was higher. Several explanations were suggested by Brock and Tilbrook (2000) for the higher rate of tap root death in small-leaved cultivars compared with large-leaved cultivars – either stolon characteristics which enabled small-leaved cultivars to be independent of the tap root, or a stronger tap root with a prolonged life span in large-leaved cultivars. Further studies would be necessary to determine the interaction between leaf size, tap root diameter and tap root death in *T. repens* x *T. uniflorum* hybrids, which could differ to the relationships seen in white clover.

It was hypothesised that increased tap root diameter could contribute to a longer life span in the hybrid tap root, but this did not appear to be a strong contributing factor (Figure 3.9). Although white clover tap root diameter tended to be smaller than both hybrids, it only differed statistically to BC$_1$ at the beginning of the primary tap root. However, having a greater diameter away from the tap root base may have played a part in the higher survival of *T. uniflorum* compared with BC$_2$ and white clover. For example, most tap root diameter measurements were higher for red clover than white clover, as was tap root survival at that time (13 months old). The tap root of *T. uniflorum* is relatively woody in nature (Dymock *et al.*, 1989), which may play a part in increased survival through mechanical resistance to decay. Comparative anatomy of white clover, *T. uniflorum* and *T. repens* x *T. uniflorum* tap roots has not been studied before, and could provide useful information to explain tap root survival, as well as other ecophysiological differences among clover types. Various factors contribute to tap root death, probably in combination, including natural ageing, disease and inadequate carbohydrate supply (Westbrooks and Tesar, 1955; Thomas, 2003). Thomas (2003) describes how the diversion of carbon to nodal root formation could limit the carbohydrate supply to the tap root, leading to its death. However, it is hypothesised that the roots of *T. repens* x *T. uniflorum* hybrids will be thicker than those of white clover due to the morphology of *T. uniflorum* roots (see Chapter 6). In that case, it may be expected that a stronger nodal root system would divert more carbon away from the tap root. Instead, tap root survival was higher in some hybrid plants, although carbohydrate supply could contribute to the eventual death of the tap root in these plants. Westbrooks and Tesar (1955) and Kilpatrick and Dunn (1961) noted the presence of *Rhizoctonia* and/or *Fusarium* species on white clover tap roots, and concluded that they played some part in tap root loss. Discolouration of the outer layer of the tap root and rotting of lateral roots, as described by Westbrooks and Tesar (1955), were noted in the current experiment. In addition, lesions were present on some tap roots, while others had split at the crown. During tap root harvesting, the presence of clover
root weevil larvae (*Sitona lepidus* Gyllenhal) and – particularly during later harvests – grass grub (*Costelytra zealandica* (White)) was noted. However, Dymock and Hunt (1989) suggested that tolerance of *T. uniflorum* to grass grub could be inherited by *T. repens* x *T. uniflorum* hybrids. Dymock et al. (1989) also reported reduced grass grub larval growth on *T. uniflorum* and some *T. repens* x *T. uniflorum* hybrids.

3.4.5 **Fungal disease and virus infection**

The susceptibility of *T. uniflorum* to root diseases has not been studied, but shoot disease in the current experiment was very low (Figure 3.23). Observations elsewhere have shown that foliar diseases are more prevalent in *T. repens* x *T. uniflorum* hybrids than in white clover (K. Widdup pers. comm.). However, while the BC1 generation did have a significantly higher mean shoot fungal disease score than BC2 and white clover in the current experiment (Figure 3.23), there was significant variability among families. Fungal disease increased markedly in some families compared with their parental cultivars, but others showed relatively little change or even reductions in the mean fungal disease score (Figure 3.24). It is therefore possible that lower susceptibility to root diseases could contribute to increased tap root survival in *T. uniflorum* and some hybrids. In addition to low fungal disease, no virus infection was observed in *T. uniflorum* (Figure 3.25). Gibson et al. (1971) noted that this species has resistance to a range of viruses. Fungal disease and virus infection were scored when present at high frequencies, but this only occurred on one occasion for each group of pathogens. More frequent scoring is recommended, to confirm the relative susceptibility of clover types, and to identify hybrid families which consistently demonstrate low susceptibility to fungal disease and viruses.

3.4.6 **Flowering**

The effects of hybridisation with *T. uniflorum* on flowering characteristics have important implications for the commercial seed production of this material. The lower height of inflorescences relative to the canopy (Figure 3.14), for both backcross generations, was explained by the observed differences in morphological characteristics (Figures 3.15 and 3.16). The shorter, less erect peduncles and, most importantly, the length of peduncles compared with the petiole would all contribute to the positioning of inflorescences within, or not far above, the canopy. This is likely to make commercial harvesting of seed difficult. While still lower than white clover, the second generation of backcrosses did show improvement in these characteristics, suggesting that successive backcrossing could be used to overcome this problem. Without selection, this could result in the loss of important *T.*
*uniflorum* characteristics that are predominant in the BC$_1$ generation, such as tap root persistence. However, it is likely that these traits are independently inherited (W. Williams, pers. comm.), and it should be possible to select for characteristics from both species. The current experiment was not managed in the same manner as a seed crop, but the relative differences among clover types could be expected to remain the same. This assumes there are no clover type differences in the response of morphological characteristics, such as peduncle length, to influences from grazing or competition from grasses.

Seed production may also be reduced in the BC$_1$ generation due to a lower density of inflorescences (Figure 3.13). In addition, observations indicate that floret number may be lower on the inflorescences of BC$_1$ plants, but this has not been measured. Hybridisation has also been shown to affect flowering characteristics in *T. repens* x *T. nigrescens* hybrids (Marshall *et al.*, 1998). In that case, higher inflorescence production was introduced from the *T. nigrescens* parent, but floret numbers and seed set were maintained.

### 3.4.7 Variability

It was expected that the hybrid types would be more variable than white clover for the traits measured in the current experiment, due to the potential variation in the combination of genes which contribute to the *T. uniflorum* portion of the hybrid genome. Instead, many characteristics, particularly morphological traits, were more variable in white clover than in the BC$_1$ generation (e.g. Tables 3.7, 3.8 and 3.9). The reason for this anomalous result is not clear. It may be due to the range of morphological types present among the white clover cultivars, from small-leaved Tahora to large leaf types such as Aran and Kopu II. However, the same cultivars, except Tahora, were represented in the BC$_1$ generation. It is unlikely that the absence of hybrids derived from this one cultivar would affect the overall variability of the generation. The BC$_2$ generation was also often more variable than the BC$_1$ generation, but contained material from a more limited number of cultivars.

### 3.5 Conclusions

- Hybridisation with *T. uniflorum* affected many white clover characteristics in the BC$_1$ generation. However, some characteristics were not changed by a second generation of back crossing. In some instances, important *T. uniflorum* characteristics (such as tap root survival) were lost in the BC$_2$ generation.
• Dry matter production was reduced as expected in the *T. repens* x *T. uniflorum* hybrids, but this may be overcome by phenotypic selection, particularly in some hybrid families.

• Nitrogen fixation was not affected by hybridisation, but confirmation of this finding at higher levels of mineral N would be useful.

• Although survival of BC₁ tap roots at the end of the experiment was lower than expected, *T. uniflorum* and the BC₁ generation had significantly higher tap root survival than white clover. This may be improved further by selection and breeding.

• Some hybrid families were more superior than others at maintaining (or improving) characteristics of their white clover parental cultivars, such as lateral spread, stolon density, growth habit and resistance to shoot fungal diseases and viruses.

• The general morphological type of the BC₁ hybrid has a smaller leaf lamina area, shorter internodes and a more prostrate growth habit compared with white clover.
Chapter 4
Root depth distribution and associated traits

4.1 Introduction

Root systems are the interface between plant and soil, where the interception and uptake of water and nutrients take place. Rooting depth, root morphology and root architecture all influence the ability of plants to access water and nutrients. Higher proportions of root mass in deeper soil layers, or a greater maximum rooting depth, could increase access to subsoil water (Grieu et al., 2001), as well as interception of mobile nutrients such as nitrate, which leach down the soil profile. Studies on the root depth distribution of white clover are limited, but have found up to 70% of the total root mass occurs in the top 100–150 mm of the profile (Caradus, 1981; Nichols et al., 2007). Uptake of water and nutrients is also influenced by root diameter (Jungk, 1996; Eissenstat, 1992) and root architecture. Root architecture controls root length density and the extent to which root systems explore the soil through branching.

If the root distribution and rooting depth of white clover could be altered, it is possible that access to water and nutrients could be improved. The root system of T. uniflorum is reported to have a number of advantages over that of white clover, and is one of the main features driving interspecific hybridisation with this species. However, although T. uniflorum is reported to have deep roots (Pandey et al., 1987), there is no data on the maximum rooting depth or root distribution of this species compared with white clover. In addition, the effects of hybridisation with white clover on both rooting depth and root depth distribution are unknown. Reports also suggest T. uniflorum has thicker roots than white clover (Pandey et al., 1987), but there is no quantitative data on root morphology or architecture.

The main objective of this study was to describe the root depth distribution of T. repens x T. uniflorum interspecific hybrids and compare this to the white clover and T. uniflorum parents, to determine what effect hybridisation may have on this characteristic. It was hypothesised that the root depth distribution of T. uniflorum and T. repens x T. uniflorum hybrids would be different to that of white clover. Linked to this was the hypothesis that the root mass of T. uniflorum and T. repens x T. uniflorum hybrids may differ in morphology to that of white clover – specifically, root diameter and root density. Root length density (RLD) and specific root length (SRL) were, therefore, measured at several depths. The second objective was to compare the maximum rooting depth of T. uniflorum, white clover and T. repens x T.
uniflorum hybrids, hypothesising that the rooting depth of *T. uniflorum* is greater than that of white clover and that this characteristic is also present in hybrid material.

### 4.2 Materials and methods

#### 4.2.1 Experimental setup

The experimental setup (Plate 4.1) follows that described in Crush *et al.* (2005b). Root depth distribution was measured in 1 m deep x 150 mm diameter tubes, made from PVC stormwater pipe. Each tube was cut in half lengthwise and the two halves taped back together with duct tape. Tubes were filled with mortar sand, leaving a 10 mm lip at the top for water and nutrient application. The sand was watered down during filling to ensure it settled evenly down the tube. Water holding capacity of the sand was calculated as 346 ml l⁻¹, by weighing the drained wet weight of five 100 g samples of air dried sand.

![Plate 4.1. Experimental setup of the root depth distribution experiment in a glasshouse at AgResearch, Ruakura Research Centre, Hamilton.](image)

The tubes were supported in a wooden frame, with removable sides to enable access to the tubes at harvest. A layer of pea gravel was laid down in the bottom of the frame and covered in 5 mm thick foam sheets, allowing drainage while preventing the loss of sand from the open bottoms of the tubes. The outside of the frame was covered with building sisalation.
(aluminiumised insulation paper, see Plate 4.1) to prevent edge effects from solar heating of the outer tubes.

The experiment was oriented west-east in a glasshouse at AgResearch, Ruakura Research Centre, Hamilton (37º 46’ 23.58"S, 175º 18’ 22.47"E) and was conducted from 10 April to 5 December 2008. Supplementary lighting was used from 6–8 am and 5–6 pm to provide a 12 hour day length throughout the experiment. Glasshouse heaters were set to operate when day/night temperatures fell below 18/12°C. Mean day/night temperatures over the duration of the experiment were 19.1/13.3°C; mean day/night temperature and maximum daytime solar radiation during each harvest period are shown in Table 4.1. Data were recorded on a weekly basis by the glasshouse control units.

Table 4.1. Weekly mean day/night temperatures and weekly mean maximum solar radiation for each harvest period. The number of days to each harvest are calculated from the first day of planting to the mid-point of the harvesting period.

<table>
<thead>
<tr>
<th>Harvest</th>
<th>Day time temperature (ºC)</th>
<th>Night time temperature (ºC)</th>
<th>Maximum solar radiation (μmol m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (70 days)</td>
<td>18.9</td>
<td>13.1</td>
<td>946</td>
</tr>
<tr>
<td>2 (119 days)</td>
<td>16.6</td>
<td>11.5</td>
<td>841</td>
</tr>
<tr>
<td>3 (170 days)</td>
<td>18.6</td>
<td>12.5</td>
<td>1184</td>
</tr>
<tr>
<td>4 (237 days)</td>
<td>21.3</td>
<td>15.3</td>
<td>1510</td>
</tr>
</tbody>
</table>

4.2.2 Plant material

The six clover entries used in this experiment included two T. uniflorum accessions, two white clover cultivars, and two BC₁ (backcross 1) populations generated by backcrossing F₁ (first filial generation) hybrids to each of the white clover parents (Table 4.2). Each BC₁ was represented by two family bulks derived using different T. uniflorum accessions as the male parent in the F₁ hybrid (Table 4.2). The F₁ hybrids were not included as these were produced through embryo culture, so seed was not available.

Seed was scarified with sandpaper and germinated on damp filter paper in Petri dishes on 7 April 2008. Germinated seed was inoculated in the Petri dishes using a suspension of “Nodulaid” moist peat inoculant for white, red and strawberry clover (Becker Underwood Pty. Ltd., Somersby, Australia). Two grams of inoculant was mixed with a solution of 20% minus N nutrient solution and 80% tap water, and 0.5 ml of the supernatant was pipetted into each dish. Seedlings were then planted in the sand tubes when the radicle was 10–20 mm
long. Any fatalities that occurred within one week of planting were replaced with spare seedlings. These had been grown on in sand pots, which allowed for development of the root system prior to transplanting, and also provided a growth medium which minimised disturbance of the roots during relocation.

White clover parents and BC$_1$ hybrids were planted out over three days from 10-12 April 2008. Due to slow and poor germination of the two $T.~uniflorum$ accessions, more seed needed to be scarified five to seven days after the initial germination. The $T.~uniflorum$ seedlings were planted out between 14 and 24 April 2008, with the majority being planted by 18 April 2008.

Table 4.2. White clover, $T.~uniflorum$, and $T.~repens \times T.~uniflorum$ BC$_1$ entries used in the root depth distribution experiment. Entry numbers correspond to the experimental design. cv = cultivar; OP = open pollinated.

<table>
<thead>
<tr>
<th>Entry number</th>
<th>Clover type</th>
<th>Description</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>White clover</td>
<td>cv. Grasslands Kopu II</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>White clover</td>
<td>cv. Crusader</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>$T.~uniflorum$</td>
<td>AZ4382$^#$ OP</td>
<td>Greek origin</td>
</tr>
<tr>
<td>4</td>
<td>$T.~uniflorum$</td>
<td>AZ4383$^#$ OP</td>
<td>Turkish origin</td>
</tr>
<tr>
<td>5</td>
<td>BC$_1$</td>
<td>Kopu II BC$_1$</td>
<td>Kopu II x 80-2$^\dagger$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kopu II x 900-4$^\ddagger$</td>
</tr>
<tr>
<td>6</td>
<td>BC$_1$</td>
<td>Crusader BC$_1$</td>
<td>Crusader x 80-2$^\dagger$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Crusader x 900-4$^\ddagger$</td>
</tr>
</tbody>
</table>

$^#$ Accession number, Margot Forde Germplasm Centre (Palmerston North, New Zealand).
$^\dagger$ 80-2 = Kopu II-2 x T66-6, where T66-6 = a specific genotype of AZ4382.
$^\ddagger$ 900-4 = Kopu II-2 x AZ4383-11.

4.2.3 Experimental design

Ten replicates of the six clover entries were measured at each of four harvests over time for a total of 240 plants (60 per harvest). For the two hybrid populations, the ten replicates were made up of five replicates each of the two family bulks. The design was based on a split plot arrangement for ease of harvesting, with each replicate containing four blocks (harvests) (Figure 4.1). Each block contained six tubes, one for each of the six clover entries.
Figure 4.1. Experimental layout of the root depth distribution experiment. 1 = Kopu II; 2 = Crusader; 3 = AZ4382; 4 = AZ4383; 5 = Kopu II BC1; 6 = Crusader BC1. a = 80-2 as F1, b = 900-4 as F1. # = replacement for entry 3.
Due to low germination, only five replicates of *T. uniflorum* AZ4382 were available. Remaining replicates were replaced with extra plants of *T. uniflorum* AZ4383. The BC$_1$ families were randomly distributed throughout the experiment but balanced within replicates so that all combinations of parental material were represented (i.e. Kopu II x 80-2 + Crusader x 80-2; Kopu II x 900-4 + Crusader x 900-4; Kopu II x 80-2 + Crusader x 900-4; Kopu II x 900-4 + Crusader x 80-2).

### 4.2.4 Nutrient solution

Plants in all tubes received a low ionic strength nutrient solution (Appendix 15). The chemistry of this solution is based on the average soil solution of a range of New Zealand pasture topsoils (Edmeades et al., 1985; Blamey et al., 1991) and has been used extensively in similar experimental systems (Crush et al., 2007; 2005b; Nichols et al., 2007). Each plant initially received 50 ml of nutrient solution three times per week, with 200 ml applied five days per week once established. 200 ml was equivalent to the water holding capacity of the top 34 mm of sand. On remaining days, the tubes were watered with a gentle spray of tap water. The nutrient solution was made up in 135 l batches with deionised water, and adjusted to a pH of 5.0–5.5 with 20% NH$_4$ solution or 30% HCl.

### 4.2.5 Plant harvests

Harvests were conducted on 19 June (harvest 1, 70 days after sowing), 7–8 August (harvest 2, 119 days), 25–29 September (harvest 3, 170 days), and 1–5 December 2008 (harvest 4, 237 days). The number of days to each harvest were calculated from the first day of planting, up to the first day of the harvest or, where harvesting took more than two days, up to the mid-point of the harvesting period. At each harvest time, 10 replicates were removed from the frame in blocks of six plants (entries). Each tube was laid flat and opened by cutting the tape and lifting off the top half (Plate 4.2). The shoot was then removed and the sand/root column cut at 50, 100, 150 and 200 mm, then in 100 mm increments from 200 mm to 1 m. Each section was washed through a 2 mm sieve in water, capturing the roots which were rinsed to remove any adhering sand. The roots and shoots were blotted and dried at 70°C overnight (Crush et al., 2010b), then weighed to determine shoot dry weight (DW), total root DW and root mass distribution with depth (root DW in each depth section).

Shoot mineral and elemental content of Crusader, Crusader BC$_1$ (80-2 and 900-4 families) and *T. uniflorum* (4382 and 4383 accessions) was analysed at harvest 4. Harvest 4 plants were used as they provided a greater amount of material for the analytical technique.
The whole shoots were ground and analysed for Al, As, B, Ca, Co, Cr, Cu, C:N, Fe, K, Mg, Mn, Mo, %N, Na, Ni, P, Pb, S, and Zn. Analyses were performed by Analytical Services, Faculty of Agriculture and Life Sciences, Lincoln University using ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometer) (Varian Australia Pty. Ltd., Melbourne).

Plate 4.2. Hybrid plant at harvest 4 (237 days) with one half of the tube removed, enabling the sand root column to be rolled out intact and be divided into depth sections.

At harvest 3, the roots between 50–100 mm and 400–500 mm were collected from all tubes and preserved in 70% ethanol. These were subsequently scanned using the WinRhizo™ software package (Regent Instruments Inc., Quebec) and total root length measured from the stored images. This was used to generate RLD (km m⁻³), based on the volume of the relevant sections. The roots were rinsed to remove ethanol and oven dried overnight at 70°C (Crush et al., 2010b). Dry weights were corrected for loss of weight due to storage in ethanol (Crush et al., 2010a) and included in the root distribution and total root DW data. Specific root length (m g⁻¹) of each sample was calculated using root length and root DW data. Measurements were made at harvest 3, as larger root systems from older plants would have been difficult to process. Plants at harvest 3 were still well established (see Results section 4.3.2.2).
4.2.6 Statistical analysis

For most data, statistical comparisons were based on clover entries or families. Shoot DW, root DW, and root:shoot ratio were log-transformed to satisfy the assumptions of analysis of variance (ANOVA). Data were first analysed for overall differences between entries, harvests, and entry x harvest interactions, using ANOVA in Minitab version 15 (Minitab Inc.). Shoot and root DW data were then analysed by ANOVA at each of the four individual harvests. All means were compared using Tukey’s multiple pair-wise comparisons to account for unbalanced data and multiple comparisons (Milliken and Johnson, 2009). Back-transformed means and estimated standard errors of the mean (SEM) (back-transformed mean x log SEM) are presented.

For shoot mineral and elemental data, clover entries were grouped into clover types, and also analysed as populations within types. Data were analysed in Genstat version 13 (VSN International Ltd.) using REML, with blocks as random effects and clover type as fixed effects. Bartlett’s test was first applied to assess the homogeneity of variances between groups, and heterogeneous data was either log-transformed, modelled using different variances for each clover type (*T. uniflorum*, Crusader BC1, Crusader), or analysed using rank analysis as appropriate. Two models were fitted to account for differences among clover types and among populations within types. Where differences among populations (4382, 4383, Crusader x 80-2, Crusader x 900-4, Crusader) were not significant at the 5% level, the model for clover type only was fitted. Results for significance tests present the predicted or transformed means from the analysis, plus or minus the standard errors. Where data was transformed, the back-transformed means are also given. These are geometric means, showing the approximate means of actual shoot mineral concentrations.

Prior to root depth distribution analysis, root DW in the four 50 mm deep sections at the top of each tube were combined into two 100 mm deep sections at 0–100 and 100–200 mm. All depth sections were then of equal size. At each harvest, root depth distribution (root DW by depth) was analysed using an exponential model (1) in SAS version 9.1 (SAS Institute Inc.).

\[
\text{Root mass} = \beta_j R_j^{\text{Depth}} \quad \text{for } j = 1, 2, ..., 6 \quad \text{where } j \text{ denotes the six clover entries.}
\]

\[\beta_j = \text{DW at 0–100 mm and } R_j = \text{rate at which DW decreases with depth.}\]

To estimate the values of \(\beta_j\) and \(R_j\) in the exponential model (1), this equation was transformed to an equivalent regression model (2) by taking natural logarithms using the GLM procedure (general linear model).
(2) \( \ln(\text{Root mass}) = \ln \beta_j + \text{Depth} \times \ln R_j = C_{1j} + C_{2j} \times \text{Depth} \)

where \( C_{1j} = \ln \beta_j \) and \( C_{2j} = \ln R_j \).

The results for \( C_{1j} \) and \( C_{2j} \) were then back-transformed to \( \beta_j \) and \( R_j \) in the exponential model (1), using \( \beta_j = \exp(C_{1j}) \) and \( R_j = \exp(C_{2j}) \).

Differences between clover entries were determined by pair-wise comparisons of the resulting values for \( \beta_j \) (DW at 0–100 mm) and \( R_j \) (rate at which DW decreases with depth) using Tukey’s pair wise comparison method as above.

In addition to the exponential model, root distribution was also assessed by comparing the proportion of total root mass at 0–100 mm, 100–200 mm and 400–500 mm from harvest 4 using ANOVA in Minitab version 15 (Minitab Inc.). Differences between clover entries were determined using Tukey’s pair wise comparison method as above. Root length density and SRL data at 50–100 mm and 400–500 mm from harvest 3 were analysed using ANOVA in Minitab version 15 (Minitab Inc.), and clover entry differences determined using Tukey’s pair wise comparison method. The change with depth was calculated from RLD and SRL at 400–500 mm as a proportion of that at 50–100 mm, and data were analysed using the two sample Wilcoxon test (Conover, 1980) for non-normally distributed data.

Differences between 80-2 and 900-4 families were examined for shoot DW, root DW, root:shoot DW ratio, RLD and SRL using ANOVA in Mintab version 15 (Minitab Inc.). Dry weight data within the Crusader BC1 and Kopu II BC1 populations were first combined over all harvests (e.g. Kopu II x 80-2, harvest 1–4 versus Kopu II x 900-4, harvest 1–4). Shoot and root DW of families with common F1 parents were then combined across populations (e.g. Crusader x 80-2 and Kopu II x 80-2 combined) to increase replication for ANOVA at harvest 4 only, when plants appeared to be well established (see Results section 4.3.2). Differences in root distribution between the families at harvest 4 were also assessed by comparing \( \beta_j \) and \( R_j \) using t-tests.

In graphs and tables, means with the same letter were not significantly different at the 5% level, using the means separation methods stated above for the respective traits. Trends nearing statistical significance (\( P=0.05-0.099 \)) are noted in the text.

Differences in variability among clover entries for shoot and root DW at harvest 4, and RLD and SRL at harvest 3, were analysed using a test for equal variances in Minitab version 15 (Minitab Inc.) with the untransformed data. This compares two variances using the F-test or
Levene’s test, depending on the normality distribution of the data. Standard deviations are presented to indicate the relative size of the variance for each entry, while significant differences between entries are indicated with lettering, as mentioned above.

4.3 Results

4.3.1 Nodulation

Pink healthy nodules were observed on plants at all harvests, including large coralloid nodules which appeared to be particularly prevalent on *T. uniflorum* plants (Plate 4.3). At harvest 4 coralloid nodules were noted on 85% of *T. uniflorum* plants, 65% of BC₁ plants and 40% of white clover plants. The coralloid nodules were present at a range of depths, including the deepest sections (0.9–1 m).

Plate 4.3. Large corraloid nodules on *T. uniflorum* at harvest 4 (237 days).

4.3.2 Plant dry weight

4.3.2.1 Over all harvests

The clover entries differed (*P*<0.001) for shoot and root total DW. For all harvests combined, shoot and root DWs of both white clover cultivars were smaller than those of the two *T. uniflorum* accessions (*P*<0.001), and also smaller than their respective BC₁ hybrids (*P*<0.001) (Figures 4.2A and 4.2B). Compared with the *T. uniflorum* accessions, Kopu II shoots and
roots were approximately 73% smaller, and Crusader shoots and roots were nearly 90% smaller. Shoots of Kopu II were 73% smaller than the Kopu II BC1 and roots were 66% smaller, while Crusader shoots were 80% smaller than the Crusader BC1 and roots were 78% smaller. In addition, the shoots of the Kopu II cultivar were 2.3 times larger than Crusader ($P=0.005$) and roots were 2.1 times larger ($P=0.003$).

Figure 4.2. Back-transformed means averaged over all harvests (± estimated SEM) for shoot dry weight (A), root dry weight (B) and root:shoot ratio (C) of the six clover entries. Means with the same letter, within parameters, show no significant differences at the 5% level.
There was no difference in overall shoot DW between the \textit{T. uniflorum} accessions and the BC\textsubscript{1} hybrids (Figure 4.2A). The root DW of the \textit{T. uniflorum} accessions and Kopu II BC\textsubscript{1} also showed no differences, but the roots of Crusader BC\textsubscript{1} were 48\% smaller ($P=0.04$) than accession 4383 (Figure 4.2B). Overall root:shoot ratio differed only for the Kopu II BC\textsubscript{1} which was approximately 20\% smaller than \textit{T. uniflorum} accession 4383, Kopu II and Crusader BC\textsubscript{1} ($P<0.033$), and 29\% smaller than Crusader ($P<0.001$) (Figure 4.2C).

**4.3.2.2 Individual harvest times**

Not all of the results from the overall comparisons held for individual harvest times, although there were significant clover entry differences in shoot and total root DW at every harvest ($P<0.001$). The clover entry x harvest interaction was also significant for both shoot ($P<0.001$) and root DW ($P=0.002$). Clover entry differences were also similar for shoot and root DW (Figures 4.3 and 4.4).

**\textit{T. uniflorum} accessions**

The \textit{T. uniflorum} accessions differed at harvest 1 for shoot DW ($P=0.049$) but not for root DW (Figures 4.3 and 4.4). Shoots of accession 4383 were 1.8 times larger than those of 4382 at this time. At all other harvests there were no differences between the \textit{T. uniflorum} accessions.

**\textit{T. uniflorum} and white clover**

The white clover cultivars were generally smaller than the \textit{T. uniflorum} accessions at most harvests (Figures 4.3 and 4.4). For example, shoot DW of accession 4383 was 4.1–19.2 times larger than that of Crusader ($P<0.002$), and root DW was 4.2-17.6 times larger than that of Crusader ($P<0.001$). The differences in shoot and root DW between \textit{T. uniflorum} and Crusader tended to increase in size over time.

The \textit{T. uniflorum} accessions also tended to be larger than Kopu II, particularly accession 4383 (Figures 4.3 and 4.4). For example, shoot DW of accession 4383 was larger than that of Kopu II at all harvests except harvest 4, when there was still a trend towards larger DW for accession 4383 ($P=0.079$). At the remaining harvests, shoot DWs of accession 4383 were 2.5–5.2 times larger than those of Kopu II ($P<0.021$) (Figure 4.3). Roots of accession 4383 were 3–5.1 times larger than those of Kopu II ($P<0.02$) (Figure 4.4). The differences in shoot and root DW between accession 4383 and Kopu II also increased in size over time, but not as markedly as between the \textit{T. uniflorum} accessions and Crusader.

Shoot and root DW of \textit{T. uniflorum} 4382 was larger than those of Kopu II only at harvest 3 ($P=0.032$), by 8.2 and 6.3 times, respectively (Figures 4.3 and 4.4). However, there were still
trends for larger shoot DW for accession 4382 at harvest 2 ($P=0.071$), and for larger root DW at harvest 2 ($P=0.076$) and harvest 4 ($P=0.061$), when compared with Kopu II.

**White clover and BC$_1$ hybrids**
The white clover cultivars were also generally smaller than their respective BC$_1$ hybrids. Crusader BC$_1$ was larger than the Crusader parent at harvest 1, 3 and 4 for both shoot and root DW. Shoot DW was 2–24.2 times larger ($P<0.007$) (Figure 4.3), and root DW was 2.2–14.8 times larger ($P<0.006$) (Figure 4.4). The magnitude of the differences between these clovers increased over time. The differences in shoot and root DW between the Kopu II BC$_1$ and the Kopu II parent took longer to develop. They did not differ at harvest 1, but the trend was for a larger shoot DW in Kopu II BC$_1$ compared with Kopu II (Figure 4.3), by 3.1–6.7 times, at harvests 2 ($P=0.061$), 3 ($P=0.045$) and 4 ($P=0.004$). There were also trends towards a larger root DW for Kopu BC$_1$ at harvests 2 ($P=0.065$) and 3 ($P=0.057$), by 2.5 and 4 times respectively, and it was 10 times larger than the Kopu II parent at harvest 4 ($P=0.003$) (Figure 4.4).

**BC$_1$ hybrids and T. uniflorum**
Root and shoot DW of *T. uniflorum* accession 4382 did not differ to either of the hybrids at any time (Figure 4.3 and 4.4). Accession 4383 also did not differ to the hybrids at the end of the experiment, but its shoot DW was often larger than that of the hybrids at the earlier harvests (Figure 4.3 and 4.4). At harvest 1, shoot DW of accession 4383 was 2 times larger than that of Crusader BC$_1$ ($P<0.001$) and 2.1 times larger than that of Kopu II BC$_1$ ($P<0.001$) (Figure 4.3); and root DW was 2.6 times larger than that of both hybrids ($P<0.001$) (Figure 4.4).

**White clover cultivars**
The white clover cultivars only differed to each other at harvest 1, when the shoots of Kopu II were 1.7 times larger than those of Crusader ($P=0.048$) and the roots of Kopu II were 1.9 times larger ($P=0.025$) (Figure 4.3 and 4.4). Kopu II shoots were 3.7 and 4.3 times larger than those of Crusader at harvests 3 and 4 respectively, and roots were 2.9 and 3.4 times larger, respectively.
Figure 4.3. Back-transformed means for shoot dry weight (± estimated SEM) of the six clover entries at harvest 1 (70 days) (A), harvest 2 (119 days) (B), harvest 3 (170 days) (C) and harvest 4 (237 days) (D). Means with the same letter, within harvests, show no significant differences at the 5% level.
Figure 4.4. Back-transformed means for root dry weight (± estimated SEM) for the six clover entries at harvest 1 (70 days) (A), harvest 2 (119 days) (B), harvest 3 (170 days) (C) and harvest 4 (237 days) (D). Means with the same letter, within harvests, show no significant differences at the 5% level.
4.3.2.3 Variability of clover entries

The variability in DW of the different clover entries was assessed at harvest 4 (Table 4.3). For both shoot and root DW, each hybrid was more variable than its respective white clover parental cultivar ($P<0.023$). Kopu II BC$_1$ and Crusader BC$_1$ did not differ significantly for DW variability, but Kopu II was more variable than Crusader ($P<0.01$). Shoot DW of the *T. uniflorum* accessions was often less variable than that of the hybrids ($P<0.023$), and shoot DW of accession 4383 was also less variable than that of Kopu II ($P=0.01$).

Table 4.3. Standard deviations for shoot and root dry weight of the six clover entries at harvest 4 (237 days). Clover entries with the same letter, within parameters, show no significant differences in variability at the 5% level.

<table>
<thead>
<tr>
<th>Clover entry</th>
<th>Shoot dry weight (g)</th>
<th>Root dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. uniflorum</em> 4382</td>
<td>2.20$^{ab}$</td>
<td>3.01$^{ab}$</td>
</tr>
<tr>
<td><em>T. uniflorum</em> 4383</td>
<td>2.11$^a$</td>
<td>2.60$^a$</td>
</tr>
<tr>
<td>Crusader BC$_1$</td>
<td>8.23$^{cd}$</td>
<td>3.42$^{ab}$</td>
</tr>
<tr>
<td>Kopu II BC$_1$</td>
<td>13.6$^d$</td>
<td>5.20$^b$</td>
</tr>
<tr>
<td>Crusader</td>
<td>1.65$^a$</td>
<td>0.88$^c$</td>
</tr>
<tr>
<td>Kopu II</td>
<td>4.58$^{bc}$</td>
<td>2.29$^a$</td>
</tr>
</tbody>
</table>

4.3.3 Shoot elemental analyses

In order to investigate the observed differences in growth among clover entries, a subset of plants at harvest 4 was analysed for shoot element contents. Significant differences among populations within clover types were found only for levels of Ca, Mn, Mo and the C:N ratio (Table 4.4). Calcium was higher in *T. uniflorum* accession 4382 than accession 4383, and both accessions had more Ca than the Crusader hybrid families and the Crusader parent. Manganese content was lower in the Crusader parent than in the other populations. While levels of Mn in the hybrid families did not differ, Mn in *T. uniflorum* accession 4382 was higher than in accession 4383. Crusader x 80-2 had a lower Mo concentration than the other populations, including Crusader x 900-4. The C:N ratio of the hybrids was lower than in all other populations, and it was higher in *T. uniflorum* accession 4382 than in both accession 4383 and the Crusader parent.

In some instances the differences in element concentrations were substantial. For example, Ca content of *T. uniflorum* accession 4382 was 70–85% higher than in the hybrid families and Crusader parent; and in accession 4383 was 42–54% higher. Manganese content of Crusader
was 44% lower than in accession 4382, 32% lower than in accession 4383, 35% lower than in Crusader x 80-2 and 24% lower than in Crusader x 900-4.

Table 4.4. Mean shoot concentrations (±SEM) with P values, for elements showing differences among populations at harvest 4 (237 days). For log-transformed data, the back-transformed mean is given in brackets. Means with the same letter, within parameters, show no significant differences at the 5% level. Units are in mg kg⁻¹ unless otherwise specified.

<table>
<thead>
<tr>
<th>Element</th>
<th>T. uniflorum 4382</th>
<th>T. uniflorum 4383</th>
<th>Crusader x 80-2</th>
<th>Crusader x 900-4</th>
<th>Crusader</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>28448 ±1150a</td>
<td>23693 ±813b</td>
<td>16190 ±1150c</td>
<td>16713 ±1150c</td>
<td>15398 ±909c</td>
<td>0.009</td>
</tr>
<tr>
<td>Mn</td>
<td>135.9 ±8.4a</td>
<td>112.6 ±5.9b</td>
<td>117.8 ±8.4ab</td>
<td>101.1 ±8.4b</td>
<td>76.7 ±6.6c</td>
<td>0.041</td>
</tr>
<tr>
<td>Mo</td>
<td>8.93 ±0.95ab</td>
<td>9.30 ±0.67b</td>
<td>3.39 ±0.95c</td>
<td>6.83 ±0.95a</td>
<td>9.04 ±0.75ab</td>
<td>0.05</td>
</tr>
<tr>
<td>C:N</td>
<td>3.08 ±0.034a</td>
<td>2.94 ±0.024b</td>
<td>2.84 ±0.034c</td>
<td>2.83 ±0.038c</td>
<td>2.96 ±0.044b</td>
<td>0.015</td>
</tr>
</tbody>
</table>
(21.65)  | (18.99)          | (17.08)         | (16.88)         | (19.24)         |          |         |

Populations did not differ for concentrations of the remaining elements, which were therefore examined for differences among clover types only. Differences were found for most elements, with the only exceptions being Mg, S, As, B and Pb (Table 4.5). The T. uniflorum parent had higher levels of Al, Fe, Co, Cr, and Ni than the Crusader BC₁ and Crusader parent.

Both T. uniflorum and Crusader BC₁ also had higher levels of Na, K, Cu and Zn than the Crusader parent. Potassium content was in turn higher in Crusader BC₁ than T. uniflorum; but Cu and Zn were higher in T. uniflorum than in Crusader BC₁. Phosphorus content of T. uniflorum was also higher than that of Crusader BC₁. The Crusader BC₁ had a higher %N content than both parents.

Again, differences between clover types were often substantial. For example, Cr and Ni contents of T. uniflorum were 6.6 and 11 times higher, respectively, than in Crusader BC₁, and 13.5 and 5.5 times higher than in Crusader. Compared with Crusader, the K content of T. uniflorum was 25% higher, and in Crusader BC₁ it was 50% higher.
Table 4.5. Mean shoot concentrations (±SEM) with P values, for elements showing differences among clover types only, at harvest 4 (237 days). For log-transformed and ranked data the back-transformed means are given in brackets. Means with the same letter, within parameters, show no significant differences at the 5% level. Units are in mg kg⁻¹ unless otherwise specified.

<table>
<thead>
<tr>
<th></th>
<th>T. uniflorum</th>
<th>Crusader BC₁</th>
<th>Crusader</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>As</td>
<td>0.3962 ±0.0346&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3635 ±0.0423&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4322 ±0.0599&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.637</td>
</tr>
<tr>
<td>B</td>
<td>36.97 ±1.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.65 ±2.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.15 ±2.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.09</td>
</tr>
<tr>
<td>Cr</td>
<td>106.01 ±11.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.17 ±5.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.84 ±5.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>K</td>
<td>14906 ±925&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17950 ±1088&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11886 ±1211&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.002</td>
</tr>
<tr>
<td>Mg</td>
<td>2800 ±80.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2950 ±123.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2642 ±292.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.447</td>
</tr>
<tr>
<td>%N</td>
<td>1.92 ±0.048&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.39 ±0.061&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.13 ±0.106&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ni</td>
<td>50.4 ±5.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.6 ±2.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.19 ±2.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P</td>
<td>1056 ±37.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>925 ±39.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1716 ±439.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.042</td>
</tr>
<tr>
<td>Al</td>
<td>6.232 ±0.117&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.437 ±0.131&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.409 ±0.144&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Co</td>
<td>-0.242 ±0.156&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.549 ±0.188&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-1.922 ±0.265&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cu</td>
<td>2.503 ±0.046&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.089 ±0.056&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.898 ±0.063&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(6.67)</td>
</tr>
<tr>
<td>Fe</td>
<td>6.769 ±0.108&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.754 ±0.126&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.737 ±0.140&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Na†</td>
<td>19.53 ±1.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.4 ±2.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.64 ±2.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S</td>
<td>7.377 ±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.271 ±0.074&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.255 ±0.082&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.388</td>
</tr>
<tr>
<td>Pb</td>
<td>-0.104 ±0.1505&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.3391 ±0.1796&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.3493 ±0.2005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.467</td>
</tr>
<tr>
<td>Zn</td>
<td>3.937 ±0.060&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.416 ±0.073&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.176 ±0.082&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N:P</td>
<td>18:1 ±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25:1 ±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26:1±1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N:S</td>
<td>12:1 ±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17:1 ±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16:1±1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
</tbody>
</table>

† mean rankings from rank analysis

4.3.4 Root system shape

The change in differences among clover entries over time for shoot and root DW results (Section 4.3.2.2) suggests there were differences in growth rate, particularly at the earlier harvests. Differences among entries were similar at harvest 3 and 4, suggesting they had stabilised by this time and more accurately represent real differences between plant entries.
For this reason, the presentation of root depth distribution results will concentrate on harvest 4 data.

Differences in root system shape occurred either through differences in shallow root mass (i.e. root mass in the top 100 mm, $\beta_j$) or differences in the rate at which root mass decreased with depth ($R_j$). As with total DWs, significant differences among clover entries changed from harvest to harvest (Table 4.6). Some of the differences between entries were evident from harvest 1 or 2, while others did not develop until harvest 3 and 4. In addition, some of the differences present at earlier harvests had disappeared by harvest 3 or 4.

Table 4.6. Root shape parameters for the six clover entries at each harvest. $\beta_j =$ root mass at 0–100 mm (g); $R_j =$ rate of decrease with depth. Means with the same letter within harvests show no significant differences at the 5% level. Harvest 1 = 70 days; harvest 2 = 119 days; harvest 3 = 170 days; harvest 4 = 237 days.

<table>
<thead>
<tr>
<th>Harvest</th>
<th>Root shape parameter</th>
<th>T. uniflorum 4382</th>
<th>T. uniflorum 4383</th>
<th>Crusader BC1</th>
<th>Kopu II BC1</th>
<th>Crusader BC1</th>
<th>Kopu II BC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$\beta_j$</td>
<td>0.042a</td>
<td>0.044a</td>
<td>0.048a</td>
<td>0.026a</td>
<td>0.036a</td>
<td>0.034a</td>
</tr>
<tr>
<td>2</td>
<td>$\beta_j$</td>
<td>0.068a</td>
<td>0.077a</td>
<td>0.110a</td>
<td>0.067a</td>
<td>0.049a</td>
<td>0.066a</td>
</tr>
<tr>
<td>3</td>
<td>$\beta_j$</td>
<td>0.483a</td>
<td>0.373ab</td>
<td>0.115c</td>
<td>0.211bc</td>
<td>0.037d</td>
<td>0.047d</td>
</tr>
<tr>
<td>4</td>
<td>$\beta_j$</td>
<td>1.894a</td>
<td>1.759a</td>
<td>1.480a</td>
<td>2.620a</td>
<td>0.065b</td>
<td>0.274c</td>
</tr>
</tbody>
</table>

| 1       | $R_j$               | 2.8E-05ab        | 0.00047b         | 1.4E-07a    | 2.7E-05ab   | 2.0E-09a    | 4.1E-06ab   |
| 2       | $R_j$               | 0.00463ab        | 0.00986b         | 0.00004c    | 0.00169a    | 0.00030ac   | 0.00004c    |
| 3       | $R_j$               | 0.0145ab         | 0.0105ab         | 0.0172ab    | 0.0077a     | 0.0314bc    | 0.0647c     |
| 4       | $R_j$               | 0.0415a          | 0.0435a          | 0.0432a     | 0.0293a     | 0.1777b     | 0.0935ab    |

In well established plants at harvest 4, there was no difference in the root shape of T. uniflorum accession 4382, accession 4383, Crusader BC1 and Kopu II BC1; but these were all different to both white clover cultivars (Table 4.6). For Kopu II this was due to a lower $\beta_j$ value ($P<0.001$), while Crusader had both a lower $\beta_j$ value ($P<0.001$) and a higher $R_j$ value (i.e. slower rate of decrease with depth) ($P<0.05$). Although the $R_j$ value of Kopu II did not differ significantly to Kopu II BC1, there was also a trend towards a slower rate of decrease with depth in the parent ($P=0.056$). The rate of decrease did not differ between the white clover cultivars, but $\beta_j$ (root mass at 0–100 mm) was lower for Crusader ($P<0.001$). Actual root dry mass distribution with depth is presented in Figure 4.5.
Figure 4.5. Root depth distribution (root dry weight by depth) (±SEM) at harvest 4 (237 days) for *T. uniflorum* 4382 (A), *T. uniflorum* 4383 (B), Crusader BC₁ (C), Kopu II BC₁ (D), Crusader (E) and Kopu II (F).

Although the white clover cultivars had significantly lower $\beta$ values than *T. uniflorum* and the hybrids, there were no differences among entries in the proportion of roots at 0–100 mm at harvest 4 (Figure 4.6A). However, the *T. uniflorum* parents had approximately 10% more of their total root mass at 100–200 mm (a total of 26%) than the white clover cultivars (a total of 16% for Crusader; and 17% for Kopu II) ($P<0.015$) (Figure 4.6B). Crusader BC₁ (23 %) also had a higher proportion of roots at 100-200 mm than the Crusader parent ($P=0.044$).

Proportions of total root mass at 400–500 mm did not differ among *T. uniflorum* and the hybrids, nor between the hybrids and their respective white clover parents (Figure 4.6C),
although there was a trend towards a higher proportion for Kopu II (7%) compared with Kopu II BC1 (4%) (P=0.074).

Figure 4.6. Proportion (±SEM) of total root mass at 0–100 mm (A), 100–200 mm (B) and 400–500 mm (C) at harvest 4 (237 days) for the six clover entries. Means with the same letter within sampling depths show no significant differences at the 5% level.
4.3.5 Root depth penetration

Roots of the hybrids had penetrated deeper than those of the white clover cultivars at all four harvests (Figure 4.7), with roots of the two *T. uniflorum* accessions being the deepest out of all the clover entries. Roots of some *T. uniflorum* plants first reached 1 m at harvest 2, compared with harvest 3 for the hybrid and white clover entries. At that point (harvest 3), more hybrid plants (35%) had reached 1 m than for the white clover cultivars (15%). Once roots reached 1 m, they accumulated at the bottom of the tubes, as shown in Figure 4.5 for harvest 4.

![Figure 4.7. Root penetration over time for the six clover entries.](image)

4.3.6 Root length density and specific root length

*Root length density*

At harvest 3, mean RLD of both hybrids at 50–100 mm was considerably greater than for their respective white clover parents (Figure 4.8A). However, while there was a trend towards higher RLD for Kopu II BC₁ (2.6 times higher) compared with Kopu II (*P*=0.061), the difference between Crusader and Crusader BC₁ was not significant. The *T. uniflorum* accessions did not differ to each other for RLD, nor did the two hybrid populations, or the white clover cultivars (Figure 4.8A).
At 400–500 mm depth, there were no differences in RLD between either of the hybrids and their respective parental cultivars, but RLD of the *T. uniflorum* accessions was usually greater than that of the other clover entries (Figure 4.8B). For example, RLD of accession 4383 was 3.2–3.7 times greater than those of Crusader BC1 (*P*=0.04), Kopu II BC1 (*P*=0.039) and Kopu II (*P*=0.026), and 9.1 times higher than Crusader (*P*=0.005). Root length density of accession 4382 at 400–500 mm was 10.4 times greater than that of the Crusader cultivar (*P*=0.019), and also tended to be greater than Kopu II (*P*=0.065), Crusader BC1 (*P*=0.083) and Kopu II BC1 (*P*=0.085), by approximately 4 times (Figure 4.8B). The SEM of accession 4382 at 400–500 mm was very large (Figure 4.8B), and there were only 5 replicates compared with 9–10 replicates for the other clover entries.

Figure 4.8. Mean (±SEM) root length density at 50–100 mm (A) and 400–500 mm (B); and mean (±SEM) specific root length at 50–100 mm (C) and 400–500 mm (D) for the six clover entries. Measurements were made at harvest 3 (170 days). Means with the same letter show no significant differences at the 5% level.

Root length density decreased with depth for all entries, but the change was not as pronounced for the *T. uniflorum* accessions (Figure 4.9A). For accession 4383 the mean RLD at 400–500 mm as a proportion of the means at 50–100 mm (17%) was greater than for Crusader BC1 (4%), Kopu II BC1 (2%), Crusader (4%) and Kopu II (3%) (*P*<0.005). For
accession 4382 the proportion of RLD at 400–500 mm (22%) was greater than that of Crusader BC1, Kopu II BC1, and Kopu II ($P<0.017$), and also tended to be higher than that of Crusader ($P=0.061$).

![Bar chart showing mean RLD and SRL for different clover entries at 400–500 mm.](image)

**Figure 4.9.** Mean ($\pm$SEM) root length density (RLD) (A) and specific root length (SRL) (B) at 400–500 mm as a proportion of the means at 50–100 mm for the six clover entries. Means with the same letter show no significant differences at the 5% level. Measurements were made at harvest 3 (170 days).

**Specific root length**

At 50–100 mm, the SRLs of the two *T. uniflorum* accessions did not differ, but both were significantly lower than in most other clover entries (Figure 4.8C). For example, SRL of accession 4382 was 53% lower than that of Crusader BC1 ($P=0.009$), 63% lower than that of Kopu II BC1 ($P<0.0001$), and 57% lower than that of Kopu II ($P=0.001$). At 400–500 mm there were no differences in SRL among all clover entries (Figure 4.8D).
The change in SRL from 50–100 mm to 400–500 mm differed among clover entries (Figure 4.9B). Both *T. uniflorum* accessions had more root length per unit of dry weight at 400–500 mm than at 50–100 mm, while the hybrids and white clover had less. These changes equate to an increase in SRL at 400–500 mm compared with 50–100 mm of 208% for accession 4382 and 163% for accession 4383. In comparison, Crusader BC1 and Kopu BC1 II had decreases with depth representing 81% and 77%, respectively, of the SRL at 50–100 mm; and SRL of Crusader and Kopu II decreased by 66% and 62%, respectively. As a proportion of the 50–100 mm zone, SRL of *T. uniflorum* accession 4382 tended to be greater than that of Crusader BC1 (*P*=0.046), Kopu II BC1 (*P*=0.032), Kopu II (*P*=0.012), and Crusader (*P*=0.061). The proportion of SRL at depth for accession 4383 was also greater than for both hybrids and both white clover cultivars (*P*<0.015).

### 4.3.6.1 Variability of clover entries

Variability of RLD and SRL was assessed in the 50–100 mm section (Table 4.7). The RLD of Crusader BC1 was more variable than that of the Crusader cultivar (*P*=0.001), but Kopu II BC1 did not differ to the Kopu II parent. Root length density of Kopu II BC1 was more variable than Crusader BC1 (*P*=0.024), and that of Kopu II was also more variable than Crusader (*P*<0.001). There were no differences among clover types in variability for SRL, although there was a trend for lower variability in accession 4383 compared with Crusader BC1 (*P*=0.079).

<table>
<thead>
<tr>
<th></th>
<th>Root length density (km m⁻³)</th>
<th>Specific root length (m g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. uniflorum</em> 4382</td>
<td>12.90abc</td>
<td>22.80⁵</td>
</tr>
<tr>
<td><em>T. uniflorum</em> 4383</td>
<td>7.72a</td>
<td>22.39⁵</td>
</tr>
<tr>
<td>Crusader BC1</td>
<td>14.44b</td>
<td>37.67⁵</td>
</tr>
<tr>
<td>Kopu II BC1</td>
<td>34.12c</td>
<td>23.70⁵</td>
</tr>
<tr>
<td>Crusader</td>
<td>3.77d</td>
<td>35.50⁵</td>
</tr>
<tr>
<td>Kopu II</td>
<td>19.27bc</td>
<td>28.66⁵</td>
</tr>
</tbody>
</table>

### 4.3.7 Differences among families within hybrid populations

The hybrid families were assessed for differences within each BC1 population. Means for shoot DW, root DW and root:shoot ratio, calculated over all harvests, did not differ between
the 80-2 and 900-4 families (Table 4.8), although there were trends towards a larger shoot DW and smaller root:shoot ratio for the 80-2 family in the Crusader BC₁ population.

Table 4.8. Log-transformed means (±SEM) for shoot dry weight (DW), root DW and root:shoot ratio, with P values, for families within BC₁ populations. Means are averaged over all harvests. Back-transformed means are presented in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Kopu II BC₁</th>
<th>Crusader BC₁</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80-2</td>
<td>900-4</td>
</tr>
<tr>
<td>Shoot DW (g)</td>
<td>-1.11</td>
<td>±0.185</td>
</tr>
<tr>
<td></td>
<td>±0.33</td>
<td>±0.23</td>
</tr>
<tr>
<td>Root DW (g)</td>
<td>-1.01</td>
<td>±0.153</td>
</tr>
<tr>
<td></td>
<td>±0.36</td>
<td>±0.27</td>
</tr>
<tr>
<td>Root:shoot</td>
<td>0.097</td>
<td>±0.049</td>
</tr>
<tr>
<td></td>
<td>±0.10</td>
<td>±0.14</td>
</tr>
</tbody>
</table>

However, at harvest 4, when plants were well established, there was a significant difference in root shape within both Kopu II BC₁ and Crusader BC₁, with family 80-2 having a higher β₃ value (root mass at 0–100 mm) (Table 4.9). As both families had the same Rᵢ value (rate of decrease in mass with depth) (see Table 4.6), the larger root mass at 0–100 mm for 80-2 would mean that this family had a significantly larger total root mass at harvest 4.

Table 4.9. Model values for β₃ (root mass at 0–100 mm) and Rᵢ (decrease in root mass with depth), with P values, for families within BC₁ populations at harvest 4 (237 days).

<table>
<thead>
<tr>
<th></th>
<th>Kopu II BC₁</th>
<th>Crusader BC₁</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80-2</td>
<td>900-4</td>
</tr>
<tr>
<td>β₃</td>
<td>3.289</td>
<td>0.010</td>
</tr>
<tr>
<td>Rᵢ</td>
<td>0.0293</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Analysis of the combined DW data (Kopu II BC₁ + Crusader BC₁) did show that both shoot DW and total root DW of the 80-2 family were greater than those of the 900-4 family at harvest 4 (Table 4.10). Shoot DW was three times larger in the 80-2 family, and root DW was two times larger.
Table 4.10. Mean log-transformed shoot and total root dry weight (±SEM) at harvest 4 (237 days), with P values, for the 80-2 and 900-4 families. Data for both BC₁ populations (Crusader BC₁ and Kopu II BC₁) are combined for each family. Back-transformed means are presented in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>80-2</th>
<th>900-4</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot dry weight (g)</td>
<td>2.76 ±0.276 (15.8)</td>
<td>1.66 ±0.276 (5.3)</td>
<td>0.011</td>
</tr>
<tr>
<td>Root dry weight (g)</td>
<td>2.13 ±0.218 (8.4)</td>
<td>1.39 ±0.218 (4.0)</td>
<td>0.028</td>
</tr>
</tbody>
</table>

Based on the combined data of the Crusader BC₁ and Kopu II BC₁ populations, RLD at 50–100 mm deep was also higher for the 80-2 family (by 2.5 times) (Table 4.11). In addition, SRL of the 80-2 family at 50–100 mm was higher than that of the 900-4 family (by 1.4 times) (Table 4.11). There were no differences between the families for RLD or SRL at 400–500 mm. Data for family differences in the change in RLD and SRL are presented in Appendix 18.

Table 4.11. Mean root length density (RLD) and specific root length (SRL) (±SEM), with P values, at 50–100 mm and 400–500 mm for 80-2 and 900-4 families at harvest 3 (170 days).

<table>
<thead>
<tr>
<th>Depth</th>
<th>80-2</th>
<th>900-4</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLD (km m⁻³)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50-100 mm</td>
<td>41.04 ±8.526</td>
<td>16.09 ±8.088</td>
<td>0.049</td>
</tr>
<tr>
<td>400-500 mm</td>
<td>0.48 ±0.125</td>
<td>0.54 ±0.119</td>
<td>0.696</td>
</tr>
<tr>
<td>SRL (m g⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50-100 mm</td>
<td>155.5 ±8.10</td>
<td>108.4 ±7.68</td>
<td>0.001</td>
</tr>
<tr>
<td>400-500 mm</td>
<td>105.6 ±15.52</td>
<td>99.2 ±14.72</td>
<td>0.767</td>
</tr>
</tbody>
</table>

4.4 Discussion

4.4.1 Plant growth

In this experimental system, *T. repens x T. uniflorum* interspecific hybrids had greater root and shoot masses than the white clover cultivars Kopu II and Crusader (Figures 4.2 – 4.4). Characteristics intermediate to the parents were expected for interspecific hybrids, particularly in early generations of backcrossing. Specifically, the hybrids were expected to be larger than *T. uniflorum* but smaller than white clover. Therefore, the small size of the white clover parents found in this experiment was unexpected. Higher growth rates of *T. uniflorum* and the BC₁ hybrid clovers may reflect some adaptation to low soil fertility or moisture deficit in the natural environment of the *T. uniflorum* parent. The tubes were well watered, which
eliminates moisture stress as a factor in this experiment. The nutrient solution used here is based on the composition of the typical soil solution in New Zealand pasture topsoils (Edmeades et al., 1985; Blamey et al., 1991), and is much less concentrated than traditional culture solutions. It is possible that white clover was nutrient limited by the use of this solution in sand culture, and that T. uniflorum was less limited because it may be adapted to resource-limited soils. This culture system has been used successfully for studying root depth distribution of forage grasses (Crush et al., 2005b; 2007), however grasses have a more finely divided root system than white clover, increasing their capacity to capture nutrients (Jackman and Mouat, 1972; Eissenstat, 1992). If low nutrient conditions were responsible for the low growth of Kopu II and Crusader in the current experiment, the performance of the hybrid clovers suggests that some adaptation to low soil fertility may have been introduced from the T. uniflorum parent.

### 4.4.2 Mineral nutrition

Reported concentrations of critical nutrient levels for white clover vary greatly depending on factors such as companion species, climate, plant age and the tissue sampled (Dunlop and Hart, 1987). It should be noted that apart from the observed growth effect, there were no signs of nutrient deficiency in any of the plants throughout this experiment. Phosphorus concentrations found in the current experiment – equivalent to 0.09–0.17% of DM (Table 4.5) – were considerably lower than critical levels of 0.3–0.4% reported for white clover in ryegrass pasture by McNaught (1970), but still generally within the ranges for white clover monoculture reviewed by Dunlop and Hart (1987) (0.1–0.25%). Similarly, the observed S concentrations of 0.14–0.16% DM (Table 4.5) were lower than critical levels reported for white clover in pasture by McNaught (1970) (0.25–0.3%) and Morton and Smith (2000) (0.23%), but within reported ranges for white clover monocultures of 0.1–0.29% (Dunlop and Hart, 1987).

N:S and N:P ratios in the current experiment, also indicate that S levels were adequate for growth of white clover, while P was not (Table 4.5). Ratios of N:P and N:S are often used to assess the adequacy of P and S, rather than absolute concentrations (Fageria, 2001). Dijkshoorn et al. (1960) and McNaught (1970) reported S deficiency in legumes and white clover, respectively, to occur where N:S ratios were greater than 18:1. Similarly, McNaught (1970) found that N:P ratios greater than 14:1 indicated P deficiency in white clover. Despite the low P supply, growth of T. uniflorum and Crusader BC₁ was greater than that of white clover. This suggests the P physiology of T. uniflorum and T. repens x T. uniflorum hybrids might differ to white clover. The critical P level of T. uniflorum, and its effect on the critical P
level of *T. uniflorum* x *T. repens* hybrids, is not known. Phosphorus concentrations and growth responses seen here could, therefore, indicate a lower critical P level for *T. uniflorum* and *T. uniflorum* x *T. repens* hybrids than for white clover. This would have a significant effect on the ability of hybrids to grow on marginal soils with low P content. Other aspects of P physiology, such as efficiency of P use and the relative size of different P fractions, could also play a part.

An adequate concentration of plant N is at least 4.5% for white clover (McNaught, 1970). Although the %N content of Crusader BC1 was significantly higher than its *T. uniflorum* and Crusader parents, all three clover types were still considerably below this critical level (1.92–2.39% DM) (Table 4.5). Despite this, *T. uniflorum* and Crusader BC1 grew well, and significantly more than the white clover cultivar, providing more evidence that this material may tolerate low fertility conditions. High P is essential for nodulation of legumes (O'Hara, 2001; Marschner, 1988), therefore the low N content in these plants may be a symptom of P deficiency. These interactions are complex, and not fully understood, but P limitation has been found to decrease nodulation and nodule growth in white clover (Almeida *et al.*, 2000; Høgh-Jensen *et al.*, 2002). Plants in the current experiment did have pink healthy nodules and, in some cases, large coralloid nodules, but nodule numbers, nodule mass and N fixation rates were not quantified. As with P, the critical %N content of *T. uniflorum* is not known. Although %N of *T. uniflorum* and Crusader BC1 was below critical white clover levels, this may still be adequate for this material.

Concentrations of K, Mg, Ca and Na differed in how they compared with reported critical levels for white clover. However, these four elements are known to interact (Fageria, 2001), with changes in concentration occurring depending on the concentrations of the other elements, to bring about a balance in cation content. For example, K decreases the concentration of Mg, Ca and Na (McNaught, 1970; Fageria, 2001). The effect of such interactions in the current experiment is unknown. However, concentrations of Ca (1.5–2.8% DM) (Table 4.4) were well above reported critical levels for white clover growth (McNaught, 1970; Dunlop and Hart, 1987; Edmeades and Perrott, 2004). Concentrations of Mg (0.26–0.3% DM) (Table 4.5) were also well above the white clover critical levels reported by McNaught (1970) (with ryegrass) and Dunlop and Hart (1987) (monoculture), and slightly above the critical minimum concentration for mixed herbage (0.2% DM) (Edmeades, 2004). Both K and Na function in osmotic adjustment of plants under water stress (Iannucci *et al.*, 2002; Zheng *et al.*, 2010). The higher levels of K and Na found here for *T. uniflorum* and
Crusader BC₁ (Table 4.5) could, therefore, indicate a greater potential adaptation to water stress environments in these plants than in white clover.

Among the micronutrients, concentrations of Fe, Mn, Mo, B, Cu and Zn were also well above critical levels for white clover (McNaught, 1970). Significantly higher concentrations of Cr, Ni, Al, Co, Cu, Fe and Zn in *T. uniflorum* suggest this species may have a greater ability to accumulate minor elements and metals (Table 4.5). However, only Cu, Mn and Zn were also higher in Crusader BC₁ than in the Crusader parent (Tables 4.4 and 4.5). High manganese levels can be toxic to plant tissues but the concentrations found here in *T. uniflorum* and the Crusader hybrid (up to 136 ppm) were still well below the white clover toxicity thresholds reported by Smith *et al.* (1983) (570 ppm) and Andrew and Hegarty (1969) (650 ppm).

Mineral profiles of *T. uniflorum* and *T. repens x T. uniflorum* hybrids have not been reported before. The current work provides the first information on mineral nutrient concentrations, and how they compare with white clover and white clover critical levels. However, much more research is required to understand the biological significance of the concentrations and differences observed here.

### 4.4.3 Root system shape and distribution

Root system shape and root depth distribution characteristics of the hybrids were more similar to the *T. uniflorum* parent (Table 4.6 and Figure 4.6) than to white clover – despite the BC₁ hybrid genomes being 75% white clover. In particular, *T. uniflorum* and the hybrids had a higher proportion of their total root mass high in the profile, shown by lower Rj values than for white clover (Table 4.6) and, in the case of *T. uniflorum*, by a higher root mass at 100-200 mm (Figure 4.6B). van Wijk (2011) modelled the effects of rooting strategies, soil type and rainfall on transpiration of herbaceous plants, and suggested that as rainfall decreases, evaporation is a more important source of water loss than drainage. As a result, roots are distributed higher in the profile to maximise water uptake. This suggests that adaptation of *T. uniflorum* to moisture stress may be a determining factor in root distribution of the hybrids. Soil type and root competition can also affect root distribution (van Wijk, 2011). Shallow rooting can also be advantageous for P acquisition as most soil P is concentrated near the surface (Ge *et al.*, 2000; Lynch and Brown, 2001). In addition to soil moisture, the root distribution of *T. uniflorum* and, therefore, the *T. repens x T. uniflorum* hybrids, may also reflect the soil fertility status of *T. uniflorum*’s natural environment.

The lower proportion of roots at 100–200 mm for white clover (Figure 4.6B) must be offset by root mass deeper in the profile as the proportion of roots at 0–100 mm did not differ to *T.
uniflorum and the hybrids (Figure 4.6A). The slower rate of decrease in root mass with depth (Table 4.6) also suggested that more roots should occur at depth for white clover than for T. uniflorum and the hybrids. There were indications that this may have been the case for Kopu II compared with Kopu II BC₁, but the proportion of root mass at depth for Crusader did not differ to that of Crusader BC₁ (Figure 4.6C).

The impact of nutrient availability on the observed patterns of root shape and distribution is unknown. However, the potential differences in critical internal P levels among the clover types are unlikely to have affected the partitioning of roots down the profile. Differences in the values of root mass at 0–100 mm, derived from the model, reflect differences in plant size and were likely to have been influenced here by the effects of nutrient supply on growth. Although the proportion of roots at 0–100 mm did not differ, the higher absolute mass of T. uniflorum and the hybrids in the upper profile in this growth system would have increased their ability to capture P.

The accumulation of roots at 1 m by at least some plants of all entries suggests the capability to penetrate to greater depths (Figure 4.5). The deeper mean maximum rooting depths of the hybrid populations at each harvest also indicates that roots of these plants may penetrate deeper overall than white clover, and therefore access deeper soil water (Figure 4.7). This trait appears to have been inherited from the T. uniflorum parent, which always had the greatest maximum rooting depth. Performance of T. repens x T. uniflorum hybrids under drought conditions is examined in Chapter 7 and 8. The faster penetration by roots of T. uniflorum and hybrid clovers, compared with the white clover parents, could aid establishment under dry soil conditions. T. uniflorum is adapted to a very dry environment. It germinates with autumn rain and requires rapid early growth to access deeper water before the soil dries out again (W. Williams pers. comm.). The seed of T. uniflorum is larger than that of white clover (Gibson et al., 1971), a trait which has been associated with larger, more vigorous, seedlings (Moot et al., 2000), and may aid establishment.

### 4.4.4 Root morphology

Generally, higher SRL values indicate thinner roots, assuming equal tissue densities (Eissenstat, 1992). Specific root length data in this experiment suggests that the T. uniflorum accessions had thicker roots at 50–100 mm than white clover (Figure 4.8C). However, the changes in SRL at 400–500 mm – increasing for the T. uniflorum accessions and decreasing for the hybrids and white clover – suggest that T. uniflorum had more of its fine root mass at depth, while hybrids and white clover had more of their fine root mass higher in the profile.
Specific root length data also suggests the roots of Kopu II were finer than those of Crusader. The thicker roots of *T. uniflorum* at 50–100 mm should be a disadvantage for nutrient uptake (Eissenstat, 1992), although finer roots at depth would increase the capture of leaching nutrients. The greater RLD observed at 400–500 mm for *T. uniflorum* would also increase interception of nutrients, through increased exploration of the growth media (Figure 4.8B). This could have contributed to the higher growth of *T. uniflorum* compared with white clover, and to its higher P content compared with the Crusader hybrid. However, it does not explain the growth difference between the Crusader hybrid and its white clover parent, which had the same SRL pattern (Figures 4.8 and 4.9). This indicates that the suggested difference in P physiology compared with white clover may have influenced growth of these plants more than differences in root morphology did.

At 50–100 mm depth, a higher RLD and SRL in the 80-2 families could have increased the nutrient uptake of these plants (Table 4.11). However, there were few differences in shoot mineral content between the 80-2 and 900-4 families, and these did not include major elements. This further highlights the potentially smaller influence of root morphology compared with inherent differences in P physiology and nutrient requirements for this hybrid material.

### 4.4.5 Genotypic variation

The presence of differences between families with different F1 parents should be considered in future screening work, as it appears the background of the F1 parents may have a considerable influence on phenotype (Tables 4.8 – 4.11). It is unclear whether these differences were a result of traits from the *T. uniflorum* parent or the original white clover parent, which both contribute 25% of the genes in the BC1 generation. The consistency of the relative differences between the 80-2 and 900-4 families suggests that the *T. uniflorum* parent was responsible. However, the two *T. uniflorum* accessions in the current experiment were the *T. uniflorum* parents of the families used, and while the accessions themselves did not show any differences in the measured characteristics (Figures 4.2 – 4.4, Table 4.6, Figures 4.8 and 4.9), there were differences between families (Tables 4.8 – 4.11). It is not known how hybridisation affects the expression of genes in this material. Further work may be necessary to understand the influence of the parents in the F1 generation, and research to specifically study differences between related families would be valuable. This would not only assist in interpreting the results of future work, but would also aid the selection of material for breeding. In the meantime, the potential effect of F1 genotypes should be taken into consideration when studying other hybrid characteristics. It is also possible that the family
differences observed here represent transgressive segregation, where hybrids express characteristics outside the normal range of either parent (Grant, 1975; Rieseberg et al., 1999).

4.4.6 The effect of variability

Several large differences in RLD and SRL were only close to statistical significance. The sizes of the standard errors were often considerable, and variability of the hybrid material may also play a part in the outcome of the analyses. For example, the significantly higher variability for Crusader BC1 compared to Crusader (Table 4.7) may be responsible for the absence of a significant difference in RLD between these entries at 50–100 mm (Figure 4.8A). For some root traits, higher replication may be necessary to account for the variability of hybrid material. As different hybrid genotypes contain different combinations of *T. uniflorum* genes, it is expected that the hybrids will be very variable. In the standardised conditions of the current experiment, shoot and root DW of the hybrid clovers were indeed more variable than those of the white clover parents (Table 4.3).

4.5 Conclusions

- Root system shape and depth distribution of *T. repens* x *T. uniflorum* interspecific hybrids were strongly influenced by *T. uniflorum*. Differences in root shape compared with white clover occurred mainly in the upper part of the profile, and may reflect the soil moisture and soil fertility of *T. uniflorum*’s natural environment. *T. uniflorum* and hybrid roots penetrated faster to depth than white clover, which could aid establishment and lead to greater maximum rooting depths – and therefore access to deeper soil water.

- Growth of the white clover cultivars was relatively poor. This was different from what was expected, and in contradiction to the growth results reported in Chapter 3. Further study is required to confirm this result (see Chapter 5).

- Shoot mineral and elemental analyses indicate that P, and perhaps N, were limiting for white clover in this system, suggesting that the hybrids may have inherited the ability to tolerate low fertility conditions from *T. uniflorum*. The mechanism by which this would occur is unknown, and further study on the P physiology of this material is required. P and N herbage contents were below white clover critical levels, but may still be adequate for *T. uniflorum* and *T. repens* x *T. uniflorum* hybrids.

- Aspects of root mass distribution and morphology could influence the ability of *T. uniflorum* and some hybrids to intercept nutrients, but differences in P physiology appear more likely to be responsible for the growth effects observed. However, root morphology
was examined for only part of the root system. More information on the relative abilities of the different clovers to intercept nutrients would be gained by study of the morphology of whole root systems.

- Differences between hybrid families, possibly arising from differences in F$_1$ parents, also warrant further investigation.
Chapter 5  
Nutrient effects on growth

5.1 Introduction

The previous experiment (Chapter 4), suggested that *T. repens* x *T. uniflorum* BC\textsubscript{1} (backcross 1) hybrids may have higher growth than white clover at low nutrient levels, particularly under limiting P. If so, this would have major implications, both for the use of these hybrids in nutrient limited environments and for the reduction of mineral fertilisers in more favourable environments. In turn this could positively affect farm profitability and reduce the environmental impacts of agriculture. The first objective of this experiment was to quantify and compare the growth of BC\textsubscript{1} hybrids and white clover cultivars at different nutrient levels. The second objective was to determine whether the growth difference between BC\textsubscript{1} hybrids and white clover under low nutrient conditions was repeatable. The low ionic strength nutrient solution which was used in the previous experiment has been successful in similar work with grasses (Crush *et al.*, 2005b; 2007), but may not be appropriate for white clover. Compared with white clover, the roots of grasses are better adapted to acquire nutrients (Jackman and Mouat, 1972; Dunlop and Hart, 1987). It was hypothesised that growth of the clover entries would not differ using a concentrated nutrient solution, but that growth of the BC\textsubscript{1} generation would be higher than that of white clover using lower concentration solutions.

As outlined in Chapter 2 (Section 2.3), soil P is one of the major limiting factors for white clover growth. This is in part due to poor competition for P against the finer, higher density (per volume of soil) roots of forage grasses, but is also attributable to aspects of the P physiology of white clover. Attempts to improve the P-efficiency of white clover in the field, through selection of superior genotypes under controlled conditions, have been unsuccessful (Caradus, 1994a; Caradus and Dunn, 2000). One factor which may contribute to inefficient use of absorbed P in white clover, compared to species such as lotus, is its high levels of stored inorganic P (P\textsubscript{i}) in the vacuoles (Hart and Jessop, 1983). The final objective of this study was, therefore, to measure shoot mineral concentrations, including P\textsubscript{i}, at these different nutrient levels and to compare BC\textsubscript{1} hybrids with white clover. It was hypothesised that clover types would differ in the shoot concentrations of some minerals but, most importantly, that concentrations of key minerals such as P, under limiting nutrient supply, would be below the levels required for adequate white clover growth. In addition, it was hypothesised that leaf P\textsubscript{i} concentrations of hybrids and white clover would differ.
5.2 Materials and methods

5.2.1 Experimental setup

The experiment was oriented north-south in a glasshouse at AgResearch, Ruakura Research Centre, Hamilton (37° 46′ 23.58"S, 175° 18′ 22.47"E) and was conducted from 2 September 2010 to 19 January 2011. Supplementary lighting was used from 6–8 am and 5–6 pm to provide a 12 hour day length, up until 1 December. Mean day/night temperatures over the duration of the experiment were 22/16°C; mean day/night temperatures and maximum daytime solar radiation are shown in Figure 5.1. Temperature and radiation data were recorded by the glasshouse control system on a weekly basis.

![Figure 5.1. Weekly mean day/night temperatures and maximum daytime solar radiation during the experimental period.](image)

PVC pots with a base diameter of 120 mm and an upper diameter of 145 mm were filled with mortar sand, leaving a 10 mm lip for water and nutrient application. The volume of sand in each pot was 1.24 l, with a water holding capacity of 410 ml l⁻¹. Water holding capacity was calculated from the drained wet weight of five 100 g samples of air dried sand.

5.2.2 Plant material

The clover entries used in the previous experiment, in which growth effects were observed, were used as the basis for the selection of plant material for the current experiment. Parental material included one T. uniflorum accession and two white clover cultivars (Crusader and
Kopu II), while the BC₁ generation was represented by four family bulks to account for the potential family differences seen in the previous experiment (Table 5.1). Two of these families were derived by backcrossing to the Crusader parent and two to the Kopu II parent. For each backcross parent (i.e. Crusader and Kopu II), two F₁’s with different *T. uniflorum* accessions as the original male parent were used. One Crusader BC₁ and one Kopu II BC₁, from the F₁ 900-4, were used in the previous experiment (Chapter 4). The *T. uniflorum* parent of this F₁ was accession AZ4383. The remaining Crusader BC₁ was derived from the F₁ 487-7, and the remaining Kopu II BC₁ was derived from the F₁ 487-9. These families were used in place of the 80-2 families from the previous experiment, for which there was no longer seed available, as they had the same *T. uniflorum* parent in the F₁ (accession AZ4382).

**Table 5.1. Clover entries used in the nutrient experiment. cv = cultivar; OP = open pollinated.**

<table>
<thead>
<tr>
<th>Entry number</th>
<th>Clover type</th>
<th>Description</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>White clover</td>
<td>cv. Crusader</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>White clover</td>
<td>cv. Grasslands Kopu II</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>T. uniflorum</em></td>
<td>AZ4383# OP</td>
<td>Turkish origin</td>
</tr>
<tr>
<td>4</td>
<td>BC₁</td>
<td>Crusader x 487-7†</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>BC₁</td>
<td>Crusader x 900-4‡</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>BC₁</td>
<td>Kopu II x 487-9†</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>BC₁</td>
<td>Kopu II x 900-4‡</td>
<td></td>
</tr>
</tbody>
</table>

# Accession number, Margot Forde Germplasm Centre (Palmerston North, New Zealand) – shortages supplemented with AZ4382 OP (Greek origin).
† 487-7 and 487-9 = Pitau-1 x AZ4382.
‡ 900-4 = Kopu II-2 x AZ4383-11.

Seed was scarified with sandpaper and germinated on damp filter paper in Petri dishes on 27 August 2010, and seedlings were planted into the pots at 10–20 mm radicle length. Planting took place over four days from 2–6 September 2010. Insufficient seed germinated for three clover entries, and these gaps were filled with spare seedlings.

Seedlings were inoculated with *Rhizobium* spp. on 8 September 2010 using a suspension of “Nodulaid” moist peat inoculant for white, red and strawberry clover (Becker Underwood Pty. Ltd., Somersby, Australia). Two grams of inoculant was mixed with a 25% sucrose solution and 1 ml of this liquid was pipetted around the base of each seedling.
5.2.3 Nutrients

Three nutrient solutions were used, to provide contrasting levels of nutrient concentration:

1. A complete Long Ashton nutrient solution based on Hewitt (1966) (Appendix 17), referred to here as “Complete”.

2. The low ionic strength solution used in the previous experiment (Chapter 4), in which the growth difference between white clover and the hybrids was observed (Appendix 15), referred to as “LIS”. The chemistry of this solution was based on the average soil solution of a range of New Zealand pasture topsoils (Edmeades et al., 1985; Blamey et al., 1991).

3. A 50% dilution of the low ionic strength solution, referred to as “1/2 LIS”.

The Complete and LIS solutions were made up in 45 l quantities, following the methods in Appendix B, and pH was maintained at 6–6.5 using additions of 20% NH₄ solution or 30% HCl. Deionised water was used to dilute the LIS 50:50 as needed. Unused solution was discarded after 10–14 days.

An equivalent volume of nutrients to that used in the first experiment was applied to each pot. The volume applied to the tubes in Chapter 4 was equal to 3.4% of the maximum waterholding capacity. For the current experiment, the equivalent volume was 17.3 ml, but for practicality, 20 ml of nutrients was applied to each pot using a 20 ml plastic scoop. This was equal to 3.9% of the potential volume of water in the sand. Nutrients were applied three days per week, and on remaining days all pots received deionised water. Initially, 50 ml of deionised water was applied to each pot on watering days. This volume was increased to 100 ml per pot from 18 November 2010, as growth rates and ambient temperatures increased. From this time, a further 80 ml of deionised water was also applied prior to the application of nutrients, so that a total of 100 ml of liquid was applied on all days.

5.2.4 Experimental design

The experimental layout was a row-column factorial design, with 10 replicates of the seven clover entries in each nutrient treatment. Plants were distributed across three tables in the glasshouse, with four replicates of each clover entry x nutrient treatment combination on each of two tables, and two replicates on the third table (Figure 5.2). Where there were shortages of seed, missing replicates of these entries were replaced with spare seedlings of other entries, as indicated in Figure 5.2. The planting order within each entry was pre-determined, so as to distribute missing plants evenly across both the layout and across nutrient treatments. The
spare plants were included in the final data analysis, and replacements for entry 3 (*T. uniflorum* 4383) were treated as an extra entry (see Table 5.1 and Figure 5.2).

Table 3 Table 2 Table 1

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>6 (5)</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>6 (5)</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 5.2. Experimental layout for the nutrient experiment, showing the arrangement of clover entries and nutrient treatments. Row and column numbers for each table are also shown. Entry numbers correspond to those shown in Table 5.1.

Complete LIS 1/2 LIS

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>6 (5)</td>
<td>3</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>7</td>
<td>6 (7)</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>3 †</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>6 (7)</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>6 (7)</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>7</td>
<td>3</td>
<td>6 (7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>5</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>7</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>6 (5)</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>3 †</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>6</td>
<td>7</td>
<td>4</td>
<td>3 †</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>6 (7)</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>6 (7)</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>7</td>
<td>3</td>
<td>6 (7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>7</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>6 (5)</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>7</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>5</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>7</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>6 (5)</td>
<td>5</td>
<td>2</td>
<td>3 †</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>6 (5)</td>
<td>5</td>
<td>2</td>
<td>3 †</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>4 (5)</td>
<td>7</td>
<td>2</td>
<td>6</td>
<td>5</td>
<td>3 †</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>4</td>
<td>6 (5)</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>14</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>6 (5)</td>
</tr>
</tbody>
</table>

5.2.5 Measurements

Prior to harvest all plants were scored visually for stress pigmentation on a scale of 1 to 5 (low to high severity), depending on the degree of red, yellow and/or bronze pigmentation of the leaves. Plants were then harvested on 18–19 January 2011. Sand was first removed from the roots by immersing the plants in water, with a second rinse to remove any remaining fine particles. Nodulation of each plant was scored on a scale of 1 to 5 (low to high nodulation). Roots and shoots were separated, with shoot material divided into leaf (petiole+lamina) and stolon where appropriate. Shoots of small plants, in which the formation of stolons had not begun, were left intact. As concentrations of P and N can differ between leaves and stolons (Caradus, 1992), only leaf material (lamina + petiole) was analysed for mineral and N concentrations. Leaf samples were frozen immediately in liquid N to halt P enzyme activity, transferred to a freezer and subsequently freeze dried for 60 hours. As stolon and root samples were not used for mineral and elemental analyses, they were oven dried overnight (15 hours). The oven temperature was set to 100°C for rapid drying, to minimise enzyme P activity (Bollons and Barraclough, 1997). All samples were weighed to obtain total shoot dry weight (DW) and root DW.
Leaf samples were ground and analysed for Al, As, C:N, Ca, Co, Fe, K, Li, Mg, Mn, Mo, %N, Na, P, Pb, S, and Zn using ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometer) (Varian Australia Pty. Ltd., Melbourne). Inorganic P was analysed using the water extraction method of Zohlen and Tyler (2004). All analyses were performed by Analytical Services, Faculty of Agriculture and Life Sciences, Lincoln University. Leaf samples of many plants were of an inadequate size for analysis (<300-400 mg for total minerals and %N), particularly in LIS and ½ LIS treatments. A subset of clover entries was therefore selected for analysis, which provided the most balanced replication across clover entries and nutrient treatments at the maximum possible replication. In addition, the entries provided groups of material related either through a white clover parent or a common F1 parent. Total mineral concentrations, including total P and inorganic P, were analysed in Kopu II, Kopu II x 487-9, Kopu II x 900-4 and Crusader x 900-4 in all treatments. Leaf %N content and the C:N ratio were analysed in all treatments in the Kopu II x 900-4 family only, due to the amount of dried material required for analysis (200 mg). The number of replicates analysed for each clover entry/nutrient treatment combination are shown in Table 5.2.

P and Pi use-efficiency were calculated as DW accumulated per unit of P or Pi (leaf DW/P concentration and leaf DW/Pi concentration). The fraction of Pi in the total P pool was also calculated.

Table 5.2. Number of replicates sampled for shoot elemental analyses, for the selected clover entries in the three nutrient treatments. LIS = low ionic strength. Replicates >10 represent extra plants used to replace missing replicates of other entries.

<table>
<thead>
<tr>
<th></th>
<th>½ LIS</th>
<th>LIS</th>
<th>Complete</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kopu II</td>
<td>8</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Kopu II x 487-9</td>
<td>6</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Kopu II x 900-4</td>
<td>11</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Crusader x 900-4</td>
<td>8</td>
<td>11</td>
<td>13</td>
</tr>
</tbody>
</table>

5.2.6 Statistical analysis

Data were analysed with linear mixed models (LMM) fitted using REML in Genstat version 13 (VSN International Ltd.), and means were compared using the 5% LSDs. Tables, plus row and column numbers within tables, were included as random effects to account for variability due to the experimental layout. All data, except for Pi and fraction Pi, were log-transformed to account for non constant variance. Results are presented on the log scale to allow for more
accurate presentation of the standard error of the mean (SEM), and back-transformed means are also shown. Spare plants that were included to replace missing replicates were included in the final analysis, with those for *T. uniflorum* accession AZ4382 representing an extra clover entry.

Shoot DW, root DW and root:shoot ratios were analysed separately for both the full data set and the plants selected for mineral and elemental analyses. This allows for more accurate conclusions to be made about the effect of shoot mineral/element concentrations on plant size.

For the full DW data set, plus nodulation and pigmentation scores, clover entries were divided into three groups based on either species (*T. uniflorum* 4383 and 4382) or white clover parent (Crusader, Crusader x 487-7 and Crusader 900-4; or Kopu II, Kopu II x 487-9 and Kopu II x 900-4). A series of LMMs were fitted sequentially, dropping insignificant terms. A 3-way model was fitted first, with clover entries nested within clover groups to determine whether the effect of nutrient treatment differed among entries within groups. This model was denoted by “(clover group|entry) x nutrient treatment”. Where this was not significant at the 5% level a 2-way model was fitted. When the clover group x entry interaction was not significant at the 5% level, this was dropped from the 2-way model and a clover entry x nutrient treatment only model was applied. In turn, if this interaction was not significant a model with additive clover entry and nutrient treatment effects was fitted (clover entry + nutrient treatment). Any insignificant individual terms (clover entry or nutrient treatment) were subsequently dropped from this model. For shoot elemental data and the subset DW data, the clover entry x nutrient treatment model was fitted, followed by the additive model or individual terms as described above.

In graphs and tables, means with the same letter were not significantly different at the 5% level, using the means separation methods stated above for the respective traits. Differences in variability among relevant clover entries for key parameters were assessed using a test for equal variances in Minitab version 15 (Minitab Inc.). This compares two variances using an F-test or Levene’s test, depending on the normality distribution of the data. Where data were log-transformed for REML, untransformed data were used in the variance tests. Standard deviations are presented to indicate the relative size of the variance for each clover entry and significant differences between clover entries are indicated with lettering, as mentioned above.
5.3 Results

5.3.1 Dry weights

Main effects for shoot DW, root DW and root:shoot ratio are presented in Table 5.3

Table 5.3. Main effects for shoot dry weight (DW), root DW and root:shoot ratio. P values are presented for the linear mixed models fitted, with insignificant terms dropped sequentially. The selected model for each parameter is shaded in grey.

<table>
<thead>
<tr>
<th>Term tested</th>
<th>3 way model</th>
<th>2 way model</th>
<th>Full model</th>
<th>Additive model</th>
<th>Additive model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clover group x entry x nutrient treatment</td>
<td>0.065</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clover group x entry</td>
<td>0.040</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clover entry x nutrient treatment</td>
<td>0.641</td>
<td>0.098</td>
<td>0.579</td>
<td>&lt;0.001</td>
<td>0.033</td>
</tr>
</tbody>
</table>

5.3.1.1 Shoot dry weight

Interactions

There was no clover group x entry x nutrient interaction for shoot DW, although there was a trend (Table 5.3) towards smaller changes in DW with nutrient treatment for some clover entries (Figure 5.3A). For example, relative to the Complete treatment, shoot DW of Kopu II x 900-4 was 53% smaller in the LIS treatment and 61% smaller in the ½ LIS, compared with 66% and 83% smaller, respectively, for Kopu II x 487-9 and 87% and 80% smaller, respectively, for Kopu II. Back-transformed means are presented in Table 5.4.
Figure 5.3. Mean log shoot (A) and root dry weight (B) (±SEM) of the eight clover entries in the Complete, LIS and ½ LIS nutrient treatments. LIS = low ionic strength. Lower case letters indicate comparisons between nutrient treatments within each clover entry; upper case letters indicate comparisons between clover entries in the relevant group (Crusader, Kopu II, *T. uniflorum*) within nutrient treatments. Means with the same letter show no significant differences at the 5% level.
Table 5.4. Back-transformed means for shoot and root dry weight of the 8 clover entries in the Complete, LIS, and ½LIS nutrient treatments. LIS = low ionic strength.

<table>
<thead>
<tr>
<th>Clover entry</th>
<th>Shoot dry weight (g)</th>
<th>Root dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complete</td>
<td>LIS</td>
</tr>
<tr>
<td>Crusader</td>
<td>1.640</td>
<td>0.174</td>
</tr>
<tr>
<td>Crusader x 487-7</td>
<td>1.353</td>
<td>0.341</td>
</tr>
<tr>
<td>Crusader x 900-4</td>
<td>1.956</td>
<td>0.361</td>
</tr>
<tr>
<td>Kopu II</td>
<td>1.976</td>
<td>0.254</td>
</tr>
<tr>
<td>Kopu II x 487-9</td>
<td>2.300</td>
<td>0.774</td>
</tr>
<tr>
<td>Kopu II x 900-4</td>
<td>2.910</td>
<td>1.355</td>
</tr>
<tr>
<td>T. uniflorum 4383</td>
<td>1.164</td>
<td>0.145</td>
</tr>
<tr>
<td>T. uniflorum 4382</td>
<td>0.613</td>
<td>0.122</td>
</tr>
</tbody>
</table>

**Overall differences among entries within clover groups**

In contrast to the clover group x entry x nutrient interaction, the clover group x entry interaction for shoot DW was significant (Table 5.3). Data showed that the Kopu II hybrids were 1.5 – 2.8 times heavier than Kopu II (based on back-transformed means) across all nutrient treatments (Figure 5.4). Kopu II x 900-4 shoot DW was also 1.8 times larger than that of Kopu II x 487-9 (Figure 5.4). There were no significant differences in shoot DW among Crusader and its hybrids, or between the two *T. uniflorum* accessions (Figure 5.4).

![Graph showing mean log shoot dry weight (SEM) of clover entries across nutrient treatments](image)

**Figure 5.4.** Mean log shoot dry weight (±SEM) of the eight clover entries across nutrient treatments. Numbers in parentheses are back-transformed means (g). Means with the same letter within groups (indicated by shading) show no significant differences at the 5% level.
5.3.1.2 Root dry weight

Interactions

The clover group x entry x nutrient interaction was significant for root DW (Table 5.3, Figure 5.3B). Root DWs of all entries were smaller in the ½ LIS and LIS treatments than in the Complete treatment, but did not differ between these two lower strength treatments. Root DW of the hybrids generally decreased less than that of the white clover parents. For example, in the LIS treatment, root DW of Kopu II x 900-4 decreased by 52% compared with the Complete treatment, while root DW of Kopu II x 487-9 and Kopu II decreased by 66% and 84%, respectively. In the ½ LIS treatment the decrease in root DW, compared with the Complete treatment, was also much smaller for Kopu II x 900-4 (-62%) than for Kopu II x 487-9 (-82%) and Kopu II (-77%). Within the Crusader group, root DW of both Crusader 900-4 (-80%) and Crusader x 487-7 (-78%) decreased less than that of the Crusader parent (-87%) between the Complete and LIS treatments. Crusader x 487-7 (-64%) decreased its root DW less than Crusader 900-4 (-85%) and Crusader (-84%) between the Complete and ½ LIS treatments. Back-transformed means are presented in Table 5.4.

Changes in nutrient treatment affected the root DW of *T. uniflorum* accession 4382 less than for accession 4383. Compared with the Complete treatment, root DW of accession 4382 decreased by 78% and 73%, in the LIS and ½ LIS treatments respectively, compared with 89% and 85%, respectively, for accession 4383 (Figure 5.3B).

Differences among entries within clover groups in each treatment

Root DW of the white clover cultivars and their respective hybrids did not differ in the Complete treatment, but there were differences in the lower strength nutrient treatments (Figure 5.3B). In the LIS treatment, root DW of Crusader x 900-4 was 1.8 times larger than that of the Crusader parent; and in the ½ LIS treatment, root DW of Crusader 487-7 was 2 times larger than Crusader x 900-4 and 2.3 times larger than Crusader. Root DW of both Kopu II x 487-9 and Kopu II x 900-4 were larger than that of Kopu II in the LIS treatment (by 3.3 and 5.2 times respectively), but Kopu II x 900-4 was larger than both Kopu II and Kopu II x 487-7 in the ½ LIS treatment (by 2.9 and 2.3 times respectively). Root DW of the two *T. uniflorum* accessions did not differ among the three nutrient treatments (Figure 5.3B)

5.3.1.3 Root:shoot ratio

The additive model showed significant overall clover entry and nutrient treatment effects for root:shoot ratio (Table 5.3). Root:shoot ratios of the *T. uniflorum* accessions were higher than those of all the other clover entries by 31–58%, and the ratio for Kopu II x 487-9 was 21% higher than that of Kopu II and 16% higher than that of Kopu II x 900-4 (Figure 5.5A). Mean
The root:shoot ratio in the $\frac{1}{2}$ LIS treatment was 12% higher than in the Complete treatment (Figure 5.5B).

**Figure 5.5.** Mean log root:shoot ratio (±SEM) by clover entry (A) and nutrient treatment (B). Numbers in parentheses are back-transformed means. LIS = low ionic strength. Means with the same letter indicate no significant differences at the 5% level.

### 5.3.1.4 Variability of clover entries

Shoot DW showed no differences in variability among clover entries in the Complete treatment, but the hybrids were generally more variable than their respective parents in the LIS and $\frac{1}{2}$ LIS treatments (Table 5.5). Root DW of the hybrids was also generally more variable than their respective parents in all treatments (Table 5.5). Where these differences were not significant, the standard deviations of the hybrids (as an indicator of variability) were still higher than those of the white clover parents. The white clover cultivars and their
respective hybrids did not differ in variability for root:shoot ratio in any of the nutrient treatments (Table 5.5).

Table 5.5. Standard deviations for shoot dry weight (DW), root DW and root:shoot ratio of Crusader and Kopu II and their respective hybrids in the Complete, LIS and ½ LIS nutrient treatments. LIS = low ionic strength. Clover entries with the same letter, within parameters, show no significant differences in variability at the 5% level within the Crusader or Kopu II groups.

<table>
<thead>
<tr>
<th></th>
<th>Complete</th>
<th>Crusader</th>
<th>Crusader x 487-7</th>
<th>Crusader x 900-4</th>
<th>Kopu II</th>
<th>Kopu II x 487-9</th>
<th>Kopu II x 900-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot DW (g)</td>
<td></td>
<td>0.843a</td>
<td>1.766a</td>
<td>1.018a</td>
<td>2.108a</td>
<td>1.553a</td>
<td>1.810a</td>
</tr>
<tr>
<td>Root DW (g)</td>
<td>0.375a</td>
<td>0.217a</td>
<td>0.202a</td>
<td>0.170a</td>
<td>0.683a</td>
<td>1.011ab</td>
<td>1.457b</td>
</tr>
<tr>
<td>Root:shoot</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.275a</td>
<td>0.117a</td>
<td>0.154a</td>
</tr>
<tr>
<td>LIS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoot DW (g)</td>
<td>0.120a</td>
<td>0.335b</td>
<td>0.263b</td>
<td>0.125a</td>
<td>0.125a</td>
<td>0.615b</td>
<td>1.413b</td>
</tr>
<tr>
<td>Root DW (g)</td>
<td>0.078a</td>
<td>0.155a</td>
<td>0.179a</td>
<td>0.176a</td>
<td>0.081a</td>
<td>0.418b</td>
<td>0.761b</td>
</tr>
<tr>
<td>Root:shoot</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.131a</td>
<td>0.088a</td>
<td>0.185a</td>
</tr>
<tr>
<td>½ LIS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoot DW (g)</td>
<td>0.148a</td>
<td>0.762b</td>
<td>0.853b</td>
<td>0.539a</td>
<td>0.523a</td>
<td>1.104b</td>
<td></td>
</tr>
<tr>
<td>Root DW (g)</td>
<td>0.097a</td>
<td>0.282a</td>
<td>0.249a</td>
<td>0.288a</td>
<td>0.317b</td>
<td>0.583b</td>
<td></td>
</tr>
<tr>
<td>Root:shoot</td>
<td>0.206a</td>
<td>0.282a</td>
<td>0.249a</td>
<td>0.138a</td>
<td>0.128a</td>
<td>0.116a</td>
<td></td>
</tr>
</tbody>
</table>

5.3.1.5 Subset of plants for mineral and elemental analyses

Shoot and root DW results from the subset of plants used for mineral and elemental analyses were similar to those for the full data set (Figure 5.6). Back-transformed means are presented in Appendix 19. As with the full data set, there was a significant clover entry x nutrient treatment interaction for root DW ($P<0.001$), but there was also a significant interaction for shoot DW ($P<0.001$). The effect of nutrient treatment was much smaller for the Kopu II hybrids than the Kopu II parent, particularly for Kopu II x 900-4 (Figures 5.6A and 5.6B). Compared with the Complete treatment, shoot DW in the LIS treatment decreased by 52% for Kopu II x 900-4, 66% for Kopu II x 487-9, 82% for Crusader x 900-4 and 91% for Kopu II. The decrease in shoot DW from the Complete treatment to the ½ LIS treatment was also much smaller for Kopu II x 900-4 (61%) than for Kopu II, Kopu II x 487-9 and Crusader x 900-4 (82–84%).
Figure 5.6. Mean log shoot (A) and root dry weight (B) (±SEM) for clover entries sampled for shoot elemental analyses in the Complete, LIS and ½ LIS nutrient treatments. LIS = low ionic strength. Lower case letters indicate comparisons between nutrient treatments within each entry; upper case letters indicate comparisons between clover entries within nutrient treatments. Means with the same letter show no significant differences at the 5% level.

Results for the root:shoot ratios in the subset of plants for elemental analyses were also similar to the full data set. There was no clover entry x nutrient interaction, but the overall clover entry ($P=0.036$) and nutrient treatment effects ($P=0.040$) were significant. The root:shoot ratio of Kopu II x 487-9 was 24% higher than that of Kopu II and 16% higher than that of Kopu II x 900-4 (Figure 5.7A). Root:shoot ratios in the ½ LIS and LIS treatments were 14% higher than in the Complete treatment (Figure 5.7B).
Figure 5.7. Mean log root:shoot ratio (±SEM) of the clover entries sampled for shoot elemental analyses, by clover entry (A) and nutrient treatment (B). Numbers in parentheses are back-transformed means. LIS = low ionic strength. Means with the same letter show no significant differences at the 5% level. In (A), lower case letters indicate comparisons between Kopu II and its respective hybrids; upper case letters indicate comparisons between Kopu II x 900-4 and Crusader x 900-4.
5.3.2 Shoot elemental analyses

Main effects from the linear mixed models fitted for shoot mineral and N concentrations are presented in Table 5.6.

Table 5.6. Table of main effects for shoot elemental analyses, with $P$ values for the linear mixed models fitted. Grey shading indicates the model used, after dropping insignificant terms, as described in Section 5.2.6. †one clover entry only, therefore only the nutrient treatment effect applies; $P_i$=leaf inorganic $P$; Fraction $P_i = P_i/Total P$.

<table>
<thead>
<tr>
<th>Term tested</th>
<th>Full model</th>
<th>Additive model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clover entry x nutrient treatment</td>
<td>Clover entry</td>
</tr>
<tr>
<td>As</td>
<td>0.690</td>
<td>0.028</td>
</tr>
<tr>
<td>Al</td>
<td>0.758</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C:N†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>0.463</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cd</td>
<td>0.056</td>
<td>0.923</td>
</tr>
<tr>
<td>Co</td>
<td>0.418</td>
<td>0.034</td>
</tr>
<tr>
<td>Fe</td>
<td>0.533</td>
<td>0.027</td>
</tr>
<tr>
<td>K</td>
<td>0.652</td>
<td>0.256</td>
</tr>
<tr>
<td>Li</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>0.538</td>
<td>0.002</td>
</tr>
<tr>
<td>Mo</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>0.599</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>%N†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na</td>
<td>0.953</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ni</td>
<td>0.379</td>
<td>0.836</td>
</tr>
<tr>
<td>P</td>
<td>0.139</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P_i</td>
<td>0.080</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fraction P_i</td>
<td>0.898</td>
<td>0.001</td>
</tr>
<tr>
<td>DW unit P</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>DW unit P_i</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Pb</td>
<td>0.617</td>
<td>0.080</td>
</tr>
<tr>
<td>S</td>
<td>0.034</td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>0.099</td>
<td>0.003</td>
</tr>
<tr>
<td>N:P†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N:S†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3.2.1 Shoot P and P_i

Shoot P and P_i concentrations, and the fraction of total P as P_i, showed clover entry effects (Table 5.6). Mean P concentration was lower in Kopu II x 900-4 than in Kopu II (by 14%) and Kopu II x 487-9 (by 26%), as was the mean P_i concentration (31% lower than Kopu II and 37% lower than Kopu II x 487-9) (Figures 5.8A and 5.8B). Kopu II x 900-4 also had lower P (by 14%) and P_i concentrations (by 29%) compared with Crusader x 900-4. Differences in the fraction of total P as P_i followed a similar pattern as for P_i concentration, with 8–12% less of the total P pool as inorganic P for Kopu II x 900-4 than for the other clover entries (Figure 5.8C). P_i concentration also had a significant nutrient treatment effect (Table 5.6), and was 6% higher in the ½ LIS treatment than in the Complete treatment (Figure 5.9).

![Figure 5.8](image)

Figure 5.8. Mean log P concentration (A), mean P_i concentration (B) and mean fraction of total P as P_i (C) (±SEM) for the clover entries analysed. Numbers in parentheses are back-transformed means. Lower case letters indicate comparisons between Kopu II and its respective hybrids; upper case letters indicate comparisons between Kopu II x 900-4 and Crusader x 900-4. Means with the same letter show no significant differences at the 5% level.
The P and P\textsubscript{i} use-efficiencies of Kopu II x 900-4 (DW per unit P and P\textsubscript{i}) were higher than for the other clover entries in the ½ LIS and LIS treatments (Figure 5.10). For example, P use-efficiency of Kopu II x 900-4 in the ½ LIS treatment was 2.3 times higher than that of Kopu II, 3.8 times higher than that of Kopu II x 487-9, and 3.7 times higher than that of Crusader x 900-4. Differences in P\textsubscript{i} use-efficiency were similar, but slightly larger.

P use-efficiency and P\textsubscript{i} use-efficiency showed significant clover entry x nutrient treatment interactions (Table 5.6), with smaller effects of nutrient treatment in the Kopu II hybrids, particularly Kopu II x 900-4 (Figure 5.10). Back-transformed means are presented in Appendix 20. Both parameters were higher in the Complete treatment than the ½ LIS and LIS treatments, for all clover entries. Compared with the Complete treatment, P use-efficiency and P\textsubscript{i} use-efficiency in the LIS treatment decreased by 47% and 48% respectively for Kopu II x 900-4, 60% and 63% respectively for Kopu II x 487-9, 90% and 91% respectively for Kopu II, and 73% for Crusader x 900-4. In the ½ LIS treatment the decreases in P use-efficiency and P\textsubscript{i} use-efficiency, compared with the Complete treatment, were still smaller for Kopu II x 900-4 (-58%), whereas those of Kopu II x 487-9 (-80% and -83%) were similar to the other clover entries (-79–82%).

Both Kopu II x 900-4 and Crusader x 900-4 showed no significant differences in P use-efficiency and P\textsubscript{i} use-efficiency between the LIS and ½ LIS treatments (Figures 5.10A and 5.10B). However, P use-efficiency and P\textsubscript{i} use-efficiency of Kopu II were 84% and 86%
higher, respectively, in the \( \frac{1}{2} \) LIS than in the LIS treatment, and P\(_i\) use-efficiency of Kopu II x 487-9 was 2.2 times higher in the LIS treatment than in the \( \frac{1}{2} \) LIS treatment.

Figure 5.10. Mean (±SEM) log P use-efficiency (g dry weight (DW) mg\(^{-1}\) kg\(^{-1}\) P) (A) and mean log P\(_i\) use-efficiency (g DW mg\(^{-1}\) kg\(^{-1}\) P\(_i\)) (B) of the clover entries sampled for elemental analyses in the Complete, LIS and \( \frac{1}{2} \) LIS nutrient treatments. LIS = low ionic strength. Lower case letters indicate comparisons between nutrient treatments within each clover entry; upper case letters indicate comparisons between clover entries within each nutrient treatment for Kopu II and its respective hybrids. Means with the same letter show no significant differences at the 5% level. * indicates significant differences between Kopu II x 900-4 and Crusader x 900-4 for the given nutrient treatment at the 5% level.
Variability of clover entries

P and P_i use-efficiencies of Kopu II x 900-4 were generally more variable than the other clover entries (Appendix 21). The only differences in variability for P and P_i use-efficiency between Kopu II x 487-9 and the Kopu II parent were in the LIS treatment, where the hybrid was also more variable.

5.3.2.2 Other minerals and elements

Clover entry x nutrient treatment interactions

Among the other minerals and elements measured, only S, Li and Mo showed significant clover entry x nutrient treatment interactions (Table 5.6). As with other interactions previously presented, the effect of nutrient treatment was much smaller for Kopu II x 900-4 than for the other clover entries (Figure 5.11). In fact, there were no significant differences in the concentrations of these elements across nutrient treatments for this entry. Back-transformed means are presented in Table 5.7.

Mean S concentrations for Kopu II x 900-4 were often lower than for the other clover entries, particularly Kopu II x 487-9 (all treatments) and Crusader x 900-4 (½ LIS and LIS treatments) (Figure 5.11A). Kopu II x 487-9 also had higher S concentrations than Kopu II in the Complete and ½ LIS treatments.

The mean Li concentrations for both the Kopu II hybrids were higher than the parent in all nutrient treatments, and levels in Kopu II x 900-4 were also higher than that of Crusader x 900-4 in the LIS and ½ LIS treatments (Figure 5.11B).

Although Kopu II x 900-4 showed no changes in Mo concentration with nutrient treatment, the remaining clover entries showed some increases in Mo concentration in the lower nutrient treatments (Figure 5.11C). There were no differences among entries in Mo concentration in the Complete treatment (Figure 5.11C), but the concentration in Kopu II x 900-4 was lower than in the other clover entries in the two lowest strength treatments. Mo concentration of Kopu II x 487-9 was also lower than that of Kopu II in the LIS treatment.
Figure 5.11. Mean log S, Li and Mo concentrations (±SEM) for clover entries sampled for elemental analyses in the Complete, LIS and ½ LIS nutrient solutions. LIS = low ionic strength. Lower case letters indicate comparisons between nutrient treatments within each clover entry; upper case letters indicate comparisons between clover entries within each nutrient treatment for Kopu II and its respective hybrids. Means with the same letter show no significant differences at the 5% level. * indicates significant differences at the 5% level between Kopu II x 900-4 and Crusader x 900-4 for the given nutrient treatment.
Table 5.7. Back-transformed means for S, Li and Mo concentrations of the clover entries sampled for elemental analyses, in the Complete, LIS and ½ LIS nutrient treatments. LIS = low ionic strength.

<table>
<thead>
<tr>
<th>Clover entry</th>
<th>Complete S (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>LIS S (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>½ LIS S (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Complete Li (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>LIS Li (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>½ LIS Li (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Complete Mo (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>LIS Mo (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>½ LIS Mo (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kopu II</td>
<td>721</td>
<td>1213</td>
<td>1031</td>
<td>0.973</td>
<td>0.605</td>
<td>0.832</td>
<td>0.416</td>
<td>3.401</td>
<td>1.224</td>
</tr>
<tr>
<td>Kopu II x 487-9</td>
<td>1230</td>
<td>1507</td>
<td>2096</td>
<td>1.897</td>
<td>2.185</td>
<td>1.481</td>
<td>0.431</td>
<td>0.573</td>
<td>1.067</td>
</tr>
<tr>
<td>Kopu II x 900-4</td>
<td>763</td>
<td>796</td>
<td>865</td>
<td>1.823</td>
<td>2.043</td>
<td>1.704</td>
<td>0.252</td>
<td>0.240</td>
<td>0.336</td>
</tr>
<tr>
<td>Crusader x 900-4</td>
<td>749</td>
<td>1245</td>
<td>1509</td>
<td>1.523</td>
<td>1.055</td>
<td>1.193</td>
<td>0.298</td>
<td>0.618</td>
<td>1.026</td>
</tr>
</tbody>
</table>

**Macronutrients**

Among the remaining major elements, Ca, Mg and Na showed significant clover entry effects (Table 5.6), with considerable differences in concentrations among some entries. The mean Ca concentration of Kopu II x 487-9 was 40% higher than that of Kopu II and 33% higher than that of Kopu II x 900-4 (Table 5.8). There were no differences in mean Mg concentration among Kopu II and its two hybrids, but mean Na concentrations of Kopu II x 487-9 and Kopu II x 900-4 were more than two times higher than those of the Kopu II parent (Table 5.8). There were no differences in mean Ca or Na concentrations between Kopu II x 900-4 and Crusader x 900-4, but the Mg concentration of Crusader x 900-4 was 20% higher than that of Kopu II x 900-4 (Table 5.8). These three minerals also showed significant nutrient treatment effects (Table 5.6 and Table 5.8).

Shoot %N was measured within one clover entry only, and was lower in the ½ LIS treatment plants than in the Complete treatment plants, while the mean leaf C:N ratio was higher in the ½ LIS treatment than in the other nutrient treatments (Table 5.8). Mean Cd, Ni and Pb concentrations did not differ among either clover entries or nutrient treatments, with back-transformed means of 0.039, 4.632 and 0.570 mg kg<sup>-1</sup> respectively.
Table 5.8. Mean (±SEM) log concentrations of elements for clover entry and nutrient treatment effects. LIS = low ionic strength. Numbers in parentheses are back-transformed means. Among clover entries, lower case letters indicate comparisons between Kopu II and its respective hybrids; upper case letters indicate comparisons between Kopu II x 900-4 and Crusader x 900-4. Means with the same letter, among clover entries or nutrient treatments, show no significant differences at the 5% level. Units are mg kg⁻¹, except for %N and ratios.

<table>
<thead>
<tr>
<th>Clover entry</th>
<th>Nutrient treatment</th>
<th>Complete</th>
<th>LIS</th>
<th>% LIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kopu II</td>
<td>Kopu II x 487-9</td>
<td>Kopu II x 900-4</td>
<td>Crusader x 900-4</td>
<td></td>
</tr>
<tr>
<td>Al</td>
<td>4.49ᵃ</td>
<td>5.10ᵇ</td>
<td>4.92ᵇᵃ</td>
<td>4.95ᵃ</td>
</tr>
<tr>
<td>±0.129</td>
<td>±0.141</td>
<td>±0.123</td>
<td>±0.124</td>
<td></td>
</tr>
<tr>
<td>(89.1)</td>
<td>(164.4)</td>
<td>(136.5)</td>
<td>(140.6)</td>
<td></td>
</tr>
<tr>
<td>As</td>
<td>-0.394ᵃᵇ</td>
<td>-0.191ᵇ</td>
<td>-0.483ᵇᵃ</td>
<td>-0.253ᵇ</td>
</tr>
<tr>
<td>±0.1421</td>
<td>±0.1484</td>
<td>±0.1355</td>
<td>±0.1376</td>
<td></td>
</tr>
<tr>
<td>(0.675)</td>
<td>(0.826)</td>
<td>(0.617)</td>
<td>(0.776)</td>
<td></td>
</tr>
<tr>
<td>C:N</td>
<td>Measured in Kopu II x 900-4 only</td>
<td>3.48ᵃ</td>
<td>3.50ᵇ</td>
<td>3.61ᵇ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.080</td>
<td>±0.081</td>
<td>±0.081</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(32.6)</td>
<td>(32.2)</td>
<td>(37.1)</td>
</tr>
<tr>
<td>Ca</td>
<td>9.69ᵃ</td>
<td>10.03ᵇ</td>
<td>9.75ᵃᵇ</td>
<td>9.78ᵃ</td>
</tr>
<tr>
<td>±0.041</td>
<td>±0.048</td>
<td>±0.037</td>
<td>±0.038</td>
<td>±0.036</td>
</tr>
<tr>
<td>(16220)</td>
<td>(22697)</td>
<td>(17103)</td>
<td>(17748)</td>
<td>(16597)</td>
</tr>
<tr>
<td>Co</td>
<td>-1.12⁴ᵃ</td>
<td>-0.978ᵇ</td>
<td>-0.798ᵃᵇ</td>
<td>-0.829ᵃ</td>
</tr>
<tr>
<td>±0.0896</td>
<td>±0.1067</td>
<td>±0.0798</td>
<td>±0.0842</td>
<td>±0.074</td>
</tr>
<tr>
<td>(0.325)</td>
<td>(0.376)</td>
<td>(0.450)</td>
<td>(0.436)</td>
<td>(0.326)</td>
</tr>
<tr>
<td>Fe</td>
<td>5.03ᵃ</td>
<td>5.33ᵇ</td>
<td>5.35ᵇᵃ</td>
<td>5.30ᵃ</td>
</tr>
<tr>
<td>±0.129</td>
<td>±0.140</td>
<td>±0.124</td>
<td>±0.126</td>
<td>±0.121</td>
</tr>
<tr>
<td>(153.4)</td>
<td>(205.6)</td>
<td>(209.8)</td>
<td>(200.3)</td>
<td>(226.8)</td>
</tr>
<tr>
<td>K</td>
<td>N/A</td>
<td>9.20ᵃ</td>
<td>9.48ᵇ</td>
<td>9.46ᵇ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.071</td>
<td>±0.071</td>
<td>±0.076</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9927)</td>
<td>(13043)</td>
<td>(12849)</td>
</tr>
<tr>
<td>Mg</td>
<td>8.57ᵃ</td>
<td>8.65ᵃ</td>
<td>8.61ᵃᵇ</td>
<td>8.79ᵇ</td>
</tr>
<tr>
<td>±0.044</td>
<td>±0.052</td>
<td>±0.040</td>
<td>±0.041</td>
<td>±0.038</td>
</tr>
<tr>
<td>(5261)</td>
<td>(5687)</td>
<td>(5481)</td>
<td>(6568)</td>
<td>(5009)</td>
</tr>
<tr>
<td>Mn</td>
<td>5.61ᵃ</td>
<td>5.85ᵇ</td>
<td>5.51ᵃᵇ</td>
<td>5.82ᵇ</td>
</tr>
<tr>
<td>±0.073</td>
<td>±0.081</td>
<td>±0.070</td>
<td>±0.071</td>
<td>±0.067</td>
</tr>
<tr>
<td>(272.6)</td>
<td>(345.8)</td>
<td>(246.2)</td>
<td>(336.0)</td>
<td>(234.6)</td>
</tr>
<tr>
<td>%N</td>
<td>Measured in Kopu II x 900-4 only</td>
<td>0.19ᵃ</td>
<td>0.14ᵇ</td>
<td>0.04ᵇ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.091</td>
<td>±0.092</td>
<td>±0.092</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.21)</td>
<td>(1.15)</td>
<td>(1.04)</td>
</tr>
<tr>
<td>Na</td>
<td>7.07ᵃ</td>
<td>8.04ᵇ</td>
<td>7.88ᵇᵃ</td>
<td>7.85ᵃ</td>
</tr>
<tr>
<td>±0.068</td>
<td>±0.080</td>
<td>±0.060</td>
<td>±0.062</td>
<td>±0.056</td>
</tr>
<tr>
<td>(1175)</td>
<td>(3100)</td>
<td>(2641)</td>
<td>(2561)</td>
<td>(3087)</td>
</tr>
<tr>
<td>Zn</td>
<td>3.82ᵃ</td>
<td>3.61ᵇ</td>
<td>3.62ᵃᵇ</td>
<td>4.01ᵇ</td>
</tr>
<tr>
<td>±0.111</td>
<td>±0.123</td>
<td>±0.104</td>
<td>±0.106</td>
<td>±0.100</td>
</tr>
<tr>
<td>(45.6)</td>
<td>(37.1)</td>
<td>(37.4)</td>
<td>(55.3)</td>
<td>(30.6)</td>
</tr>
<tr>
<td>N:P</td>
<td>N/A</td>
<td>2.55ᵃ</td>
<td>2.68ᵇ</td>
<td>2.69ᵃ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.133</td>
<td>±0.131</td>
<td>±0.132</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(12.8:1)</td>
<td>(14.5:1)</td>
<td>(14.8:1)</td>
</tr>
<tr>
<td>N:S</td>
<td>N/A</td>
<td>2.47ᵃ</td>
<td>2.64ᵇ</td>
<td>2.75ᵇ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.143</td>
<td>±0.142</td>
<td>±0.142</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(11.8:1)</td>
<td>(14:1)</td>
<td>(15.6:1)</td>
</tr>
</tbody>
</table>
**Micronutrients and metals**

Clover entry differences for micronutrients and metals (Table 5.6) all involved significantly higher concentrations in hybrid entries (Table 5.8). Both Kopu II x 487-9 and Kopu II x 900-4 were higher than Kopu II for Al (by 84% and 53% respectively) and Fe (by 34% and 37% respectively). Kopu II x 900-4 also had 39% more Co than Kopu II. Kopu II x 487-9 had higher mean concentrations of Mn (by 41%) and As (by 34%) than Kopu II x 900-4, and its mean Mn concentration was also 27% higher than Kopu II. Mean concentrations of As and Zn were higher in Crusader x 900-4 than in Kopu II x 900-4, by 26% and 48% respectively.

### 5.3.3 Nodulation and stress pigmentation scores

#### 5.3.3.1 Nodulation

There was a significant ($P=0.017$) clover group x entry x nutrient interaction for nodulation scores. The mean scores of both Crusader and Kopu II x 900-4 did not change significantly with nutrient treatment (Figure 5.12).

![Figure 5.12. Mean nodulation scores (±SEM) for the eight clover entries in the Complete, LIS and ½ LIS nutrient treatments. LIS = low ionic strength. Lower case letters indicate comparisons between nutrient treatments within each clover entry; upper case letters indicate comparisons between clover entries in the relevant group (Crusader, Kopu II, T. uniflorum) within nutrient treatments. Means with the same letter show no significant differences at the 5% level.](image-url)
For most other clover entries, nodulation scores in the LIS treatment were higher than in the Complete treatment, but those in the ½ LIS treatment did not differ to either the LIS or Complete treatments (Figure 5.12).

There were no differences in the mean nodulation score between the *T. uniflorum* accessions in any nutrient treatment, but the Crusader and Kopu II groups did show some differences (Figure 5.12). Nodulation scores of both Kopu II x 487-9 and Crusader x 487-7 were lower than their respective parents in the Complete treatment (Figure 5.12). However, mean nodulation scores of both of the Kopu II hybrids were higher than the Kopu II parent in the LIS treatment, and the mean score of Crusader x 900-4 was also higher than that of Crusader. In the ½ LIS treatment, there were no differences among the Kopu II group, but the mean nodulation score of Crusader x 487-7 was lower than that of the Crusader parent.

### 5.3.3.2 Pigmentation

There was no difference in the effect of nutrient treatment on stress pigmentation among entries within clover groups, but there was a clover group x entry effect (*P*<0.001). Mean pigmentation scores did not differ between the *T. uniflorum* accessions or among Crusader and its hybrids, but were lower in Kopu II x 900-4 than in Kopu II and Kopu II x 487-9 (Figure 5.13).

![Figure 5.13. Mean pigmentation scores (±SEM) for the eight clover entries. Means with the same letter within clover groups (indicated by shading) show no significant differences at the 5% level.](image-url)
5.4 Discussion

5.4.1 Hybrid responses to applied nutrients

These results confirm the observations reported in Chapter 4. In the LIS treatment, which was the same nutrient solution used in the previous experiment, shoot and root DWs of three of the four hybrid families studied were higher than that of their white clover parents (Figure 5.3). As predicted, growth of all clover entries was reduced in the two lower strength solutions, and there were no differences in growth among clover entries within groups in the Complete treatment (Figure 5.3). However, the effect of decreasing nutrient solution strength was smaller for many of the hybrid entries than for the white clover parents. In addition to the LIS treatment, shoot and root DW of two of the four hybrid families were also higher than the white clover parental cultivars in the more dilute ½ LIS treatment.

Kopu II x 900-4 was consistently less affected than other clover entries by changes in nutrient treatment. Sulphur, Li and Mo concentrations, plus nodulation, of Kopu II x 900-4 did not change significantly with decreasing nutrient solution strength (Figures 5.11 and 5.12). In particular, changes in nutrient treatment affected the growth of Kopu II x 900-4 less than Kopu II and Kopu II x 487-9 (Figures 5.3 and 5.6), and Kopu II x 900-4 had higher growth at lower nutrient concentrations than Kopu II x 487-9. This probably explains the lower stress pigmentation in Kopu II x 900-4 compared with Kopu II x 487-9 and the Kopu II parent (Figure 5.13). Kopu II x 900-4 and Crusader x 900-4 share a common F₁ parent, but were often very different (Figures 5.6, 5.8, 5.10 and 5.11), suggesting that T. uniflorum parentage may not be the sole, or strongest, influence on performance of the hybrids in this experiment. The superior performance of Kopu II x 900-4 may be evidence of transgressive segregation, wherein hybrids express characteristics outside the normal range of either parent (Grant, 1975; Rieseberg et al., 1999).

The responses of the Crusader hybrids to changes in nutrient treatment were different to those shown by the Kopu II hybrids (Figures 5.3 and 5.6). Although growth of both the Crusader hybrids was also less affected than that of the Crusader parent in the LIS treatment, only Crusader x 900-4 was larger than the parent. In contrast, in the ½ LIS treatment it was Crusader x 487-7 which was less affected, and grew larger than, the parent and Crusader x 900-4.

In the previous experiment (Chapter 4), shoot and root DWs combined over all harvests were higher for T. uniflorum than for the white clover parents. The statistical models used in the current experiment did not compare the growth of T. uniflorum with the white clover parents.
However, with the same LIS nutrient solution as used previously, the absolute means of *T. uniflorum* were generally similar to or smaller than those of Kopu II and Crusader (Table 5.4). Differences in culture conditions in the current experiment, compared with Chapter 4, including pot size, may have affected the growth of *T. uniflorum* but not white clover. The two experiments were conducted for different durations and at different times of the year (April–December for the first experiment (Chapter 4), and September–January for the current experiment). This would have differentially affected plant developmental rates in the experimental material, through factors such as temperature regimes and solar radiation levels. Daylength is unlikely to have had an effect, as supplementary lighting was used to give a 12 hour daylength throughout the previous experiment, and also up to December in the current experiment.

In contrast to *T. uniflorum*, hybrid families may still have been able to express their growth potential due to differences in gene expression compared to the parents. While it is generally expected that hybrid plants will be intermediate in characteristics to the parents, transgressive segregation can occur, as mentioned above. In the current experiment, instances where hybrid families exhibit superior characteristics compared with both white clover and *T. uniflorum*, may represent transgressive segregation. This is likely to occur in *T. repens x T. uniflorum* hybrids, given the widespread occurrence of transgressive segregation in interspecific hybrids (e.g Nasrallah *et al.* (2000); Rosenthal *et al.* (2002); Kirk *et al.* (2011)), and the high frequencies of transgressive traits that are reported (e.g Rosenthal *et al.* (2002)).

### 5.4.2 Mineral nutrition – phosphorus and nitrogen

Mineral nutrition in the clover entries analysed was similar to that reported in the previous experiment (Section 4.3.3). Most of the essential elements in the current experiment were above critical levels reported for white clover growth with the exceptions, again, of P and N (Figure 5.8 and Table 5.8). Overall mean P concentrations (equivalent to 0.08–0.11% DM) were similar to the lower end of concentrations found in the previous experiment. They were still considerably lower than critical levels reported by McNaught (1970) for white clover in ryegrass pasture (0.3–0.4%), and most were just below the ranges reviewed by Dunlop and Hart (1987) for white clover monoculture (0.1–0.25%). Despite this, Kopu II x 900-4 was able to accumulate higher DWs than related clover entries. The mean P concentration in this entry was not only low, but significantly lower than the already sub-optimal concentrations in the Kopu II parent and Kopu II x 487-9, as well as Crusader x 900-4, suggesting it is even more tolerant of low tissue P concentrations than other hybrid families (Figure 5.8).
However, the mean Pi concentration and the fraction of Pi as a percentage of total P were also lower in Kopu II x 900-4 than in the other entries, suggesting its superior growth could be attributable, at least in part, to less sequestration of Pi in the vacuoles (Figure 5.8). Kopu II x 900-4 was also more efficient than the other entries, accumulating more leaf DW per unit of P and Pi at the two lowest strength treatments (Figure 5.10). Kopu II x 487-9 also had higher P and Pi use efficiencies than white clover in the LIS treatment but not in the ½ LIS treatment, in a similar pattern to DW. Again, this demonstrates differences among the hybrids in their ability to tolerate different levels of nutrient deficiency. Compared with white clover, higher variability for P use-efficiency and Pi use-efficiency in the hybrid entries suggests that it may be possible to select for this trait in *T. repens* x *T. uniflorum* hybrids, particularly Kopu II x 900-4 (Appendix 21).

Efflux of inorganic P from the roots of agricultural plants has a major influence on net P uptake (Cogliatti and Santa Maria, 1990; Elliott *et al.*, 1984). Dunlop and Phung (1999) found P efflux rates in white clover were three times higher than in perennial ryegrass, and concluded that this may have a considerable influence on the ability of white clover to compete for soil P. Higher leaf P concentrations in white clover than in *T. repens* x *T. uniflorum* hybrids may be required as a buffer against high P efflux rates. Efflux of P from *T. uniflorum* roots has not been studied and could be a focus for future work. Differences in the P physiology of white clover and the *T. repens* x *T. uniflorum* hybrids may relate to edaphic conditions during the evolution of the species. No information exists on nutrient status at centres of origin for either parental species.

White clover is, however, typical of species adapted to fertile environments (Hart and Jessop, 1983), with a large response to added P, high tissue P concentrations, and low growth at low soil P supplies. Plants from fertile habitats also have a relatively high root absorption capacity for nutrients (Chapin, 1980). In contrast, species from infertile habitats are slow growing, with a small response to added nutrients and a low root absorption capacity (Chapin, 1980). Instead, such plants tend to maximise nutrient acquisition through higher root:shoot ratios and root mycorrhizae (Chapin, 1980). In the current experiment, higher root:shoot ratios for the *T. uniflorum* accessions may reflect such an adaptation, and suggest that its mineral nutrition could be indicative of infertile habitats (Figure 5.5A). There is little published information on the eco-geography of *T. uniflorum*, but general locations where seed has been collected from in the past have been described as having poor soils (Forest Service of Parnitha, 2012). Similarly, higher root:shoot ratios in the lower strength nutrient treatments reflect the expected reallocation of biomass to acquire limiting resources (Figures 5.5B and 5.7B). The
higher proportion of root biomass for Kopu II x 487-9, compared with its white clover parent and Kopu II x 900-4, may have been inherited from *T. uniflorum* (Figures 5.5A and 5.7A).

Shoot %N was only analysed in Kopu II x 900-4 hybrids, due to the relatively large amount of dried material needed for analysis. However, within this clover entry, %N in all treatments was considerably lower than the critical level of 4.5% required for white clover (McNaught, 1970). As discussed previously (Section 4.42), these low N concentrations may be a symptom of P deficiency, due to the effects of low P on nodulation and N fixation (Almeida et al., 2000; Cadisch et al., 1993). In most clover entries, nodulation increased significantly in the LIS solution compared with the Complete solution, almost certainly in response to decreased supplies of mineral N (Figure 5.12). However, the same effect was not seen in the ½ LIS solution, suggesting that the nutrient supply in this treatment was inadequate to support increased nodulation. The critical %N content of *T. uniflorum* is unknown, therefore %N of *T. repens* x *T. uniflorum* hybrids in this experiment may still have been adequate for growth, at least for Kopu II x 900-4. Unlike other hybrids, nodulation of Kopu II x 900-4 did not change with nutrient treatment, suggesting this is relatively insensitive to changes in fertility of the growing medium (Figure 5.12).

### 5.4.3 Mineral nutrition – other elements

Mean S concentrations were also below the critical levels reported for white clover in pasture (McNaught, 1970; Morton and Smith, 2000), and most clover entries in the Complete treatment were below the range reported for white clover monocultures (Dunlop and Hart, 1987) (Table 5.7). However the N:S ratios suggest S was adequate for growth – at least in the Kopu II x 900-4 entry – as they were below the ratio (18:1) reported to indicate S deficiency by McNaught (1970) and Dijkshoorn et al. (1960) (Table 5.8). Sulphur concentrations of the Kopu II hybrids often differed to the other clover entries, and to each other (Figure 5.11), but further work would be required to determine whether they have different growth responses to added S.

As discussed in Chapter 4, the interpretation of Ca, K, Mg and Na concentrations is difficult due to the interaction of these minerals (Fageria, 2001). However, concentrations were generally above the critical levels reported for white clover growth (McNaught, 1970; Dunlop and Hart, 1987; Edmeades, 2004; Edmeades and Perrott, 2004). In the current experiment, K concentrations did not differ among clover entries, but Na was still considerably higher in the Kopu II hybrids than in the Kopu II parent (Table 5.8), as was found in Chapter 4. This reinforces the potential adaptation of the hybrids to water stress (see Chapters 7 and 8),
through the role of Na in osmotic adjustment (Iannucci et al., 2002; Zheng et al., 2010). As in
the previous experiment, some minor elements and metals, such as Al, Co and Li, also
accumulated more in hybrid material (Tables 5.7 and 5.8). Lithium is generally not considered
to be essential for plant growth, however Shkolnik (1984) reviews a number of studies into
the role of Li, including positive effects on photosynthesis, involvement in alkaloid
production, and high levels in marine plants as well as some terrestrial species. It is an alkali
metal like K and Na and may, therefore, play some role in osmotic adjustment under water
stress.

5.5 Conclusions

- The growth effect seen in the previous experiment was repeatable. Shoot and root
  DWs of some hybrid families were higher than white clover using the same nutrient
  solution as in Chapter 4, as well as with a more dilute solution.

- Not all hybrid families responded in the same manner to changes in nutrient treatment,
  with some being less affected than other hybrids and their white clover parents,
  particularly Kopu II x 900-4. Differences between nutrient treatments suggest that this
  family may be able to tolerate lower nutrient concentrations than other families.

- P and N appeared to be the limiting elements for growth, although low N contents may
  reflect the effects of P deficiency on nodulation and N fixation. Other elements were
  within normal ranges for white clover growth.

- Growth of some hybrid families was higher than white clover and other hybrids at low
  external and internal P concentrations, and some families had higher P and P$_i$ use-
  efficiencies, particularly Kopu II x 900-4. This may be influenced by differences in P$_i$
  sequestration compared with white clover.

- Further study on P response curves and critical N levels for *T. repens* x *T. uniflorum*
  hybrids would provide valuable information for breeding and selection of material for
  specific field environments.

- Given the differences seen among the hybrids, more families should be screened for P
  physiology related traits. Variability of the hybrid material suggests it should be
  possible to select for some traits, particularly in conjunction with soil nutrient status.

- The natural environment of *T. uniflorum* may have a considerable influence on the
  mineral nutrition of the hybrids, but there was also evidence of transgressive
segregation. Given the paucity of published information, further investigation into the edaphic environment of *T. uniflorum* may assist with understanding the mineral nutrition of the hybrids, and could also enable identification of other useful traits.
Chapter 6
Root system structure

6.1 Introduction
The influence of root morphology and architecture on the ability of plants to intercept water and nutrients, and conversely the influence of soil fertility on root architecture, has been widely reported, e.g. by Lynch (1995), Sorgonà and Cacco (2002), Fitter et al. (2002), and Dunbabin et al. (2003b). Root morphology incorporates root length, diameter, surface area, volume, and number of root tips. In contrast, root architecture characterises the structure of the root system, and can be divided into measurements of its individual components (links) or description of the overall branching pattern (topology). As with root morphology, the length, surface area, and average diameter of links are measured as overall values for the whole root system. These parameters can also be measured on the basis of specific link types, according to their position within the root system (Fitter, 1987, 1991). The functional implications of morphological and architectural measurements provide valuable information on the adaptive characteristics of root systems, and are unknown for T. uniflorum and T. repens x T. uniflorum hybrids.

In Chapter 4 it was hypothesised that the root morphology of T. uniflorum and T. repens x T. uniflorum hybrids differs from that of white clover, but the results suggested that differences were predominantly between T. uniflorum and the other clover types. This data was produced from a mixture of nodal and tap root systems, at different rooting depths. The first objective of this experiment, which expands on this previous work, was to quantify and compare the morphological characteristics of whole root systems of white clover, T. uniflorum, and T. repens x T. uniflorum BC1 (backcross 1) hybrids. The second objective was to quantify and compare the root architecture parameters of this material. Finally, the third objective was to compare the change in root length over time of white clover, T. uniflorum and T. repens x T. uniflorum BC1 hybrids, as a measure of growth. It was hypothesised that the root morphology, architecture and growth of T. uniflorum and the T. repens x T. uniflorum hybrids differs to that of white clover. Such differences may arise from functional adaptation to differing levels of soil resources.
6.2 Materials and methods

6.2.1 Experimental setup

The experiment was conducted from 19 May to 15 July 2008 in a glasshouse at AgResearch, Ruakura Research Centre, Hamilton (37° 46′ 23.42″S, 175° 18′ 22.95″E). Supplementary lighting was used from 6–8 am and 4–6 pm to ensure a daylength of 12 hours. Mean weekly day/night temperatures over the course of the experiment were 15.7/10.5°C, and mean weekly maximum day time solar radiation was 704 μmol m$^{-2}$ s$^{-1}$. Temperature and radiation data were recorded by the glasshouse control system on a weekly basis.

The experimental setup followed that used by Care (1999) and has been used previously to study the root morphology and root hairs of white clover (Care, 1999; Jahufer et al., 2006; Care et al., 1998). Plants were grown hydroponically in 45 l tubs (350 x 600 x 250 mm) (Plate 6.1). A low ionic strength nutrient solution, as for Chapters 4 and 5 (Appendix 15), was used as the growing medium; maintained at a pH of 5.0–5.5 using additions of 20% NH$_4$ solution and 30% HCl. The solution was changed weekly at first, and increased to twice weekly as nutrient uptake increased and plant growth caused the solution pH to drop. Compressed air was delivered to each glasshouse table through a manifold, which was in turn connected to stainless steel rods in each tub, providing aeration through bubbling.

Plate 6.1. Hydroponics system used in the root system structure experiment.
The tubs were covered with opaque lids which prevented light from entering the nutrient solution and stimulating algal growth. Holes in the lids supported 80 mm diameter x 45 mm high pots, and any unused holes were covered with foil to block light. The plants were held within the pots by a rubber disc, slit to a central hole around the seedling stem, and a hole in the base of the pot allowed the roots out to the nutrient solution (Plate 6.2). This system enabled the plants to be removed and replaced without damaging the roots, thus also allowing for multiple measurements over time.

Plate 6.2. Root growth of clover plants in the hydroponics system used in the root system structure experiment.

6.2.2 Plant material

The plant material used was the same as in Chapter 4, excluding *T. uniflorum* accession AZ4382, giving a total of seven clover entries (Table 6.1).

Seed was scarified with sandpaper and germinated on damp filter paper in Petri dishes, and inoculated with rhizobia as in Chapter 4. The seedlings were transplanted to pots of mortar sand in a glasshouse at approximately 10 mm radicle length. Plants received a low ionic strength nutrient solution three times per week (Appendix 15), and were transplanted to the experimental setup sequentially as the first trifoliate leaves fully opened.
Table 6.1. Clover entries used in the root system structure experiment. cv = cultivar; OP = open pollinated.

<table>
<thead>
<tr>
<th>Entry number</th>
<th>Clover type</th>
<th>Description</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>White clover</td>
<td>cv. Grasslands Kopu II</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>White clover</td>
<td>cv. Crusader</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>T. uniflorum</em></td>
<td>AZ4383# OP Turkish origin</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>BC1</td>
<td>Kopu II-7 x 80-2†</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>BC1</td>
<td>Kopu II-NC51-R3-3 x 900-4‡</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>BC1</td>
<td>Crusader-5 x 80-2†</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>BC1</td>
<td>Crusader-10-2 x 900-4‡</td>
<td></td>
</tr>
</tbody>
</table>

# Accession number, Margot Forde Germplasm Centre (Palmerston North, New Zealand).
† 80-2 = Kopu II-2 x T66-6, where T66-6 = a specific genotype of AZ4382.
‡ 900-4 = Kopu II-2 x AZ4383-11.

6.2.3 Experimental design

The design consisted of ten replicates (tubs) on two glasshouse tables (five replicates per table). Each replicate contained one plant from each of the seven clover entries (Figure 6.1), with the entries allocated to positions at random.

Figure 6.1. Experimental layout for the root system structure experiment. 1 = Kopu II; 2 = Crusader; 3 = *T. uniflorum*; 4 = Kopu II x 80-2; 5 = Kopu II x 900-4; 6 = Crusader x 80-2; 7 = Crusader x 900-4.

6.2.4 Scanning of root systems

The root system of each plant was scanned at the time of transplantation (week 0), then at seven day intervals up to week 4, and finally at week 6. Roots were spread out on a flatbed.
scanner (Epson Expression® 1680) so that they did not touch or overlap. A greyscale image was captured with dedicated root image analysis software (WinRhizo™, Regent Instruments, Quebec, Canada), using lighting from above to eliminate shadows (Figure 6.2). Images were stored in .tiff format.

![Figure 6.2. Example of a scanned BC1 hybrid at week 4, on which root morphological and architectural measurements were made.](image)

### 6.2.5 Measurements

The captured images were analysed with WinRhizo™, using greyscale to distinguish the roots (darker pixels) from the background (lighter pixels). This produced a direct measurement of root morphological and architectural characteristics. Morphological measurements included
total root length, average root diameter, root surface area and number of root tips. For practicality, root length data are presented in cm. The number of root tips per cm was calculated using total root length and number of tips. At the end of the experiment, root and shoot dry weight (DW) (Appendix 22) were obtained after oven drying overnight at 70°C (Crush et al., 2010b). Root DW was then used to derive specific root length (SRL, m root g⁻¹ root DW), number of root tips per mg of DW (tips mg⁻¹), and tissue density (g DW cm⁻³) at the end of the experiment. Data for root length, surface area, and number of tips are presented in Appendix 23.

Root architecture was measured up to week 4 only, as the large size of root systems at week 6 made accurate measurements impossible. As an overall measure of branching patterns, topological indices were calculated for each clover entry at each time as described in Chapter 2 (Section 2.2), from the slope of log altitude (longest individual path length) over log magnitude (total number of external links) (Figure 6.3A). Other data presented include mean link length, surface area and average link diameter of root systems overall; plus mean length, surface area and average diameter of individual link types (Figure 6.3B), based on Fitter (1987) and described in Section 2.2.

![Figure 6.3. Definitions of root architecture using an example root system of magnitude 7 (total number of external links) and altitude 6 (longest path length) (A). Individual link types are shown in (B), where BL = base link; II = internal link; EI = external–internal link; and EE = external–external link. Analysis based on axes (C) divides the root system into axes of connected links of increasing order from the taproot (order 0) to primary laterals (order 1), secondary laterals (order 2) etc.](image)

In addition, the mean total length and surface area of root axes were determined based on connected links of the same order, from order 0 (tap root) up to order 3 (tertiary laterals)
(Figure 6.3C). In the analysis, continuation of the current order at a fork was determined by which of the two subtending links had the largest link magnitude (number of subtending external links), with the order increasing for the remaining link. Although some 4th order lateral roots were present they were not included in the analysis (see Section 6.2.6). The average diameter of the links belonging to each order was also determined. For each root system, the total number of lateral roots (order 1 and greater) was used to calculate the mean proportion of primary (1st order), secondary (2nd order) and tertiary (3rd order) lateral roots for each clover entry. Data for number of links, altitude, external path length, and %EI links are presented in Appendix 24.

6.2.6 Statistical analysis

Clover entry comparisons were made in two groups, based on white clover parentage – i.e. *T. uniflorum*, Kopu II, and the Kopu II hybrid families; or *T. uniflorum*, Crusader and the Crusader hybrid families. For conciseness, most comparisons were made at week 4 and 6 only (root morphology) or at week 3 and 4 only (root architecture). Total root length, total surface area, and average diameter at week 4 and 6, plus link length, link surface area and average link diameter at week 3 and 4, were analysed using a linear mixed modelling (LMM) approach. This took account of potential within-plant correlations from repeated measurements over time. Growth in root length from week 0–1, 1–2, 2–3, 3–4, 4–6, and overall from week 0–6 were also analysed using LMM. Number of tips and tips cm\(^{-1}\) at week 4 and 6, plus number of links, altitude, external pathlength and %EI links at week 3 and 4, were analysed using a generalised estimating equations (GEE) approach. This accounts for both repeated measurements over time, as well as the negative binomial distribution of count data. The number of tips per mg DW also involved count data, but was determined at one time only (week 6). Hence, generalised linear modelling (GLM) was used, to account for negative binomial distributions.

Length, surface area and average diameter of II, EE and EI links (internal, external-external, external-internal) at week 4 were also analysed using LMM to account for within-plant correlations arising from multiple numbers of each link type. As there was only one BL (base link) for each plant, this data was analysed via analysis of variance (ANOVA). Length, surface area and diameter of order 1, 2 and 3 axes (1\(^{st}\), 2\(^{nd}\) and 3\(^{rd}\) order lateral roots) at week 4 were also analysed using LMM to account for multiple numbers of each axis within plants. As there was only one order 0 axis per plant, and only four observations of 4\(^{th}\) order laterals, this data was analysed using ANOVA. The number of lateral roots in orders 1 to 3 were analysed using GLM to account for the negative binomial distribution of the data, and the
percentage of lateral roots in each order was analysed via ANOVA. Due to limited observations, number and percentage of 4th order laterals could not be analysed. Specific root length, tissue density and DW data at the end of the experiment were analysed using ANOVA.

Topological indices (slope of log altitude on log magnitude) at week 0 to week 4 were compared by multiple regression analysis (log altitude on log magnitude) in Genstat version 11 (VSN International Ltd.). This describes the overall topology of the root system, between the extremes of herringbone and dichotomous branching. Linear mixed modelling and GEE were conducted in SAS version 9.1 (SAS Institute Inc.), GLM was conducted using R version 2.8.1 (R Core Team, 2012), and ANOVAs were performed in Genstat version 11 (VSN International Ltd.) or Minitab version 15 (Minitab Inc.). In graphs and tables, means with the same letter were not significantly different at the 5% level, using the means separation methods stated above for the respective traits. Trends nearing statistical significance ($P=0.05-0.099$) are noted in the text.

### 6.3 Results

#### 6.3.1 Root morphology

Mean average root diameter of *T. uniflorum* was relatively constant over the course of the experiment, but the diameter of the other clover entries increased between week 4 and 6 (Figures 6.4A and 6.4B).

At week 4, the mean average root diameter of *T. uniflorum* was 12–22% larger than that of the other clover entries ($P<0.051$), but at week 6 it was 10% smaller than Kopu II x 80-2 ($P=0.004$), 8% smaller than Crusader ($P=0.018$) and 14% smaller than Crusader x 900-4 ($P<0.001$) (Figure 6.4A and 6.4B). There was also a trend towards smaller mean average root diameters for Kopu II x 900-4 compared with Kopu II x 80-2 (7% smaller) ($P=0.087$) at week 4, and for *T. uniflorum* compared with Kopu II (6% smaller) ($P=0.09$) at week 6.
Figure 6.4. Mean root diameter of clover entries in the Kopu II (A) and Crusader (B) based groups. Statistical comparisons were made at week 4 and 6. Means with the same letter show no significant differences at the 5% level.
The mean number of tips cm\(^{-1}\) of root length was higher for Kopu II x 80-2 than for Kopu II, Kopu II x 900-4 and \textit{T. uniflorum} at both week 4 (+13–22%) and week 6 (+15–32%) \((P<0.030)\) (Figure 6.5A). At week 6, the mean number of tips cm\(^{-1}\) of root length was also 15% higher for Kopu II than for Kopu II x 900-4 \((P=0.021)\).

\textit{T. uniflorum} had 14–23% fewer tips cm\(^{-1}\) of root length than Crusader \((P=0.048)\) and the Crusader hybrids \((P<0.017)\) at week 4. Crusader \((P=0.084)\) and Crusader x 900-4 \((P=0.065)\) also tended to have fewer tips per cm\(^{-1}\) compared with Crusader x 80-2, by approximately
10% (Figure 6.5B). At week 6 there was also a trend for fewer tips cm\(^{-1}\) of root (-8–14%) for \textit{T. uniflorum} compared with Crusader x 80-2 \((P=0.027)\), Crusader x 900-4 \((P=0.035)\), and Crusader \((P=0.09)\) (Figure 6.5B).

On a DW basis, \textit{T. uniflorum} had fewer tips per unit of DW compared with the other clover entries (Figure 6.6), by 23% for Kopu II \((P<0.001)\), 30% for Kopu II x 80-2 \((P<0.001)\), 13% for Kopu II x 900-4 \((P=0.051)\), 20% for Crusader \((P=0.002)\), 33% for Crusader x 80-2 \((P<0.001)\), and 24% for Crusader x 900-4 \((P<0.001)\).

![Figure 6.6](image-url)

**Figure 6.6.** Mean number of root tips per unit root dry weight (±SEM) for clover entries in the Kopu II (A) and Crusader (B) based groups at week 6. Means with the same letter show no significant differences at the 5% level.

Both 80-2 families tended to have more tips per unit DW than the other clover entries (Figure 6.6). In addition to the differences to \textit{T. uniflorum} mentioned above, there were also trends
towards a higher number of tips per mg of root DW for Kopu II x 80-2 compared with Kopu II x 900-4 (24% more), and for Crusader x 80-2 compared with the Crusader parent (20% more) \( (P=0.008) \) and Crusader x 900-4 (13% more) \( (P=0.068) \).

Specific root length at week 6 was approximately 17% lower for \textit{T. uniflorum} than for Kopu II and Kopu II x 900-4 (Figure 6.7A). In contrast, SRLs of Crusader, Crusader x 900-4 and \textit{T. uniflorum} were all lower than Crusader x 80-2 by 15%, 13% and 24% respectively (Figure 6.7B).

![Figure 6.7](image_url)

**Figure 6.7.** Mean specific root length (SRL) (±SEM) of clover entries in the Kopu II (A) and Crusader (B) based groups at week 6. Means with the same letter show no significant differences at the 5% level.
There were overall clover entry effects for root tissue density at week 6, within both the Kopu II ($P=0.002$) and Crusader ($P<0.001$) based groups. In both cases, the tissue density of *T. uniflorum* was higher than all other clover entries (Figure 6.8), by 24–58%.

![Figure 6.8](image_url)

**Figure 6.8.** Mean root tissue density (±SEM) of clover entries in the Kopu II (A) and Crusader (B) based groups at week 6. Means with the same letter show no significant differences at the 5% level.

Growth in the mean total root length between time periods did not differ among clover entries up to week 4 (Figures 6.9A and 6.9B). However, the increase in root length of *T. uniflorum* between week 4 and week 6 was over 80% smaller than for the other clover entries ($P<0.001$). The growth in root length of both Kopu II hybrid families was approximately 28% lower than that of the Kopu II parent ($P=0.024$) (Figure 6.9A), but the Crusader hybrids did not differ to Crusader (Figure 6.9B). As a result of these differences at week 4–6, the growth in root length...
of all the hybrid families over the 6 week period of the whole experiment was 79–86% higher than that of \( T. \text{uniflorum} \) (\( P<0.001 \)) (Figure 6.9). The total increase in root length for both Kopu II x 80-2 (\( P=0.005 \)) and Kopu II x 900-4 (\( P=0.002 \)) was 26–29% lower than for Kopu II (Figure 6.9A), but the Crusader hybrids did not differ to Crusader for this trait (Figure 6.9B).

![Figure 6.9](image-url)

**Figure 6.9.** Growth in total root length at each time period for clover entries in the Kopu II (A) and Crusader (B) based groups. Means with the same letter within each time period show no significant differences at the 5% level.
6.3.2 Root architecture

6.3.2.1 Link analysis

Mean average link length of Kopu x 900-4 and, in particular, *T. uniflorum* was often longer than that of the other clover entries. At week 3, the mean average link length of Kopu II x 80-2 was 20% shorter than that of Kopu II (*P*=0.008), 21% shorter than that of Kopu II x 900-4 (*P*=0.006) and 23% shorter than that of *T. uniflorum* (*P*=0.001) (Figure 6.10A). At week 4, the mean average link length of Kopu x 80-2 was also 16% shorter than for Kopu II x 900-4 (*P*=0.033) and 25% shorter than for *T. uniflorum* (*P*<0.001); while the Kopu II parent was 15% shorter than *T. uniflorum* (*P*=0.019) (Figure 6.10A).

![Mean average link length (A and B), average surface area (C and D), and average diameter (E and F) of clover entries in the Kopu II (A, C, E) and Crusader (B, D, F) based groups. Statistical comparisons were made at week 3 and 4. Means with the same letter show no significant differences at the 5% level.](image)
Within the Crusader based group, there was a trend at week 3 towards shorter mean average link lengths for the 80-2 family (-16%) ($P=0.024$) and 900-4 family (-13%) ($P=0.069$) compared with *T. uniflorum* (Figure 6.10B). At week 4, mean average link lengths of Crusader ($P=0.002$), Crusader x 80-2 ($P<0.001$) and Crusader x 900-4 ($P<0.001$) were all shorter than that of *T. uniflorum*, by 20%, 29% and 22% respectively (Figure 6.10B). The mean average link surface area and diameter of *T. uniflorum* was larger than that of the other entries ($P<0.001$) at both week 3 and week 4 (Figures 6.10C-F). Mean average link surface area of both of the white clover parents did not differ to their respective hybrids, at either week 3 or week 4 (Figures 6.10C and 6.10D). There were no differences in link diameter among Kopu II and the Kopu II hybrids (Figure 6.10E), but link diameter of Crusader x 900-4 was 9% thicker than Crusader ($P=0.049$) and 14% thicker than Crusader x 80-2 ($P=0.004$) at week 3, and also 3% thicker than Crusader x 80-2 ($P=0.037$) at week 4 (Figure 6.10F).

In particular, the mean average II link length of *T. uniflorum* at week 4 was 15–37% higher ($P<0.041$) compared with all other clover entries (Figures 6.11A and 6.11B), but there were fewer differences for EE and EI link length (Appendices 26 and 27). Mean average II and EE link lengths of Kopu II ($P=0.008$ and $P=0.032$ respectively) and Kopu II x 900-4 ($P=0.012$) at week 4 were also 18–24% higher than those of Kopu II x 80-2 (Figure 6.11A and Appendix 26). The mean average link surface area and diameter of *T. uniflorum* was 23–73% and 14–35% higher ($P<0.05$), respectively, than the means of the other clover entries for II, EE and EI links (Figures 6.11C-F and Appendices 26 and 27).
Figure 6.11. Mean length (A and B), surface area (C and D), and average diameter (E and F) of internal–internal (II) links for clover entries in Kopu II (A, C, E) and Crusader (B, D, F) based groups at week 4. Means with the same letter show no significant differences at the 5% level.

6.3.2.2 Axis analysis

Overall, the mean length of the main tap root (axis order 0) at week 4 did not differ among clover entries, but that of *T. uniflorum* was 22–25% shorter than both the 900-4 hybrid families when comparing the LSDs at the 5% level (Appendix 28). There were no differences among the white clover parents and their respective hybrids for tap root length, surface area or link diameter (Appendix 28). Data for mean length, surface area and average link diameter of primary, secondary and tertiary laterals, are presented in Appendices 29 to 31.

At week 4, both Kopu II x 900-4 and *T. uniflorum* had a higher proportion of 1<sup>st</sup> order (primary) lateral roots, and a lower proportion of 2<sup>nd</sup> order (secondary) lateral roots, than Kopu II and Kopu II x 80-2 (Figure 6.12A). There were 42% and 38% more primary laterals
for *T. uniflorum* than for Kopu II and Kopu x 80-2, respectively, while Kopu x 900-4 had 24% and 19% more primary laterals than Kopu II and Kopu x 80-2, respectively.

![Mean % of laterals](image)

**Figure 6.12.** Mean % (±SEM) of primary (order 1), secondary (order 2) and tertiary (order 3) lateral roots for clover entries in Kopu II (A) and Crusader (B) based groups at week 4. Within lateral orders, means with the same letter show no significant differences at the 5% level.

Compared with Kopu II and Kopu x 80-2, there were 37% and 32% fewer secondary lateral roots, respectively, for *T. uniflorum*; and 20% and 15% fewer secondary lateral roots, respectively, for Kopu x 900-4. *T. uniflorum* also had a lower proportion of 3rd order (tertiary) laterals than Kopu II and Kopu II x 80-2 (-5–6%) (Figure 6.12A).

There were no differences in the proportion of lateral root orders among Crusader and the Crusader hybrids, but *T. uniflorum* had 34–42% more 1st order and 31–38% fewer 2nd order laterals than Crusader and the Crusader hybrid families (Figure 6.12B).
6.3.2.3 Topology

The topological index (TI) of *T. uniflorum* was relatively constant over the course of the experiment, while the TI of the white clover parents and BC$_1$ families decreased over time (Figures 6.13A and 6.13B).

![Graph A](image1)

![Graph B](image2)

**Figure 6.13. Changes in topological indices of clover entries in Kopu II (A) and Crusader (B) based groups, from week 0 to week 4.**

The TI of *T. uniflorum* and Kopu II x 900-4 were generally higher than those of Kopu II and Kopu II x 80-2 ($P<0.033$) (Table 6.2). Among the Crusader based group, the TI of *T. uniflorum* also tended to be higher than Crusader and Crusader x 80-2 ($P<0.008$), and that of Crusader x 900-4 was higher than Crusader x 80-2 ($P<0.035$). The TI of *T. uniflorum* was also higher than both the 900-4 families at the later measurement times, particularly compared with Crusader x 900-4 ($P<0.041$).
Table 6.2. Topological indices of clover entries in Kopu II (A) and Crusader (B) based groups, from week 0 to week 4. At each time, values with the same letter show no significant differences at the 5% level.

<table>
<thead>
<tr>
<th>Clover entry A.</th>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kopu II</td>
<td>0.92a</td>
<td>0.87a</td>
<td>0.84a</td>
<td>0.81a</td>
<td>0.76a</td>
</tr>
<tr>
<td>Kopu II x 80-2</td>
<td>0.92a</td>
<td>0.88a</td>
<td>0.83a</td>
<td>0.81a</td>
<td>0.79a</td>
</tr>
<tr>
<td>Kopu II x 900-4</td>
<td>0.98b</td>
<td>0.96b</td>
<td>0.91b</td>
<td>0.88b</td>
<td>0.85b</td>
</tr>
<tr>
<td>T. uniflorum</td>
<td>0.94ab</td>
<td>0.93b</td>
<td>0.93b</td>
<td>0.92b</td>
<td>0.91c</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clover entry B.</th>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crusader</td>
<td>0.95a</td>
<td>0.90ab</td>
<td>0.85a</td>
<td>0.81ab</td>
<td>0.77ab</td>
</tr>
<tr>
<td>Crusader x 80-2</td>
<td>0.92b</td>
<td>0.87a</td>
<td>0.82a</td>
<td>0.78a</td>
<td>0.76a</td>
</tr>
<tr>
<td>Crusader x 900-4</td>
<td>0.96a</td>
<td>0.92b</td>
<td>0.86a</td>
<td>0.84b</td>
<td>0.81b</td>
</tr>
<tr>
<td>T. uniflorum</td>
<td>0.94ab</td>
<td>0.93b</td>
<td>0.93b</td>
<td>0.92c</td>
<td>0.91c</td>
</tr>
</tbody>
</table>

6.4 Discussion

6.4.1 Root diameter and specific root length

In most cases, the roots of T. uniflorum were thicker than those of the other entries (Figures 6.4 and 6.10, Appendices 26, 27, 29 and 30). As thicker roots have a lower surface area to soil volume ratio, they are generally therefore less efficient at ion absorption than thinner roots (Jungk, 1996). For example, Wissuwa (2003) predicted that increasing the fineness of rice roots by 22% would produce a three-fold increase in the uptake of P. Crush et al. (2008) found that a white clover genotype selected for long, fine roots had a higher rate of P uptake per unit root DW, and a higher root %P concentration, than one selected for short, thick roots. The reason for the increase in average root diameter for the white clover cultivars and hybrids, compared with T. uniflorum, between week 4 and week 6 (Figure 6.4), is unclear. The consistency of this response across cultivars and hybrids suggests it is genetically based, and it may reflect developmental differences among clover entries. This may be due to differences in temporal changes in thickening of larger roots, with slower growth meaning that this thickening occurs later in T. uniflorum.

In general, plants with a high average root diameter can be expected to have a low SRL (less root length per unit DW), and vice versa. However, this is not always the case, as average root
diameter is strongly influenced by the thickest roots, whereas SRL is mainly influenced by fine roots (Boot, 1989). While the mean average root diameter of \textit{T. uniflorum} was higher than that of the other clover entries at week 4 (Figure 6.4), there were few differences in the mean link diameter of the thickest axis (the tap root, order 0) (Appendix 28). However, there were consistent patterns in the differences in average root diameter versus SRL at week 6. These observations suggest that \textit{T. uniflorum} had similar thick roots but less fine root mass than Kopu, Kopu x 900-4 and Crusader x 80-2; and less thick roots but a similar fine root mass as Crusader, Crusader x 900-4 and Kopu II x 80-2 (Figures 6.4 and 6.7). Crusader x 80-2 had similar thick roots and more fine root mass compared with Crusader and Crusader x 900-4 (Figures 6.4 and 6.7).

However, interpretation of the SRL results is complicated by tissue density. Higher SRL generally reflects more fine root mass, assuming tissue densities are equal (Eissenstat, 1992). Due to the higher tissue density in \textit{T. uniflorum} (Figure 6.8), it is possible that the lower SRL does not reflect a lower fine root mass compared with Kopu II, Kopu II x 900-4 and Crusader x 80-2 – that is, shorter root length per unit weight is offset by higher tissue density rather than larger diameter. Similar tissue densities in Crusader and the Crusader hybrids do confirm that the higher SRL of Crusader x 80-2 reflects more fine root mass, compared with Crusader and Crusader x 900-4 (Figures 6.8 and 6.7).

**6.4.2 Root architecture**

Numerous studies document the importance of root system architecture and topology in the interception of soil nutrients by plants (Dunbabin \textit{et al.}, 2003b; Lynch, 1995), although similar studies on white clover are limited. The topological index and proportion of lateral root orders observed in the current experiment show that \textit{T. uniflorum} is more herringbone-like (simply branched) than white clover (Figures 6.12 and 6.13, Table 6.2). Similar results for Kopu II x 900-4 indicate that this root topology can be introduced into at least some hybrid families. The higher proportion of EI links for Kopu II x 900-4 provides further evidence for a more herringbone topology (Fitter, 1986) compared with Kopu II and Kopu II x 80-2 (Appendix 24). Herringbone root systems require more carbon to produce and maintain than dichotomous systems, due to the number of high magnitude links which have large diameters in order to accommodate the conducting vessels necessary to support flow to, and from, high numbers of external links (Fitter, 1996). Even so, Fitter (1987) predicted that herringbone root systems are more efficient at exploiting the soil, especially for mobile nutrients with high diffusivities, e.g. nitrate. In dichotomous systems, there is more overlap in the depletion zones of neighbouring roots, leading to lower exploitation efficiency. This has
been confirmed by modelling (Fitter et al., 1991). However, in contrast to Fitter (1987), Nielsen et al. (1994) used modelling to show that acquisition efficiency of immobile P was higher in herringbone, rather than dichotomous, systems. More recent studies suggest that, in addition to ion diffusivity, mass flow and the plasticity of root responses to heterogeneous nutrient supplies also influence the ability of differing root architectures to intercept nutrients (Dunbabin et al., 2003b). Optimal root architecture may differ depending on the ions involved and the uniformity of its distribution in the soil (Dunbabin et al., 2003b; Fitter et al., 2002).

Fitter et al. (1991) also found that exploitation efficiency is correlated with long interior and exterior link lengths, most likely because the greater distance between branches results in less overlap in the depletion zones of neighbouring roots. In the current experiment, mean II, EE and EI link lengths of T. uniflorum were all greater than those of the other entries (Figure 6.11, Appendices 26 and 27). Compared with the other clover entries, T. uniflorum and in some cases Kopu II x 900-4, also had less frequent branching (Figures 6.5 and 6.6), which reflects the longer II link lengths that were observed (Figure 6.11). In contrast, Kopu x 80-2 and Crusader 80-2 had more frequent branching than some of the other entries (Figures 6.5 and 6.6), with concomitant shorter II link lengths (Figure 6.11). This supports the dichotomous topology suggested for these families, along with the potential decrease in exploitation efficiency of such systems through overlap of the depletion zones of neighbouring roots.

Several studies have confirmed the theory, at least for dicotyledons, that plants grown at low fertility would be more herringbone-like than when grown at high fertility (Fitter et al., 1988; Taub and Goldberg, 1996). In contrast, grasses appear to maintain a herringbone topology, regardless of nutrient supply (Taub and Goldberg, 1996; Fitter and Stickland, 1991). It has also been shown that herringbone root systems are characteristic of plants from infertile/poor resource soils (Taub and Goldberg, 1996; Fitter et al., 1988; Fitter and Stickland, 1991), although Taub and Goldberg (1996) only found this relationship when plants were grown with low soil resources (nutrients and water). As there was no nutrient limitation in the current experiment, and the hydroponic growth medium eliminates the effect of ion diffusion, this suggests the root system topology and architecture observed for T. uniflorum is likely to represent adaptation to the edaphic conditions in which this species naturally occurs. Topology suggests this adaptation may have been inherited by the Kopu II x 900-4 hybrid family (Figure 6.13 and Table 6.2). The indirect associations between link length and topological index, found by Fitter and Stickland (1991) (longer link length in slow growing species) may also support the connection between soil fertility and topology/architecture for
as do the negative relationships that have been suggested between nutrient supply and link length (Fitter and Stickland, 1991; Fitter et al., 1988).

Fitter (1987) concluded that herringbone root systems are less transport efficient than dichotomous systems due to inherently higher external path lengths, which result in a greater distance for transport from the point of absorption to the shoot. In the current experiment, herringbone-like *T. uniflorum* had a shorter exterior path length than the other entries (Appendices 24E and 24F) (and also the shortest maximum individual path length, or altitude (Appendices 24C and 24D)), suggesting it may actually have a higher transport efficiency. However, the longer mean link length of *T. uniflorum* may offset its shorter exterior path length (Figure 6.10). Fitter (1996) later suggested that as there are few gains in transport efficiency after several orders of branching in dichotomous systems, herringbone topologies are, in fact, often more efficient. Link length is also associated with transport efficiency, but the higher mean surface areas of long links, as seen in the current experiment (Figure 6.10), should offset their increased cost through increasing the absorption area (Fitter, 1996). Resistance to flow is another important factor in transport efficiency. The higher link diameters of *T. uniflorum* suggest the presence of larger conducting vessels (Figure 6.10), which provide less resistance to flow. However, no information is available for the comparative root anatomy of *T. uniflorum*, *T. uniflorum* × *T. repens* hybrids and white clover, and this could be a subject for future investigation.

### 6.4.3 Tissue density and growth

In addition to being characteristic of low fertility environments, herringbone systems are also predicted to be slow growing (Fitter, 1996). A lower increase in root length for *T. uniflorum* in the current experiment does indicate that root growth in this species is slower than in white clover (Figure 6.9). This is supported by the high tissue density of *T. uniflorum* compared with the other entries (Figure 6.8), as high tissue densities are also associated with slow growth rates for both roots (Wahl and Ryser, 2000) and leaves (Garnier and Laurent, 1994) – where leaf tissue density (DW per leaf area) is analogous to SLM reported in Chapter 7. Tissue density is also related to soil fertility, with increases recorded at low nutrient supply, and vice versa (Arredondo and Johnson, 2011; Robinson et al., 1999). The uniform nutrient supply in the current experiment suggests the tissue density of *T. uniflorum* may reflect edaphic adaptations. Ryser (1996) found that species common to high nutrient environments had a low tissue density, and were larger after one growing season than species typical of low nutrient environments. However, the species from low fertility environments, which had high tissue density, were larger after two growing seasons. Again, this suggests low soil fertility in
the natural habitat of *T. uniflorum*, but there is no published information on this. The higher tissue density of *T. uniflorum* may also result in greater root longevity (Ryser, 1996; Arredondo and Johnson, 2011). While both the Kopu II hybrid families also exhibited slower root length growth than the Kopu II parent (Figure 6.9A), tissue density did not vary among these three clover entries (Figure 6.8A).

### 6.4.4 Comparison with published information on *Trifolium*

Fitter and Stickland (1991) observed similarities in root system architecture within taxonomic groups, suggesting that not all root architecture patterns reflect current adaptations. However, common historical effects in *Trifolium* may have been modified by subsequent adaptation to specific environments, especially given the wide geographical range and varying habitats of the genus. There have been very few studies on root system architecture and topology of *Trifolium* species. Nichols *et al.* (2007) concluded that the root system topology of white clover is strongly fixed genetically, as topological indices changed very little with nine generations of inbreeding, although there were minor increases in the alternative measure of branching (%EI links). Topological indices in that study of 0.71 for white clover and 0.69–0.75 for the inbred clover entries were slightly lower than the minimum values in the current experiment, but in general were more similar to the white clover culitvars than to *T. uniflorum* and some hybrids (particularly 900-4 families). A previous study by Crush *et al.* (2005a) also found little effect of inbreeding on the topological indices of white clover, and indicated that genetic control of white clover root architecture in general is very complex. In that study, mean topological indices were 0.95 in solution culture and 0.93 in sand culture, similar to the values observed in the current experiment.

Other root architecture studies on *Trifolium* species have involved *T. pratense* and *T. dubium* Sibth.. Fitter *et al.* (1988) concluded that varying N or P supply independently had little effect on the topology of *T. pratense*, but root systems in low nutrient treatments were more herringbone-like than in high nutrient treatments. When N and P were varied at the same time, Fitter and Stickland (1991) also observed a more herringbone-like nature under low nutrient supply, for both *T. pratense* and *T. dubium*. In contrast to the general pattern, the interior link length of *T. pratense* and *T. dubium* decreased under low nutrient supply, and EI link length also decreased for *T. pratense* (Fitter and Stickland, 1991). In addition to soil fertility, the relationship between root system architecture/topology and soil moisture may also be important, particularly for species, such as white clover, which are very sensitive to decreases in soil moisture. Consistent with the theory that root systems will be more herringbone-like under low soil resource conditions, Fitter (1986) found that roots of *T. pratense* became more
herringbone-like with decreasing water supply, but interior link length did not vary greatly. However, the opposite effect has been observed for white clover in the field (Fitter and Stickland, 1992), where plants became more herringbone-like with increasing soil moisture, although these results may have been confounded by waterlogging.

The response of the root architectural and morphological parameters of \( T.\ uniflorum \) and \( T.\ repens \times T.\ uniflorum \) hybrids to changes in nutrient supply or soil moisture is unknown. For that matter, there is little published information on similar responses in white clover, although Dinh et al. (2012) recorded stimulation of root growth under low P conditions. However, the effects of such changes are well documented in other species. Future investigation of the responses of \( T.\ uniflorum \) and \( T.\ repens \times T.\ uniflorum \) hybrids to both contrasting and heterogeneous nutrient supplies would provide valuable information on the functional/ecological implications of their specific root system structures, and assist with the development of populations for targeted agricultural environments.

6.5 Conclusions

- Roots of \( T.\ uniflorum \) were generally thicker than those of the other clover entries, which may affect the efficiency with which it intercepts nutrients. The interaction of root diameter with nutrient supply in this material is unknown.

- Root systems of \( T.\ uniflorum \) were more herringbone-like than white clover and some hybrids, with long link lengths, suggesting this species may be adapted to an environment with low natural soil fertility.

- The Kopu II x 900-4 family was also more herringbone-like than white clover and the 80-2 family, indicating that the topology and architecture of \( T.\ uniflorum \) can be inherited by some \( T.\ repens \times T.\ uniflorum \) hybrids, which may provide some adaptation to low soil fertility.

- The white clover parents and 80-2 hybrid families had similar topological indices, but the number of tips per unit root length and DW suggests the 80-2 families had a higher frequency of branching than the white clover parents.

- These contrasting architectures suggest potential adaptation to different soil conditions, but their true functional significance is as yet unknown. The interaction of root architecture with contrasting nutrient supply, and heterogeneous supply in time and space, requires further study.
Chapter 7
Morphological and growth responses to water stress

7.1 Introduction

As mentioned in previous chapters, the desirable characteristics of white clover (productivity, feed quality and nitrogen fixation) are offset by a number of limitations, including inferior growth and persistence under drought and permanent dryland conditions (Knowles et al., 2003; Brock et al., 2003). This limits the environments in which white clover can be utilised, and also renders it vulnerable to periodic drought events in otherwise favourable environments.

Numerous impacts of drought have been reported for white clover, including decreased dry matter (DM) production, senescence of shoots, and stolon death (Barbour et al., 1996; Brock and Kim, 1994; Knowles et al., 2003). Some of these impacts are directly attributable to the negative effects of drought, whereas others are associated with protective plant responses to water stress. Drought resistance in plants is a combination of tolerance (e.g. production of biochemical compounds, osmotic adjustment) and avoidance mechanisms (e.g. annual life cycle, decreased leaf size, stomatal closure) (Levitt, 1980). Attempts to improve the drought resistance of white clover have involved selection of dryland ecotypes (Woodfield and Caradus, 1987; van den Bosch et al., 1993), selection for large nodal roots (Caradus and Woodfield, 1998) and increased stolon density (MacFarlane et al., 1990). Grazing management also influences the impact of drought on white clover (Brock and Kim, 1994).

*Trifolium uniflorum* is a Mediterranean species, and it is described as xerophytic (Tela Botanica, 2012). However, the response to drought of *T. uniflorum* and *T. repens* x *T. uniflorum* hybrids has not been studied before. If *T. uniflorum* does possess characteristics which impart some degree of drought resistance, then *T. repens* x *T. uniflorum* hybrids could perform better under drought than white clover. This chapter investigates the effects of water stress on morphological and growth characteristics of *T. repens* x *T. uniflorum* hybrids compared with white clover cultivars. It is also expected that the hybrids will have inherited physiological and biochemical mechanisms from *T. uniflorum*, which enable this to occur. These physiological and biochemical responses are reported in Chapter 8.

The first objective of this experiment was to quantify and compare the effect of drought on *T. repens* x *T. uniflorum* hybrids and white clover cultivars, by measuring DM production. It
was hypothesised that drought would have a smaller effect on DM yields of BC1 (backcross 1) and BC2 (backcross 2) hybrids than on white clover. The second objective was to quantify and compare the effect of drought on the stolon morphology of \( T. \text{repens} \times T. \text{uniflorum} \) hybrids and white clover cultivars. Again, it was hypothesised that drought would have a differential effect on the stolon morphology of BC1 hybrids, BC2 hybrids and white clover. The final objective was to quantify and compare the effect of drought on \( T. \text{repens} \times T. \text{uniflorum} \) hybrids and white clover cultivars for other parameters which could affect growth and influence their relative abilities to tolerate dry conditions. This includes traits such as lateral spread, stolon density, root production and flowering. It was hypothesised that some of these parameters, under water stress, may differ between the hybrids and white clover.

### 7.2 Materials and methods

#### 7.2.1 Experimental area

This experiment was established in a rain shelter at Plant and Food Research, Boundary Rd, Lincoln (43° 37′ 22.92"S, 172° 28′ 6.65"E) (Plate 7.1). The rain shelter is located on a Templeton silt loam (New Zealand Soil Bureau, 1968) (Udic Ustochrept, USDA soil taxonomy), and is oriented in a north-south direction. It contains four bays, and the experiment was set up at the northernmost end of the site, occupying 12 m x 18 m within the total rain shelter area of 12 m x 55 m. The experimental area was sprayed four weeks prior to planting, with 2.5 l ha\(^{-1}\) Roundup\(^{\text{R}}\) (glyphosate) and 3.5 l ha\(^{-1}\) of Buster\(^{\text{R}}\) (glufosinate-ammonium), to remove the existing perennial ryegrass sward.

Plate 7.1. Plant and Food Research rain shelter, parked in the fine weather position. The red arrow indicates the location of the experimental site.
The shelter moved over the experimental area when sensors at the southern end of the facility detected rain, and pulled back again once the sensors became dry. A rain bucket inside the northern end of the experimental area logged no precipitation during the course of the experiment, indicating that no significant rain affected the experiment because of possible shelter malfunction or the time taken for the shelter to move into place. Maximum daily temperatures and total daily rainfall at the site, from establishment of the plants until harvest, are shown in Figure 7.1. Data were collected from the Broadfield meteorological station, 200 m east of the site (43° 37′ 28.43"S, 172° 28′ 13.5"E).

![Graph showing daily temperature and rainfall from 1/10/2009 to 30/03/2010 with a peak on 15/10/2009.]

**Figure 7.1.** Maximum daily temperature and total daily rainfall at the experimental site, from establishment until harvest. The arrow indicates the start of rain shelter operation, after which all subsequent rainfall was excluded from the experiment. Data collected from the Broadfield meteorological station.

### 7.2.2 Plant material

BC₁ families were selected based on three main criteria:

1. Maintenance of DM scores over the summer of 2008/2009 in the key traits experiment (Chapter 3).

2. Known performance of the white clover parents in dryland conditions – this included cultivars that perform well or were bred for dry environments, as well as those that perform poorly.

3. White clover parent leaf size (a range of small–medium, medium and large).
Several families selected from AgResearch experiments were also included. Corresponding white clover parental cultivars and, where available, BC₂ families were included to provide sequences of related material with increasing proportions of *T. uniflorum* genes (e.g. Kopu II, Kopu II BC₂, Kopu II BC₁). Additional families were added to increase the number of families in the BC₂ pool. *T. uniflorum* was not included, as it establishes poorly from cuttings.

A total of 16 clover entries were used, consisting of BC₁ families, BC₂ families, and white clover cultivars. A brief description of each entry is given in Table 7.1, with entry numbers corresponding to those used in the key traits experiment (Chapter 3). For full details of the hybrid entries see Appendices 1 and 2.

**Table 7.1. Description of clover entries used in the drought experiment. Entry numbers correspond to those in the key traits experiment (Chapter 3). cv = cultivar; OP = open pollinated.**

<table>
<thead>
<tr>
<th>Entry number</th>
<th>Clover type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>BC₁</td>
<td>Crusader x 80-2</td>
</tr>
<tr>
<td>6</td>
<td>BC₁</td>
<td>Crusader x 900-4</td>
</tr>
<tr>
<td>8</td>
<td>BC₁</td>
<td>Kopu II x 900-4</td>
</tr>
<tr>
<td>9</td>
<td>BC₁</td>
<td>Kopu II x 80-2</td>
</tr>
<tr>
<td>10</td>
<td>BC₁</td>
<td>Sustain x 82-3</td>
</tr>
<tr>
<td>11</td>
<td>BC₁</td>
<td>Tribute x 900-4</td>
</tr>
<tr>
<td>12</td>
<td>BC₁</td>
<td>Trophy x 902-6</td>
</tr>
<tr>
<td>13</td>
<td>BC₂</td>
<td>Crusader x (Crusader x 900-5)</td>
</tr>
<tr>
<td>14</td>
<td>BC₂</td>
<td>Kopu II x (Kopu II x 902-1)</td>
</tr>
<tr>
<td>15</td>
<td>BC₂</td>
<td>902-1-OP-4 x Trophy</td>
</tr>
<tr>
<td>17</td>
<td>BC₂</td>
<td>Durana x (Crusader x 902-1)</td>
</tr>
<tr>
<td>19</td>
<td>White clover</td>
<td>cv. Crusader</td>
</tr>
<tr>
<td>20</td>
<td>White clover</td>
<td>cv. Grasslands Kopu II</td>
</tr>
<tr>
<td>21</td>
<td>White clover</td>
<td>cv. Grasslands Sustain</td>
</tr>
<tr>
<td>23</td>
<td>White clover</td>
<td>cv. Grasslands Tribute</td>
</tr>
<tr>
<td>24</td>
<td>White clover</td>
<td>cv. Trophy</td>
</tr>
</tbody>
</table>
For each clover entry, the six best performing plants (genotypes) in the key traits experiment were identified, based on DM yields during the preceding summer, and stolon tip cuttings were taken from each of these in July 2009. The cuttings were trimmed to 2–3 nodes and one fully expanded leaf (FEL), and then planted in sand trays to which a Long Ashton nutrient solution (Hewitt, 1966) (Appendix 17) was applied three times per week. Once roots had established, the cuttings were transplanted to 40 x 40 x 120 mm root trainers of sand/peat potting mix in a glasshouse (1 August 2009). After 5 weeks, the plants were trimmed and moved to a tunnel house, then outside, for hardening off. Plants were then transplanted into the field by hand on 1 October 2009. Spacing was 660 x 600 mm, to fit the layout of the irrigation system. Each plant was placed in the same relative position (angle and distance) to the surrounding water emitters.

7.2.3 Irrigation system

Plots were irrigated using a trickle irrigation system. Each plot was fed by a manifold with 13 mm lateral lines spaced 220 mm apart. The system was set up in every plot to ensure the physical effects of the irrigation lines on growth were the same across irrigation treatments. Emitters were spaced 300 mm apart along each lateral line and were offset on adjacent laterals to ensure even watering of the area. As residual water in the tubing was heated by the sun, this was run to waste before each irrigation event to prevent scorching of the foliage.

7.2.4 Experimental design

There were six replicates and two watering treatments in the experiment (Figure 7.2). The treatment which was irrigated is referred to here as the Watered treatment, and the treatment from which irrigation was withheld is referred to as the Stressed treatment. A split plot design was used, with each replicate containing one Watered and one Stressed plot. Plots were 3.6 m x 3.95 m in total, with a 400 mm gap between the top and bottom of neighbouring plots and a 1 m gap at the sides. The 12 plots were laid out in a 3 x 4 arrangement. Treatments within replicates were assigned at random, as were the 16 clover entries within each plot. Two clones of each entry were planted in every replicate (one Watered and one Stressed) to enable genotypic effects to be considered. Border plants were used around the outside of every plot to prevent edge effects. These were established in the same manner as the experimental plants, from cuttings taken from white clover plants in an existing field experiment.
7.2.5 Watering treatments and maintenance

The site was left open to rain for five weeks after planting, to aid establishment. However, as Templeton silt loam has good water storage ability it is difficult to impose stress conditions from field capacity (S. Maley, pers. comm.). Therefore, operation of the rain shelter began in early November 2009 to begin drying down the soil. To help plant establishment, five millimetres of irrigation was applied to all plants on 24 November before the different watering treatments were imposed on 8 December 2009 (Plate 7.2A). After that time the Stressed plots received no further irrigation for the remainder of the experiment, and Watered plots were irrigated weekly to replace PET (potential evapotranspiration) +10–20 mm until the end of the experiment (23 March 2010) (Plate 7.2B). The additional water was added to reduce the development of mild moisture stress in Watered plants. Soil moisture and leaf water potential readings were used to decide the amount of additional water to apply.
Plate 7.2. Experimental site at the first irrigation (8 December 2009) (A) and the end of the experiment (23 March 2010) (B). In B, plots at the bottom of the picture are from the Watered treatment, and the rain shelter can be seen in the fine weather position, at the top, right of the picture.
Penman’s potential evapotranspiration (PET) data was obtained from the Broadfield meteorological station, located 220 m east of the experimental site. Data were usually available up to the day before irrigation. Total PET from and including the previous irrigation day was therefore calculated, and any additional PET added to this value. The corresponding irrigation volume was then calculated and applied. Figure 7.3 shows the irrigation applied at each time interval, along with the corresponding PET, plus the weekly rainfall and PET prior to operation of the rain shelter.

![Figure 7.3. Irrigation applied to the Watered treatment plots, with Penman’s potential evapotranspiration (PET) for the corresponding time interval between irrigation events. Weekly rainfall and PET between planting and the start of rainshelter operation (A) are also shown. B = 5 mm of irrigation applied to all plots prior to the start of watering treatments (C).](image)

Soil moisture was monitored weekly, with measurements made the day before irrigation. Neutron probe access tubes were installed in the centre of each plot, prior to planting, allowing measurements of soil moisture in 0.1 m increments from 0.25 to 0.95 m using a neutron probe (Troxler Electronic Industries Inc., North Carolina, USA) (Plate 7.3). Soil moisture in the top 0.2 m was monitored using time domain reflectometry (TDR). TDR rods were installed 150 mm from each neutron probe tube, and soil moisture was measured using a Trace TDR machine (Everest Interscience Inc., Tustin, USA).

Regular weeding of the plots was carried out to prevent establishment of volunteer clovers and weeds. Experimental and border plants were trimmed on 16–18 February 2010 to maintain the separation of individual plants in the Watered treatment. Stolons of Watered
plants were cut back to the youngest rooted node, and the remaining herbage was trimmed to stolon height. Plants in the Stressed treatment received only a light trim as soil moisture was limiting for regrowth.


7.2.6 Measurements

For most parameters, measurements were made on all plants. However, physiological (see Chapter 8) and root measurements were only made on a subset of plants. This was due either to the amount of time needed to do the measurements and/or the physiological effects of changes in environmental conditions over small periods of time. In these instances, a subset of related entries was measured, which included a white clover cultivar and its corresponding BC₁ and BC₂ hybrids. Kopu II was chosen as it is a large-leaved cultivar, and such germplasm is known to be more drought sensitive than small-leaved types (Barbour et al., 1996). Entry 9 was selected from the two available Kopu II BC₁ families. This sequence will be referred to as the Kopu II subset. The Kopu II, Kopu II BC₁ and Kopu II BC₂ plant in every plot was measured (36 plants in total). In the full data set, comparisons are between clover ‘types’, and in the Kopu II subset, comparisons are between clover ‘entries’.
7.2.6.1 Dry matter yield
Dry matter was scored for all plants every 4–6 weeks from early November onwards, on a visual scale of 1–9 (smallest–largest). At the end of the experiment all shoot material, including rooted stolons, was removed. Herbage was oven dried at 80°C for 48 hours and then weighed.

7.2.6.2 Stolon morphology, leaf and chlorophyll index measurements
Stolon morphological measurements were made on two stolons from each plant near the end of the experiment (17–19 March 2010), following the methodology in Chapter 3, to determine internode length, stolon diameter, leaf lamina area, petiole length, specific leaf area (SLA) (mm$^2$ mg$^{-1}$) and specific leaf mass (SLM) (mg mm$^{-2}$). Prior to removal of the stolons, chlorophyll measurements were made on the central trifoliate leaflet of the second fully expanded leaf (FEL) (relative to the stolon tip) using a SPAD-502 chlorophyll meter (Konica Minolta Sensing Inc., Osaka, Japan). Measurements in SPAD values are correlated with chlorophyll and N content (Konica Minolta, 2012; Vistoso et al., 2012). The two leaves measured on each plant were averaged.

7.2.6.3 Roots
At the final harvest, roots were sampled from the Kopu II subset. Soil cores of 100 mm diameter x 100 mm deep were taken over the original centre of each plant to sample the oldest roots. The cores were stored at 5°C, before being washed through a 2 mm sieve to extract the roots, which were preserved in 70% ethanol.

A thin cross-section was cut from the base (i.e. at the stolon) of the thickest original nodal root from each sample. These were scanned on an Epson Expression® 1680 flat bed scanner, and their cross-sectional area measured using the image analysis program WinRhizo™ (Regent Instruments Inc., Quebec) to determine root thickness. All root material was rinsed well to remove ethanol then oven dried at 60°C for 24 hours to obtain the root sample dry weight (DW). Stolon material from the cores was also oven dried and added to the total shoot DW.

7.2.6.4 Lateral spread and rooted width
The maximum lateral spread of all the plants was measured near the start of the experiment (10 November 2009) and at the end of the experiment (23 March 2010) (Plate 7.4A). The rooted width of the maximum lateral spread (distance anchored by nodal roots) was also measured at the final harvest (Plate 7.4B), and was used to calculate the rooted proportion of each plant.
Plate 7.4. Measurement of maximum lateral spread as indicated by the white bar (A) and maximum rooted width (distance anchored by nodal roots) of the same plant measured after harvest (B).
7.2.6.5 Other growth parameters

Prior to trimming in February 2010, the Stressed plants were scored for a variety of growth parameters – growth habit, growth pattern (see Plate 7.5), density, foliage colour (specifically, presence of stress associated discoloration), and the amount of visible stolon versus leaf (Table 7.2). All plants were scored for extent of senescence (Table 7.2) at the end of the experiment on 23 March 2010 (see Appendix 32 for examples).

Plate 7.5. Examples of the score values for growth pattern scoring: 1 = Compact (A), 2 = Tight shape but spreading (B), 3 = Tight middle with stolons spreading at the edges (C), 4 = Spreading (D), 5 = Spreading and open (E).
Table 7.2. Score system for growth parameters in the drought experiment. Senescence was scored for all plants and other parameters were scored in the Stressed treatment only.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score value</th>
<th>Score range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth habit</td>
<td>1 – 7</td>
<td>Prostrate – erect</td>
</tr>
<tr>
<td>Growth pattern</td>
<td>1 – 5</td>
<td>Compact – spreading and open</td>
</tr>
<tr>
<td>Stolon density</td>
<td>1 – 5</td>
<td>Tight – sparse</td>
</tr>
<tr>
<td>Foliage colour</td>
<td>1 – 5</td>
<td>Green – predominantly coloured</td>
</tr>
<tr>
<td>Visible stolon:leaf ratio</td>
<td>1 – 6</td>
<td>Mostly leaf – mostly stolon</td>
</tr>
<tr>
<td>Senescence</td>
<td>1 – 10</td>
<td>Minimal – whole plant dead</td>
</tr>
</tbody>
</table>

7.2.6.6 Flowering

Inflorescence numbers on each plant were counted on 21 December 2009, 7 January 2010 and 2 February 2010. Plants in the Stressed treatment only were counted on 21 March 2010. Due to the extent of the lateral spread of plants in the Watered treatment at that time it was not possible to access individual plants without causing damage to those around them, and other samples and measurements were still to be taken at the final harvest. As additional water stress from flowering is more specifically a function of seed fill, rather than merely numbers of inflorescences, the inflorescences were also scored according to seed fill stages as shown in Table 7.3 and Plate 7.6.

Table 7.3. Description of score values used to assess the flowering stages of inflorescences.

<table>
<thead>
<tr>
<th>Score</th>
<th>Seed fill stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>At least 50% of florets open and available for pollination</td>
</tr>
<tr>
<td>2</td>
<td>50% of florets deflexed</td>
</tr>
<tr>
<td>3</td>
<td>&gt;50% of florets deflexed</td>
</tr>
<tr>
<td>4</td>
<td>Totally deflexed – peduncle green (drawing on water and carbohydrates)</td>
</tr>
<tr>
<td>5</td>
<td>Totally deflexed – peduncle yellow at the top, green at the bottom (residual water and carbohydrates returning to the stolon)</td>
</tr>
<tr>
<td>6</td>
<td>Totally deflexed – peduncle yellow (minimal draw on water and carbohydrates)</td>
</tr>
</tbody>
</table>
Plate 7.6. Examples of flowering scores: 1 = at least 50% of florets open; 2 = 50% of florets deflexed; 3 = >50% of florets deflexed; 4 = fully deflexed, peduncle green; 5 = fully deflexed, peduncle yellow at base; 6 = fully deflexed, peduncle yellow.

7.2.7 Statistical analysis

Soil moisture, DM scores and stolon measurements were analysed using a linear mixed modelling approach (LMM) in SAS version 9.1 (SAS Institute Inc.) to account for correlations among measurements within the same plants or over time. Replicates were included as block effects. For soil moisture, a separate LMM analysis was carried out for each depth and a trend in soil moisture over time was fitted for each treatment using: \( a - b \times \text{Days} \), where \( a = \) soil moisture at day 0 (19 November 2009) and \( b = \) constant rate of change in soil moisture. The significance level of the rate of change in each trend was determined, as was the difference in the rate of change between treatments. The analyses of stolon measurements took account of clover type and watering treatment effects as well as the clover type x watering treatment interaction.

All other data were analysed via analysis of variance (ANOVA) in Genstat version 11 (VSN International Ltd.). Data were analysed for clover type/entry and watering treatment effects, as well as the clover type/entry x watering treatment interaction, using replicates as block effects. The individual standard errors of the mean (SEM) are presented. Data for percentage rooted width was log-transformed to satisfy the assumptions of ANOVA, and the back-
transformed means and estimated standard errors (back-transformed mean x log SEM) are presented. Relationships between key parameters and changes in shoot DW under water stress were tested using regression and Pearson’s correlation in Minitab version 15 (Minitab Inc.).

Significant differences among clover types or entries and watering treatments, at the 5% level, were determined using the means separation methods stated above for the respective traits. Where measurements were made in one treatment only, the differences between means are indicated using lettering – means with the same letter were not significantly different at the 5% level, using the means separation methods stated above for the respective traits. Lettering is not used where data for both watering treatments are presented, as this generates some inappropriate pair-wise comparisons. Trends towards significance ($P=0.05–0.099$) are noted in the text.

Differences in variability among clover types or entries, within watering treatments, for key parameters were analysed using a test for equal variances in Minitab version 15 (Minitab Inc.), which compares two variances using the F-test or Levene’s test, depending on the normality distribution of the data. Standard deviations are presented to indicate the relative size of the variance for each clover type, and significant differences are indicated with lettering, as mentioned above.

### 7.3 Results

#### 7.3.1 Soil moisture

The changes in soil moisture over time differed significantly between the two watering treatments at every depth (Figure 7.4). Soil moisture in the Watered treatment increased over time at 0.20–0.45 m (although the change at 0.45 m appears very small), but did not change at the 0.55–0.85 m depths. In the Stressed treatment, soil moisture decreased over time at all depths.
Figure 7.4. Trendlines fitted to mean soil moisture over time for the Watered (dashed line) and Stressed (solid line) treatments from 0.20-0.95 m. At each depth, $P$ values indicate the significance level for differences in the trends between watering treatments. For each trendline, a significant change in soil moisture over time, starting from 19/11/09 (day 0) is indicated by * ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$).
7.3.2 Dry matter yield

7.3.2.1 Total shoot dry weight
Total shoot DW at the end of the experiment differed both among clover types (P<0.001) and between watering treatments (P<0.001). Overall, shoot DW of the BC1 and BC2 generations was 40% and 35% smaller, respectively, than white clover (P<0.05), but did not differ to each other. When averaged across clover types, plants in the Stressed treatment were 62% smaller than in the Watered treatment. There was also a clover type x watering treatment interaction (P<0.001), with the decrease in total shoot DW under water stress being smaller for BC1 (-119 g) than BC2 (-219 g) and white clover (-341 g) (Figure 7.5). The water stress-induced decrease in shoot DW of BC2 was also smaller than that of white clover. Relative to the Watered treatment, the shoot DW of the BC1 generation decreased by 47%, compared with 68% for BC2 and 69% for white clover.

Figure 7.5. Mean total shoot dry weight (±SEM) for BC1, BC2 and white clover in the Watered and Stressed treatments.

In the Watered treatment, shoot DWs of both BC2 and white clover were larger than BC1 by 26% and 95% respectively, but they did not differ to BC1 in the Stressed treatment (Figure 7.5). Shoot DW of white clover was larger than BC2 in both watering treatments, by just over 50%. Data for shoot DW variability is presented in Appendix 33.
**Kopu II subset**

The Kopu II subset also showed overall clover type \((P=0.023)\) and watering treatment \((P<0.001)\) effects for total shoot DW. Kopu II BC\(_1\) shoot DW was significantly smaller than both Kopu II BC\(_2\) and Kopu II, by 34% and 40% respectively \((P<0.05)\), while shoot DW in the Stressed treatment was 63% smaller than in the Watered treatment. There was also a clover type x watering treatment interaction \((P=0.020)\). The shoot DW of Kopu II BC\(_1\) did not differ between watering treatments, whereas the DWs of Kopu II BC\(_2\) and Kopu II decreased by 78% and 62%, respectively, in the Stressed treatment compared with the Watered treatment (Figure 7.6).

![Figure 7.6. Mean total shoot dry weight (±SEM) of Kopu II BC\(_1\), Kopu II BC\(_2\) and Kopu II in the Watered and Stressed treatments.](image)

As a result, shoot DW of Kopu II BC\(_2\) and Kopu II were larger than Kopu II BC\(_1\) in the Watered treatment, by more than 95%, but they did not differ to Kopu II BC\(_1\) in the Stressed treatment (Figure 7.6). Shoot DW of Kopu II BC\(_2\) and Kopu II did not differ in either treatment. Data for shoot DW variability is presented in Appendix 34.
7.3.2.2 Shoot dry matter scores

The mean shoot DM scores of white clover, based on visual assessment, were initially larger than BC1 and BC2 in both watering treatments ($P<0.014$) (Figures 7.7A and 7.7B), and continued to be larger than both hybrids in the Watered treatment ($P<0.002$) (Figure 7.7A). However, in the Stressed treatment there were no differences in DM score between BC1 and white clover from the 1 February 2010 sampling date onwards. There were also no differences between BC2 and white clover in the Stressed treatment at the last sampling date (Figure 7.7B).

Dry matter scores of BC1 and BC2 did not differ to each other at first in the Watered treatment, but BC2 was larger than BC1 at the end of the experiment on 23 March 2010 ($P=0.041$) (Figure 7.7A). The DM scores of the hybrids were also similar initially in the Stressed treatment, but there was a trend for lower DM scores for BC2 compared with BC1, from the 1 February 2010 sampling date onwards (Figure 7.7B).

Clover type differences for mean DM scores on 23 March generally reflected those for total shoot DW at the end of the experiment, although shoot DW may have been more accurate at detecting the difference between BC2 and white clover in the Stressed treatment.

There was a clover type x watering treatment x date interaction for mean DM score ($P=0.017$). Watering treatment had no effect on mean DM scores initially, but all clover types were lower in the Stressed treatment than the Watered treatment from the 9 January 2010 sampling date onwards ($P<0.001$) (Appendix 35 and Figure 7.7). The clover type x watering treatment interaction was present from 1 February 2010, at which time the decrease in mean DM score under water stress was smaller for the BC1 generation than for white clover ($P=0.047$) (see Appendix 35 and Figure 7.7). At the final two sampling dates, the effect of water stress on BC1 was smaller than on BC2 ($P=0.016$ and 0.011) and white clover ($P<0.001$), which did not differ to each other (Appendix 35 and Figure 7.7).
Figure 7.7. Mean shoot dry matter scores (±SEM) for BC1, BC2 and white clover in the Watered (A) and Stressed (B) treatments on 10 November and 14 December 2009, 9 January, 1 February, 8 March and 23 March 2010. Scored on a scale of 1 (smallest) to 9 (largest). # indicates the start of the watering treatment effect; ## indicates the start of the clover type x watering treatment interaction.
7.3.3 Stolon morphology, leaf and chlorophyll index measurements

7.3.3.1 Internode length
The Stressed treatment decreased \((P<0.001)\) the internode length of all clover types compared with the Watered treatment (Figure 7.8A), but the effect of drought was lower for the BC1 generation compared with the BC2 generation \((P=0.006)\) and white clover \((P=0.011)\). Internode length decreased by 31\% for BC1, 46\% for BC2 and 38\% for white clover. In the Watered treatment, internode length of BC1 was 33-40\% shorter than BC2 and white clover \((P<0.001)\) (Figure 7.8A), but in the Stressed treatment both BC1 \((P=0.001)\) and BC2 \((P=0.054)\) were shorter than white clover (by 33\% and 20\%, respectively).

The correlation analyses showed that in the Kopu II subset, genotypes with longer internodes in the Watered treatment had larger decreases in total shoot DW under water stress \((P=0.005, R^2=0.395)\) (Appendix 36). Shoot DW also tended to decrease more \((P=0.08, R^2=0.2033)\) in genotypes which had larger decreases in internode length under water stress.

7.3.3.2 Stolon diameter
Water stress did not affect the stolon diameter of any of the clover types (Figure 7.8B), although the stolon diameter of white clover tended to decrease by 7\% \((P=0.091)\). The treatment effect for white clover tended to be different to that of the BC1 generation \((P=0.065)\). In the Watered treatment, stolon diameter of white clover also tended to be larger (by 7\%) than BC1 \((P=0.060)\), but there were no differences among clover types in the Stressed treatment (Figure 7.8B).

7.3.3.1 Petiole length
Petiole length was shorter \((P<0.001)\) in the Stressed treatment compared with the Watered treatment for all clover types (Figure 7.8C), but compared with white clover the reduction was smaller for the BC1 \((P=0.019)\) and BC2 \((P=0.043)\) generations. Mean petiole length decreased in the Stressed treatment by 83 mm for BC1 and BC2, and 103 mm for white clover, which was equivalent to 68\%, 74\% and 76\%, respectively. In the Watered treatment, the mean petiole length of white clover was 12\% higher than BC1 \((P=0.016)\) and 22\% higher than BC2 \((P<0.001)\), but there were no differences among clover types in the Stressed treatment (Figure 7.8C). In the Kopu II subset, shoot DW decreased less under water stress for genotypes which had smaller decreases in petiole length \((P=0.016, R^2=0.3496)\).
Figure 7.8. Mean (±SEM) internode length (A), stolon diameter (B), petiole length (C) for BC1, BC2 and white clover in Watered and Stressed treatments.
7.3.3.2 Leaf lamina area

The Stressed treatment also reduced \((P<0.001)\) the mean leaf lamina area of all clover types (Figure 7.8D), but the reduction was smaller for BC1 compared with BC2 \((P=0.051)\) and white clover \((P<0.001)\), and the reduction for BC2 also tended to be smaller than for white clover \((P=0.054)\). Leaf lamina area decreased by 486 mm\(^2\) for the BC1 generation, by 681 mm\(^2\) for the BC2 generation, and by 888 mm\(^2\) for white clover, which was equivalent to 65%, 73% and 74% respectively. In the Watered treatment, leaf lamina area of white clover was 62% higher than BC1 \((P<0.001)\) and 29% higher than BC2 \((P<0.001)\), and the BC2 generation was 25% higher than the BC1 generation (Figure 7.8D). However, there were no differences in leaf lamina area among clover types in the Stressed treatment (Figure 7.8D).

Genotypes with a smaller leaf lamina area in the Watered treatment had smaller decreases in shoot DW under water stress \((P=0.038, R^2=0.0651)\). There were also significant correlations between the effects of water stress on lamina area and shoot DW in both the full data set \((P=0.013, R^2=0.1261)\) and the Kopu II subset \((P=0.001, R^2=0.5724)\) – decreases in shoot DW under water stress were smaller in genotypes which had smaller decreases in leaf lamina area.

7.3.3.3 Specific leaf area

Specific leaf area of all clover types was lower \((P<0.001)\) in the Stressed treatment compared with the Watered treatment (Figure 7.8E), but the decrease was smaller for the BC1 generation than for white clover \((P=0.002)\). The decrease in SLA was 12.8 mm\(^2\) mg\(^{-1}\) for BC1, 14.1 mm\(^2\) mg\(^{-1}\) for BC2 and 16.7 mm\(^2\) mg\(^{-1}\) for white clover, which was equivalent to 41%, 44% and 46% respectively. In the Watered treatment, SLA of white clover was 17% higher than BC1 \((P<0.001)\) and 13% higher than BC2 \((P<0.001)\), but there were no differences among clover types in the Stressed treatment (Figure 7.8E).

7.3.3.4 Specific leaf mass

Water stress increased \((P<0.001)\) the SLM of all clover types (Figure 7.8F), but there were no differences among clover types in the size of this increase (39–46%). Specific leaf mass of white clover was lower than for the hybrids in both treatments (Figure 7.8F). In the Watered treatment, SLM of white clover was 17% lower than for the BC1 generation \((P<0.001)\) and 13% lower than for the BC2 generation \((P=0.024)\), and in the Stressed treatment it was 6% lower than BC1 \((P=0.047)\) and 9% lower than BC2 \((P=0.013)\) (Figure 7.8F).
Figure 7.8 continued. Mean (±SEM) leaf lamina area (D), specific leaf area (E), specific leaf mass (F) for BC₁, BC₂ and white clover in Watered and Stressed treatments.
7.3.3.5 Variability of clover types

Some stolon morphological parameters of the BC1 generation were less variable than white clover, particularly in the Watered treatment (Table 7.4). The BC1 generation was less variable than white clover for stolon diameter, petiole length and leaf lamina area in the Watered treatment, and for stolon diameter and leaf lamina area in the Stressed treatment. In contrast, SLA and SLM of the BC1 generation were more variable than white clover in the Watered treatment.

Variability for stolon morphological parameters of the BC2 generation was usually similar to white clover. The exceptions were petiole length in both the Watered (less variable) and Stressed treatments (more variable), and SLM in the Watered treatment (more variable) (Table 7.4). The BC1 generation was, in turn, more variable than BC2 for SLM and SLA in the Watered treatment, but less variable than the BC2 generation for stolon diameter and leaf lamina area. There were few differences in variability between the hybrids in the Stressed treatment.

Table 7.4. Standard deviations for stolon morphological characteristics of BC1, BC2 and white clover in the Watered and Stressed treatments. Clover types with the same letter within parameters and watering treatments show no significant differences in variability at the 5% level.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Watered</th>
<th>Stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BC1</td>
<td>BC2</td>
</tr>
<tr>
<td>Internode length (mm)</td>
<td>6.79a</td>
<td>7.54a</td>
</tr>
<tr>
<td>Stolon diameter (mm)</td>
<td>0.29a</td>
<td>0.53b</td>
</tr>
<tr>
<td>Petiole length (mm)</td>
<td>35.11a</td>
<td>38.81a</td>
</tr>
<tr>
<td>Leaf lamina area (mm²)</td>
<td>243.40a</td>
<td>468.50b</td>
</tr>
<tr>
<td>Specific leaf area (mm² mg⁻¹)</td>
<td>6.62a</td>
<td>4.38b</td>
</tr>
<tr>
<td>Specific leaf mass (mg mm⁻²)</td>
<td>0.0264a</td>
<td>0.0043b</td>
</tr>
</tbody>
</table>
7.3.3.6 Chlorophyll index measurements

There was no overall clover type effect for mean SPAD values (chlorophyll index) (51.6–52.6 SPAD units), which also showed no differences among clover types within each watering treatment (Figure 7.9). Overall, the SPAD value was higher ($P<0.001$) in the Stressed treatment (more chlorophyll) than the Watered treatment, by 26%, but the clover type x watering treatment interaction was not significant. Mean SPAD values of all clover types increased under water stress (Figure 7.9).

Figure 7.9. Mean SPAD (±SEM), for BC1, BC2 and white clover in the Watered and Stressed treatments.

7.3.4 Senescence

There was an overall clover type effect ($P=0.005$) for mean senescence score on 23 March 2010. Mean senescence for the BC1 generation (2.10) was lower ($P<0.05$) than for both BC2 (2.85) and white clover (2.87), which did not differ to each other. Mean senescence score did not differ among clover types in the Watered treatment (Figure 7.10), but in the Stressed treatment senescence was lower in the BC1 generation than in the BC2 generation and white clover. Shoot DW decreased less ($P=0.007$, $R^2=0.0747$) under water stress in genotypes which had smaller increases in senescence.
There was also an overall watering treatment effect ($P<0.001$), with the mean senescence score being higher in the Stressed treatment (3.05) than in the Watered treatment (2.01). The clover type x watering treatment interaction was also significant ($P=0.032$), with a smaller effect of water stress on senescence for BC$_1$ than for white clover (Figure 7.10). In fact, mean senescence score of the BC$_1$ generation did not differ between watering treatments, while that of BC$_2$ and white clover was higher in the Stressed treatment than the Watered treatment (Figure 7.10).

### 7.3.5 Roots

#### 7.3.5.1 Root cross-sectional area

There was an overall clover type effect on root cross-sectional area ($P=0.012$), across treatments. Kopu II BC$_1$ (0.896 cm$^2$) was 43% higher than Kopu II BC$_2$ (0.627 cm$^2$) and 84% higher than Kopu II (0.487 cm$^2$) ($P<0.05$). Root cross-sectional area of Kopu II BC$_2$ and Kopu II did not differ to each other. There was also an overall watering treatment effect on mean root cross-sectional area ($P=0.026$), which decreased by 31% in the Stressed treatment (0.547 cm$^2$) compared with the Watered treatment (0.793 cm$^2$). However, there were no differences between watering treatments for individual clover types, and there was also no clover type x watering treatment interaction (Figure 7.11). In the Watered treatment, there were no differences among clover types, but in the Stressed treatment the mean root cross-sectional area of Kopu II BC$_1$ was higher than Kopu II (Figure 7.11), based on the 5% LSD.
7.3.5.2 Root dry weight

Root DW showed no overall clover type or watering treatment effects, but there was a clover type x watering treatment interaction ($P=0.037$). The difference between watering treatments was significant for Kopu II BC$_1$ but not for Kopu II BC$_2$ or Kopu II (Figure 7.12). In the Stressed treatment, mean root DW of Kopu II BC$_1$ increased by 59% compared with the Watered treatment, and was 72% higher than Kopu II BC$_2$. 

![Figure 7.11. Mean basal root cross-sectional area (±SEM) of Kopu II BC$_1$, Kopu II BC$_2$ and Kopu II in the Watered and Stressed treatments at the end of the experiment.](image)

![Figure 7.12. Mean root dry weight (±SEM) in 100 mm diameter x 100 mm deep cores for Kopu II BC$_1$, Kopu II BC$_2$ and Kopu II plants in the Watered and Stressed treatments at the end of the experiment.](image)
7.3.6 Other measurements

7.3.6.1 Lateral spread

At the end of the experiment, on 23 March 2010, there were both overall clover type \((P<0.001)\) and watering treatment effects \((P<0.001)\) on lateral spread. Mean maximum lateral spread of all clover types differed significantly. White clover (770 mm) was 19\% larger than BC\(_2\) (645 mm), and 33\% larger than BC\(_1\) (580 mm) \((P<0.05)\). Lateral spread of the BC\(_2\) generation was 12\% larger than that of the BC\(_1\) generation \((P<0.05)\). Within watering treatments, lateral spread of the BC\(_1\) generation was smaller than that of BC\(_2\) (-14\%) and white clover (-30\%) in the Watered treatment, and both hybrid generations were 16–22\% smaller than white clover in the Stressed treatment (Figure 7.13). Overall, the Stressed treatment (590 mm) decreased the maximum lateral spread by 19\% compared with the Watered treatment (720 mm). The lateral spread of all clover types decreased under water stress, based on the 5\% LSD, but there was no clover type x watering treatment interaction (Figure 7.13).

![Figure 7.13. Mean lateral spread (±SEM) of BC\(_1\), BC\(_2\) and white clover on 23 March 2010 in the Watered and Stressed treatments.](image)

Genotypes with a smaller lateral spread in the Watered treatment had smaller \((P=0.001, R^2=0.1757)\) decreases in total shoot DW under water stress. There were also significant correlations between the effects of water stress on lateral spread and shoot DW in both the full data set \((P<0.001, R^2=0.4011)\) and the Kopu II subset \((P=0.006, R^2=0.3882)\) – decreases in shoot DW under water stress were smaller in genotypes which had smaller drought-induced decreases in lateral spread.
The increase in lateral spread from the start of rain exclusion to the end of the experiment (10 November 2009 to 23 March 2010) also showed an overall clover type effect ($P<0.001$), with all types differing significantly to each other. The increase in lateral spread of white clover (530 mm) was 24% higher than that of BC$_2$ (425 mm), and 44% higher than that of BC$_1$ (370 mm) ($P<0.05$). The increase for the BC$_2$ generation was 16% higher than that of BC$_1$ ($P<0.05$). There was also an overall watering treatment effect on the increase in lateral spread ($P<0.001$). Water stress reduced the increase by 26% in the Stressed treatment (370 mm) compared with the Watered treatment (500 mm).

The increase in lateral spread was 21–30% lower for all clover types under water stress, based on the 5% LSD, but there was no clover type x watering treatment interaction (Figure 7.14). In the Watered treatment the increase in spread of white clover was 23% larger than that of BC$_2$, which was in turn 22% larger than BC$_1$ (Figure 7.14). In the Stressed treatment the increase in spread of white clover was still larger than that of BC$_1$ (+36%) and BC$_2$ (+26%), but the hybrids did not differ to each other (Figure 7.14).

Figure 7.14. Mean increase in lateral spread (±SEM) of BC$_1$, BC$_2$ and white clover between 10 November 2009 and 23 March 2010, in the Watered and Stressed treatments.
### 7.3.6.2 Percentage rooted width

There was no overall clover type effect on the percentage rooted plant width (proportion of the maximum lateral spread anchored by nodal roots), which was approximately 30%.

However, there was both an overall watering treatment effect ($P<0.001$) and a clover type x watering treatment interaction ($P<0.001$). Rooted width in the Stressed treatment was 12%, which was lower than that in the Watered treatment (78%). This treatment difference for the BC$_1$ generation (-58%) was smaller than for the BC$_2$ generation (-68%) and white clover (-78%) (Figure 7.15). BC$_2$ and white clover did not differ in their response to water stress.

In the Watered treatment, the percentage rooted width of the BC$_1$ generation (71%) was smaller than that of white clover (89%) (Figure 7.15). However, in the Stressed treatment, the percentage rooted width of the BC$_1$ generation (13%) was significantly larger than both BC$_2$ (11%) and white clover (10%) (Figure 7.15). The BC$_2$ generation and white clover did not differ in either treatment.

![Figure 7.15. Mean back-transformed percentage rooted width (± estimated SEM) of BC$_1$, BC$_2$ and white clover in Watered and Stressed treatments at the end of the experiment.](image)

### 7.3.6.3 Growth form

There were clover type effects for mean growth habit, growth pattern and stolon density scores ($P<0.001$) measured in the Stressed treatment. Mean growth habit score was higher (more erect) for white clover than for the BC$_1$ and BC$_2$ generations (Figure 7.16A). The growth pattern score of white clover was also higher than that of BC$_2$ (more spreading and open), which was in turn higher than that of BC$_1$ (Figure 7.16B). Stolon density of the BC$_1$
generation was higher than both BC2 and white clover, which did not differ to each other (Figure 7.16C).

![Bar chart for growth habit (A), growth pattern (B) and stolon density (C) for BC1, BC2 and white clover plants in the Stressed treatment on 15 February 2010 (see Table 7.2 for details of the scoring system). Means with the same letter within parameters show no significant differences at the 5% level.](image)

Figure 7.16. Mean scores (±SEM) for growth habit (A), growth pattern (B) and stolon density (C) for BC1, BC2 and white clover plants in the Stressed treatment on 15 February 2010 (see Table 7.2 for details of the scoring system). Means with the same letter within parameters show no significant differences at the 5% level.

Stress colouration of the foliage did not differ significantly among clover types (data not shown). There was also no overall clover type effect for stolon:leaf ratio, but when means were compared using the LSD at the 5% level, then the ratio of the BC1 generation was smaller than that of the BC2 generation (Figure 7.17).
7.3.7 Flowering

7.3.7.1 Number of inflorescences and fully deflexed inflorescences

There were overall clover type effects at all sampling dates for both the total number of inflorescences and the total number of fully deflexed inflorescences ($P<0.001$). The BC$_2$ generation had a higher number of inflorescences (+54–126%) and totally deflexed inflorescences (+60–129%) than white clover at all sampling dates and in both watering treatments (Figure 7.18). The BC$_1$ generation also had a higher number of inflorescences (+23–47%) and totally deflexed inflorescences (+43–77%) in both treatments than white clover in December 2009 and January 2010, but these two clover types did not differ in February or March 2010 (Figure 7.18). BC$_1$ and BC$_2$ did not differ for both parameters in either watering treatment at the first two sampling dates, but BC$_1$ had fewer inflorescences (-41%) and deflexed inflorescences (-34%) in the Watered treatment in February 2010 (Figure 7.18). In the Kopu II subset, genotypes with fewer totally deflexed inflorescences in the Watered treatment in February tended to have smaller decreases ($P=0.85$, $R^2=0.1742$) in total shoot DW under water stress (Appendix 37).

Overall watering treatment effects did not occur until February 2010 ($P<0.001$). At this time, the total number of inflorescences was 40–50% lower in the Stressed treatment than the Watered treatment for the BC$_1$ and BC$_2$ generations (Figure 7.18A). The number of totally deflexed inflorescences was also 40% lower in the Stressed treatment than the Watered
treatment for the BC$_2$ generation (Figure 7.18B). However, there was no effect of watering treatment on white clover at any of the sampling dates. No clover type x watering treatment interactions occurred for either total number of inflorescences or total number of totally deflexed inflorescences throughout the experiment. There was a significant correlation ($P=0.003$, $R^2=0.0989$) between changes in total shoot DW under water stress and changes in the number of inflorescences under water stress in February – genotypes with smaller decreases in shoot DW also had smaller decreases in inflorescences.

Figure 7.18. Mean number of inflorescences (A) and mean number of totally deflexed inflorescences (B) (±SEM) in late December 2009, January 2010, February 2010 and late March 2010, for BC$_1$, BC$_2$, and white clover in the Watered (W) and Stressed (S) treatments. * denotes significant treatment differences within sampling dates for the clover types marked.
7.3.7.2 Flowering categories

Differences among clover types within flowering categories varied over time. In particular, both hybrid generations had 40–109% more inflorescences in category 4 (totally deflexed, peduncle green) than white clover at the first two sampling times (Figures 7.19A and 7.19B), for both treatments, but BC1 and white clover did not differ at the last two sampling dates (Figures 7.19C and 7.19D). BC1 and BC2 also had higher numbers of inflorescences than white clover (+41–76%) in category 3 (>50% deflexed) at the December 2009 sampling (Figure 7.19A). After that time, generally only BC2 was higher (+57–153%) than white clover (Figures 7.19B to 7.19D). In February, genotypes in the Kopu II subset with fewer inflorescenses in category 3 in the Watered treatment had smaller decreases ($P=0.034$, $R^2=0.2514$) in total shoot DW under water stress (Appendix 38).

![Bar chart](image)

**Figure 7.19.** Mean number of inflorescences (±SEM) by category (see Table 7.3 for descriptions of each category), in late December 2009 (A) and January 2010 (B), for BC1, BC2 and white clover in the Watered (W) and Stressed (S) treatments. * denotes significant treatment effects for the clover types marked.
Figure 7.19 continued. Mean number of inflorescences (±SEM) by category (see Table 7.3 for descriptions of each category), in February 2010 (C) and late March 2010 (D), for BC1, BC2 and white clover in the Watered (W) and Stressed (S) treatments. * denotes significant treatment effects for the clover types marked.

The watering treatment effect predominantly appeared at the February sampling time, when it was significant for categories 1, 3, 4 and 5 ($P<0.05$). In these categories, the numbers of inflorescences in the Stressed treatment were 42–79% lower than in the Watered treatment for the BC2 generation (Figure 7.19C). The BC1 generation also had 37–67% fewer inflorescences in the Stressed treatment than in the Watered treatment for categories 3 and 4 (Figure 7.19C). There were also clover type x watering treatment interactions in categories 1 ($P=0.011$) and 3 ($P=0.012$) at the February 2010 sampling time (Figure 7.19C). The effect of water stress on inflorescence numbers in these categories was larger for the BC2 generation (-75–79%) than for the BC1 generation (-63–67%) and white clover (-58–61%), which did not differ to each other.
7.4 Discussion

Soil moisture analyses showed that the watering regime used in this experiment created significant differences in soil moisture between the two watering treatments (Figure 7.4). In the Stressed treatment, final soil moisture at 0.2 m and 0.25 m was below or approximately equal to the wilting point reported for these soil horizons in Templeton silt loam (Martin et al., 2003). Down to 0.75 m the soil moisture was generally greater than the wilting point (12.1% v/v for 0.29–0.70 m) (Martin et al., 2003), but still no more than 50% of the field capacity. A zone of lower soil moisture at 0.55 m and 0.65 m was below or only slightly above the wilting point, which may reflect the presence of sand lenses in this soil (Pollock et al., 2009). Although soil moisture increased with watering higher up in the profile, final estimated mean soil moisture contents did not exceed the field capacity for this soil reported by Martin et al. (2003).

7.4.1 Dry matter production and growth

In accordance with the hypotheses outlined in the Introduction, the T. repens x T. uniflorum BC₁ generation was less affected by the drought stress than the BC₂ generation and white clover, for many of the parameters measured. These traits are likely to reflect adaptations in the T. uniflorum parent to its natural environment. Little information is published on this, although it is described as xerophytic (Tela Botanica, 2012). In particular, total shoot DW of the BC₁ generation decreased significantly less than the other clover types, as was predicted (Figures 7.5 and 7.6). Although the smaller shoot DW decrease of the BC₂ generation suggested it was also less affected by water stress than white clover, these two clover types had a similar proportional change in DW.

The expected decreases, due to water stress, in a number of stolon morphological parameters, including internode length, leaf lamina area, and specific leaf area, were also significantly smaller for the BC₁ hybrids than in the BC₂ generation and white clover (Figure 7.8). This suggests that under water stress, the BC₁ generation was able to maintain higher turgor and, therefore, higher cell expansion and growth of organs than the BC₂ generation and white clover. These factors may have contributed to the maintenance of DW in the BC₁ generation, and decreases in some stolon morphological traits were correlated with decreases in shoot DW. In addition, the correlations suggest that the more compact lateral spread and smaller size of some morphological traits for the BC₁ generation, also contributed to smaller decreases in shoot DW. Smaller decreases in lateral spread were also correlated with smaller decreases in DW under moisture stress. The impacts of watering treatment on growth were
seen relatively rapidly, and impacted white clover before the BC2 and BC1 generations (Figure 7.7 and Appendix 35). Differences among clover types in variability for stolon morphological parameters were very similar to those observed in the key traits experiment (Chapter 3). SPAD values increased with drought, as might be expected due to the effect of decreasing leaf size on %N. A similar increase in SPAD values under water stress was reported in a white clover population (full-sib progeny and parents) by Ballizany et al. (2012b). However, the data suggest that the differences in the response of leaf size to water stress in the current experiment did not produce differences in the N content of the leaves as SPAD values, which are correlated with shoot N concentrations (Vistoso et al., 2012), did not differ among clover types (Figure 7.9). Ballizany et al. (2012b) also found no significant genotypic variance in SPAD values in their white clover breeding population. Mean SPAD values in the Watered treatments of both studies were similar.

The higher stolon density of the BC1 generation in the Stressed treatment (Figure 7.16C) is also likely to have contributed to its smaller reduction in DW, compared with the BC2 generation and white clover. It may also contribute to persistence on farm, as suggested by MacFarlane et al. (1990) for white clover under grazing in dry hill country. The results from scoring showed that the growth pattern of the BC1 generation in the Stressed treatment was compact and dense, while white clover was spreading and open, with the BC2 generation being intermediate to these but tending to be more like white clover (Figure 7.16B). These differences appear similar to the “non-viney” and “viney” types described by Gibson et al. (1963) and Beinhart et al. (1963), and the “leafy” and “non-leafy” ladino clover types described by Yamada (1958). The “non-viney” clover types had higher stolon branching, and proved to have better persistence and productivity. In particular, they experienced lower stolon losses during summer than the “viney” types (Gibson et al., 1963). Yamada (1958) also found that the “leafy” types were more drought resistant, which was attributed to better root development. The stolon:leaf ratios in the current experiment indicate that the BC1 generation was more “leafy” (i.e. non-viney) than the BC2 generation (Figure 7.17). In combination with the growth habit scores (Figure 7.16A), which confirmed the more erect habit of white clover reported in Chapter 3, these parameters suggest the growth form of the BC1 clover types under water stress is a compact, dense, prostrate plant with a relatively high volume of leaf to stolon. Measurements of maximum lateral spread at the end of the experiment confirmed that the BC1 generation was more compact than white clover in both treatments, and more compact than BC2 in the Watered treatment (Figure 7.13). Thomas (1984) suggested that plasticity for growth habit may be a desirable characteristic for white clover under drought – specifically, the development of a prostrate habit, which is less prone to defoliation under
grazing. The inherently more prostrate growth habit of the *T. repens* x *T. uniflorum* hybrids may therefore contribute to drought resistance in grazing situations. In a mixed sward, under moisture stress, it may also enable hybrids to recover more rapidly than more erect grasses, which may be prone to more severe defoliation.

While stolon morphological characteristics of the BC$_2$ generation were similar to white clover in the key traits experiment (Chapter 3), under comparable conditions in the Watered treatment of the current experiment those of white clover were usually larger than those of BC$_2$ (Figure 7.8). There were also generally no differences in maximum lateral spread among BC$_1$, BC$_2$ and white clover in the key traits experiment, but in the Watered treatment of the current experiment the three clover types were all different (white clover > BC$_2$ > BC$_1$) (Figure 7.13). The use of grazing and a companion grass in the key traits experiment, where the clover type differences were absent, could suggest that white clover and the BC$_2$ generation may be less tolerant of grazing and competition with grass than the BC$_1$ generation. Alternatively, differences between experiments could reflect differences in seasonal growth or phenology among clover types. In Chapter 3, differences between white clover and the hybrids, for stolon morphological measurements and lateral spread, did vary throughout the year.

Similarly, at the end of the current experiment, shoot DW of the BC$_2$ generation in the Watered treatment was significantly larger than the BC$_1$ generation (Figures 7.5 and 7.6), whereas there was no difference in DM scores in the key traits experiment. Dry matter scores in the Watered treatment of the current experiment, at the last two sampling dates, were also higher for BC$_2$ than for BC$_1$ (Figure 7.7), indicating that the differences are due to clover type rather than differences in methodology between the two experiments (DW versus DM scores). This may also reflect some limitation on growth of the BC$_2$ generation by grazing and/or competition. The absence of competition for light from grass in the drought experiment may also have reduced the need for expansion of leaves and internodes in the BC$_2$ generation.

### 7.4.2 Senescence

Turner (1991) concluded that senescence was equally as important as decreases in the growth of stolons and leaves in contributing to decreased biomass production of white clover under water stress. The lower senescence shown by the BC$_1$ generation is, therefore, also likely to have contributed to the smaller decreases in DM production under drought (Figure 7.10), and there were correlations between senescence and DW decreases. Leaf senescence is commonly reported in white clover drought studies, and represents a response by the plant to increase
survival. Turner (1990b) concluded that white clover carries out osmotic adjustment to maintain stolon survival at the expense of leaf biomass. Senescence of older leaves is also a strategy to limit water loss through reducing leaf area, which may also recycle nutrients to younger tissues (Chaves et al., 2003). However, in the most severe cases of senescence in the current study (usually white clover, with some BC2 plants) there was also stolon death, suggesting such strategies were inadequate to cope with the severity of the stress that was imposed.

7.4.3 Roots

The higher overall root cross-sectional area of Kopu II BC1 and, particularly, the higher area compared with Kopu II in the Stressed treatment (Figure 7.11) may provide improved drought resistance to the hybrid over the white clover parent, as thicker roots are known to be more tolerant of dry conditions in the field in rice (Ekanayake et al., 1985) and white clover (Caradus and Woodfield, 1998). Generally, root DW increases under drought to maximise water uptake (Chaves et al., 2003). Such an increase was seen in the current experiment for Kopu II BC1, but not for Kopu II BC2 or Kopu II (Figure 7.12). The root DW of Kopu II BC1 was also higher than Kopu II BC2 under water stress. The changes in biomass allocation and greater root DWs may have enabled Kopu II BC1 to meet the high demands for water from a higher transpiration rate (see Chapter 8). This is similar to the drought resistance of “leafy” clover genotypes via superior root systems, which was suggested by Yamada (1958). Blaikie and Mason (1990) found that the relationship between root and shoot biomass of white clover was highly correlated, and although this was disrupted by water stress (increase in root relative to shoot), it was re-established over time. In comparison, Kopu II BC1 may be able to maintain a higher root:shoot ratio under water stress. When combined with results from other experiments in this study, this suggests that T. uniflorum and some T. repens × T. uniflorum hybrids may have a greater capacity to increase allocation to root biomass under limiting soil conditions. For example, in Chapter 5, one hybrid family had a higher root:shoot ratio across nutrient treatments, compared with other hybrids and the white clover parents. This may have been inherited from T. uniflorum, which also had higher root:shoot ratios than the other clover entries. In contrast, the root:shoot ratios of T. uniflorum and some hybrid families were lower than those of other clover entries when there was no nutrient limitation (Chapter 6). In the current experiment, the BC1 hybrids in general were also able to establish roots across a greater proportion of the width of the plant under water stress, compared with the BC2 generation and white clover (Figure 7.15), which would increase total root mass for access to
water. The potential impacts of carbohydrate allocation on the root growth of hybrids under water stress will be discussed in Chapter 8.

### 7.4.4 Flowering

The effects of water stress on the BC$_2$ generation may have been exacerbated by high numbers of deflexed inflorescences, in which seed fill was occurring (Figure 7.18B). In particular, numbers of inflorescences were higher in categories 3 and 4, in which the draw on water and carbohydrates would be highest (W. Williams, pers. comm.) (Figure 7.19). Correlation analyses supported the link between constitutive numbers of deflexed inflorescences and shoot DW (Appendices 37 and 38). As white clover nodes can be either reproductive or vegetative, an increase in reproductive nodes (inflorescences) decreases the number of vegetative nodes producing leaves and branches (Thomas, 1987a). Higher total numbers of inflorescences may, in themselves, affect DM production of the BC$_2$ generation, regardless of water stress. Greater numbers of inflorescences in the BC$_2$ generation, compared with white clover, supports the result reported in Chapter 3, where the mean flowering score of BC$_2$ was also significantly higher than white clover. Changes in clover type differences over time for total number of flowers and numbers in particular flowering categories occurred in both treatments, and may represent differences in temporal flowering patterns (particularly for the BC$_2$ generation) rather than responses to water stress.

### 7.5 Conclusions

- The hypotheses outlined in the Introduction were supported, suggesting that $T. \text{repens}$ x $T. \text{uniflorum}$ hybrids are more drought-resistant than white clover.

- Dry matter production of the hybrids, particularly the BC$_1$ generation, was affected significantly less than white clover by water stress. In addition, DM production of the BC$_1$ was affected later by water stress than the BC$_2$ generation and, particularly, white clover.

- Stolon morphological characteristics of the BC$_1$ generation were also less affected by water stress than white clover. This may have influenced productivity under drought and suggests the BC$_1$ hybrids were better able to maintain cell turgor and growth.

- Leaf and stolon senescence in the BC$_1$ generation was also less affected by water stress, and correlation analyses showed this may have contributed to smaller decreases in shoot DW.
• The results suggest that Kopu II BC$_1$, at least, has root characteristics and root responses which may have enabled it to maintain a higher rate of water uptake under water stress.

• Under water stress the BC$_1$ hybrids were compact, dense and prostrate, with potentially higher volumes of leaf compared with stolon, while white clover (and to a lesser extent the BC$_2$ hybrids) were more spreading and open. A smaller lateral spread was related to smaller decreases in total shoot DW under water stress.

• The effect of water stress on BC$_2$ hybrids may have been influenced by different parameters to the other clover types, such as flowering numbers and patterns.
Chapter 8
Physiological and biochemical responses to water stress

8.1 Introduction

The previous chapter showed that growth and morphological characteristics of \textit{T. repens} x \textit{T. uniflorum} hybrids, particularly in the \textit{BC$_1$} generation, were less affected by water stress than those of white clover. This chapter investigates physiological and biochemical factors which may have contributed to this drought resistance. Such characteristics are likely to have been introgressed from the \textit{T. uniflorum} parent, which is adapted to a Mediterranean environment.

As outlined in Chapter 2 (section 2.4), there are several key characteristics which influence plant resistance to drought, as well as productivity during drought. These traits include photosynthetic parameters such as net photosynthesis, stomatal conductance, transpiration, and internal CO$_2$ concentration (Chaves \textit{et al.}, 2002); the production of protective compounds (Hofmann and Jahufer, 2011); and water use efficiency (WUE) (Farquhar and Richards, 1984). However, there is no information on such traits in \textit{T. uniflorum} or \textit{T. repens} x \textit{T. uniflorum} hybrids, or their comparison to white clover. The first objective therefore, was to measure water relations parameters involved in responses (and resistance) to drought, in white clover and \textit{T. repens} x \textit{T. uniflorum} hybrids. It was hypothesised that the drought response of some of these traits in the hybrids would differ to the response of the same traits in white clover. Recent findings point at the importance of phenolic compounds, such as flavonoids, for drought resistance in white clover (Ballizany \textit{et al.}, 2012a; 2012b). The second objective was, therefore, to quantify and compare the effect of drought on the accumulation of protective phenolic compounds in white clover and \textit{T. repens} x \textit{T. uniflorum} hybrids. It was hypothesised that the hybrids may produce higher levels of these compounds than white clover under drought. As $^{13}$C discrimination ($\Delta$) is correlated with WUE (Farquhar and Richards, 1984), the third objective was to quantify and compare $^{13}$C assimilation and discrimination, as a measure of WUE in the hybrid and white clover material. These were expected to differ among \textit{BC$_1$}, \textit{BC$_2$} and white clover types.

Finally, white clover is highly valued for its high nutritive quality, and some feed quality parameters, such as digestibility, are known to be influenced by water stress (Buxton and Casler, 1993). Therefore, the final objective was to quantify and compare the feed quality of
T. repens x T. uniflorum hybrids and white clover cultivars in both well watered and water stressed conditions. It was hypothesised that feed quality would not differ among clover types under well watered conditions, but that drought would affect feed quality parameters differentially among clover types.

8.2 Materials and methods

Plant material, experimental design, maintenance and watering treatments were those described in Chapter 7 (see Section 7.2).

8.2.1 Measurements

As mentioned in Chapter 7, water potential, midday chlorophyll fluorescence and photosynthesis measurements were made only on the Kopu II subset of plants (Kopu II, Kopu II BC2 and Kopu II BC1). This was due to the amount of time needed to do the measurements and/or the physiological effects of changes in environmental conditions over small periods of time. All plants were measured for all other parameters, and data were analysed for both the full data set and the Kopu II subset. In the full data set comparisons are between clover ‘types’, while in the Kopu II subset comparisons are between clover ‘entries’.

8.2.1.1 Water relations

Photosynthesis

On 9 March 2010, after approximately three months of no watering, net photosynthesis (Pn, μmol CO2 m⁻² s⁻¹), stomatal conductance (g, mol H₂O m⁻² s⁻¹), transpiration (E, mmol H₂O m⁻² s⁻¹) and internal CO₂ concentration (Ci, μmol CO₂ mol⁻¹) were measured on plants in the Kopu II subset. Measurements took place between 10:30 am and 1 pm on a clear, sunny day with stable weather conditions, using a LI-6400 infrared gas analyser (LI-Cor Biosciences Inc., Lincoln, Nebraska). The measurements were made on the central trifoliolate leaflet of one 2nd fully expanded leaf (FEL) (relative to the stolon tip) from each plant, and adjusted for the leaf area within the measuring cuvette. Physiological WUE (mmol CO₂ mol⁻¹ H₂O) was then calculated as Pn over E.

Water potential

Plant water status was assessed on two occasions in December 2009 and once each in early January and February 2010, by measuring leaf water potential (Ψ) on plants across each plot using a pressure bomb (Soilmoisture Equipment Corp., Santa Barbara, USA). Weekly measurements were then made from 10 February 2010 on the Kopu II subset. One 2nd FEL on each plant was excised with 4–5 cm of petiole and measured immediately. Where possible, measurements were carried out during the afternoon, midway between irrigation days.
**Chlorophyll fluorescence**

Chlorophyll fluorescence measurements were made using a Mini-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Pre-dawn (3–5 am) measurements made on three plants per plot on 11 January 2010 indicated that no stress was occurring. Subsequent measurements were therefore made at midday on leaves dark adapted for 20 minutes. These measurements were carried out approximately weekly on 11 February, 27 February, 3 March, 10 March and 18 March 2010, using the central trifoliolate leaflet of one 2nd FEL from each plant. Due to the time required for dark adaptation, only the Kopu II subset was monitored. Pre-dawn fluorescence in all plants was measured near the end of the experiment (10 March 2010) to determine whether wider genotypic differences were occurring.

**8.2.1.2 Biochemistry**

**Phenolic compounds and $^{13}$C discrimination**

All surviving plants were sampled for measurement of phenolic compounds and $\delta^{13}$C at the end of the experiment (24 March 2010). Fully expanded trifoliolate leaf laminae were taken from each plant – 10 per plant in the Watered treatment and 20 per plant in the Stressed treatment. These were immediately frozen in liquid N and stored at -30ºC. Sampling took place between 11:15 and 11:45 am.

The samples were finely ground in liquid N using a mortar and pestle. For the phenolic compounds analysis, 50 mg (±2 mg) of the ground material was weighed into centrifuge tubes, to which 3 ml of methanol-distilled water-acetic acid (79:20:1) was added (Hofmann and Jahufer, 2011). The samples were vortexed for 10 seconds and extracted overnight in the dark. After extraction they were vortexed for a further 10 seconds, then centrifuged at 4000 rpm for five minutes. Two millilitres of the supernatant were then syringe filtered into amber HPLC vials. Extracted samples were stored at -20°C until analysed on an integrated HPLC machine (Agilent 1100 series, Agilent Technologies, Germany) (Hofmann and Jahufer, 2011). Each sample was run for 47 minutes at 0.8 ml min$^{-1}$, using an injection volume of 10 μl and two solvents – A (1.5% orthophosphoric acid) and B (acetic acid-acetonitrile-orthophosphoric acid-distilled water (20:24:1.5:54.5)). The solvent gradient moved from 80% A and 20% B, to 67% B at 30 minutes, 90% B at 33 minutes, 100% B at 39.3 minutes, and back to 20% B at 41 minutes. Rutin standards (quercetin 3-rutinoside $\text{C}_{27}\text{H}_{30}\text{O}_{16}$ dissolved in methanol) at 0, 10, 25, 50 and 100 ppm were used to calibrate the readings. Three sets of standards were used at the beginning, middle and end of each run of 84 samples. Thus, the levels of phenolic compounds are expressed here as rutin equivalents (Olsen *et al.*, 2010; Ryan *et al.*, 2002). Quercetin glycoside and kaempferol glycoside peaks were identified from the online spectra,
based on shape and wavelength of the peaks (Markham, 1982), and total levels of each flavonol (mg g\(^{-1}\)) were calculated for each sample. The quercetin to kaempferol ratio of each sample was then calculated. Hydroxycinnamic acid peaks were also identified from their online spectra. Regressions were made between phenolic compound concentrations and shoot DW.

\(^{13}\)C /\(^{12}\)C isotopic composition (\(\delta^{13}\)C) relative to the standard (V-PDB) was measured in all samples by Analytical Services, Faculty of Agriculture and Life Sciences, Lincoln University, using EA-CF-IRMS (Elemental Analyser – Continuous Flow Isotope Ratio Mass Spectrophotometry) (PDZ Europa Ltd., United Kingdom). \(^{13}\)C discrimination (\(\Delta\)) was then calculated using the following equation (Farquhar et al., 1982):

\[
\Delta = \frac{\delta_{\text{source}} - \delta_{\text{product}}}{1 + \delta_{\text{source}}/1000}
\]

where \(\delta_{\text{source}} = \delta^{13}\)C of the air, assumed to be -8‰ (Hall et al., 1994) and \(\delta_{\text{product}} = \delta^{13}\)C of the sample.

**Feed quality**

Leaf samples were taken from each plant at the end of the experiment (25 March 2010) and analysed for feed quality parameters using near infrared spectrophotometry (NIRS) (Foss NIRSystems Inc., Silver Spring, USA). Samples were analysed for %OM (organic matter), %ADF (acid detergent fibre), %NDF (neutral detergent fibre), %DMD (dry matter digestibility), %DOMD (digestible organic matter in dry matter), %OMD (organic matter digestibility), %CHO (carbohydrate) and %Protein. Sampling took place between 1–4:30 pm, and then 8:30–10 am on the following day. Due to the diurnal variation in carbohydrate content, only three replicates were assessed for %CHO. Laminae and a short amount of petiole were removed and sealed in ziplock bags, before being transported to the laboratory and frozen at -32\(^\circ\)C. Only healthy leaves were sampled, and sufficient were taken to obtain 2–5 g of dried material. Samples were subsequently freeze dried for 48 hours, weighed, then ground to a fine powder using an electric mill. The sample DWs were included in the total plant DWs.

**8.2.2 Statistical analysis**

Water potential and midday chlorophyll fluorescence were analysed using a linear mixed modelling approach (LMM) in SAS version 9.1 (SAS Institute Inc.) to account for correlations among measurements within the same plants or over time. Replicates were
included as block effects, and analyses took account of clover entry and watering treatment effects, as well as the clover entry x watering treatment x date interaction. All other data were analysed via analysis of variance (ANOVA) in Genstat version 11 (VSN International Ltd.). Data were analysed for clover type/entry and watering treatment effects, as well as the clover type/entry x watering treatment interaction, using replicates as block effects. Significant differences among clover types or entries and treatments were determined using the least significant difference (LSD) method at the 5% level. The individual standard errors of the mean (SEM) are presented. Differences trending towards significant ($P=0.05-0.099$) are noted in the text. Relationships between key parameters and their changes under water stress were tested using regression and Pearson’s correlation in Minitab version 15 (Minitab Inc.). Other regressions were made using Microsoft Office Excel 2007.

Differences in variability among clover types or entries, within watering treatments, for key parameters were analysed using a test for equal variances in Minitab version 15 (Minitab Inc.). This compares two variances using the F-test or Levene’s test, depending on the normality distribution of the data. Standard deviations are presented to indicate the relative size of the variance for each clover type, while significant differences ($P<0.05$) among clover types are indicated with lettering.
8.3 Results

8.3.1 Gas exchange

8.3.1.1 Net photosynthesis

In the Kopu II subset there was an overall clover entry effect ($P=0.028$) on $P_n$, which was 26–29% higher in Kopu II BC1 (23.9 μmol m$^{-2}$ s$^{-1}$) than in Kopu II BC2 (17.6 μmol m$^{-2}$ s$^{-1}$) and Kopu II (17 μmol m$^{-2}$ s$^{-1}$), which did not differ to each other. Overall, watering treatment also affected $P_n$ ($P=0.001$), which decreased 34% in the Stressed treatment (15.5 μmol m$^{-2}$ s$^{-1}$) compared with the Watered treatment (23.5 μmol m$^{-2}$ s$^{-1}$).

Although there was no clover entry x watering treatment interaction, LSD$_{0.05}$ comparisons showed that $P_n$ did not differ between watering treatments for Kopu II BC1, but decreased under water stress by 48% for Kopu II BC2 and 44% for Kopu II (Figure 8.1). As a result, while there were no differences in $P_n$ among clover entries in the Watered treatment, $P_n$ of Kopu II BC1 was over 80% higher than Kopu II BC2 and Kopu II in the Stressed treatment (Figure 8.1).

![Figure 8.1. Mean net photosynthesis (±SEM) of Kopu II, Kopu II BC1 and Kopu II BC2 in the Watered and Stressed treatments on March 9 2010.](image_url)

For most parameters reported in this chapter, variability did not differ among clover types or entries, so all variability data are presented in Appendices 50 and 51. Net photosynthesis of Kopu II BC1 was more variable than Kopu II in the Stressed treatment ($P=0.023$), while both Kopu II BC1 ($P=0.007$) and Kopu II BC2 ($P=0.009$) were more variable than Kopu II in the Watered treatment (Appendix 50).
8.3.1.2 Stomatal conductance

Stomatal conductance \((g)\) in the Kopu II subset also showed an overall clover entry effect \((P=0.028)\), and was 37–39% higher in Kopu II BC\(_1\) (0.438 mol m\(^{-2}\) s\(^{-1}\)) than Kopu II BC\(_2\) (0.268 mol m\(^{-2}\) s\(^{-1}\)) and Kopu II (0.277 mol m\(^{-2}\) s\(^{-1}\)). As with \(P_n\), there was also an overall effect of watering treatment \((P<0.001)\), with a 63% lower \(g\) in the Stressed treatment (0.178 mol m\(^{-2}\) s\(^{-1}\)) compared with the Watered treatment (0.477 mol m\(^{-2}\) s\(^{-1}\)).

Stomatal conductance of all clover entries decreased by 50–74% in the Stressed treatment compared with the Watered treatment, based on the 5% LSD, but there was no clover entry x watering treatment interaction, and there were no differences among clover entries in both treatments (Figure 8.2).

![Figure 8.2. Mean stomatal conductance (±SEM) of Kopu II BC\(_1\), Kopu II BC\(_2\), and Kopu II in the Watered and Stressed treatments on March 9 2010.](image)

8.3.1.3 Internal CO\(_2\) concentration

There was no overall clover entry effect for \(C_i\) in the Kopu II subset, although LSD\(_{0.05}\) comparisons showed that \(C_i\) of Kopu II BC\(_1\) (265.5 μmol mol\(^{-1}\)) was higher than Kopu II (249 μmol mol\(^{-1}\)), by 7%. Overall, \(C_i\) was 20% lower in the Stressed treatment (226.8 μmol mol\(^{-1}\)) than in the Watered treatment (284.9 μmol mol\(^{-1}\)) \((P<0.001)\), but there was no clover entry x watering treatment interaction. Based on the 5% LSD, \(C_i\) of Kopu II BC\(_1\) decreased by 19%, Kopu II BC\(_2\) decreased by 16% and Kopu II decreased by 26%, in the Stressed treatment compared with the Watered treatment (Figure 8.3). There were no differences among clover entries in the Watered treatment, but \(C_i\) of Kopu II BC\(_1\) was 12% higher than Kopu II in the Stressed treatment (Figure 8.3), based on the 5% LSD.
8.3.1.4 Transpiration

There was an overall clover entry effect in the Kopu II subset for mean E ($P=0.015$), which was 55–59% higher ($P<0.05$) in Kopu II BC$_1$ (4.42 mmol m$^{-2}$ s$^{-1}$) than in Kopu II BC$_2$ (2.85 mmol m$^{-2}$ s$^{-1}$) and Kopu II (2.79 mmol m$^{-2}$ s$^{-1}$). Overall, the watering treatment effect was also significant ($P<0.001$), with a 49% decrease in E in the Stressed treatment (2.26 mmol m$^{-2}$ s$^{-1}$) compared with the Watered treatment (4.45 mmol m$^{-2}$ s$^{-1}$).

The clover entry x watering treatment interaction was not significant. However, LSD$_{0.05}$ comparisons showed that E of Kopu II BC$_1$ did not change with water stress, while that of Kopu II BC$_2$ and Kopu II decreased by 60% (Figure 8.4). Consequently, there were no differences among clover entries in the Watered treatment, based on the 5% LSD, but E of Kopu II BC$_1$ was higher than that of Kopu II BC$_2$ and Kopu II in the Stressed treatment, by 119% and 127% respectively (Figure 8.4).

The variability for E of both Kopu II BC$_1$ ($P=0.005$) and Kopu II BC$_2$ ($P=0.034$) was higher than that of Kopu II in the Watered treatment, and E of Kopu II BC$_1$ was more variable than that of Kopu II BC$_2$ ($P=0.001$) and Kopu II ($P=0.002$) in the Stressed treatment (see Appendix 50).
8.3.1.5 *Physiological water use efficiency*

There was no overall clover entry effect in the Kopu II subset for mean physiological WUE, which was similar for Kopu II BC1 (6.22 mmol mol⁻¹), Kopu II BC2 (6.64 mmol mol⁻¹) and Kopu II (6.81 mmol mol⁻¹). The overall watering treatment effect was significant \((P<0.001)\), with water stress increasing mean physiological WUE by 34% in the Stressed treatment (7.52 mmol mol⁻¹) compared with the Watered treatment (5.60 mmol mol⁻¹).

Although the clover entry x watering treatment interaction was not significant, LSDₐ₀.₀₅ comparisons showed that mean physiological WUE efficiency increased in the Stressed treatment for Kopu II BC₁ (34%) and Kopu II (47%), but not for Kopu II BC₂ (Figure 8.5). Despite this, there were no differences in physiological WUE among clover entries in either watering treatment (Figure 8.5), based on the 5% LSD. There were also no differences among clover entries in the variability of physiological WUE, in either watering treatment (see Appendix 50).
Figure 8.5. Mean physiological water use efficiency (±SEM) of Kopu II BC1, Kopu II BC2 and Kopu II in the Watered and Stressed treatments on 9 March 2010.

There was a significant negative correlation ($P=0.016$) between physiological WUE and shoot DW in the Kopu II subset, across treatments, with physiological WUE increasing as shoot DW decreased (Figure 8.6). However, no such relationship was found within the individual watering treatments (data not shown).

Figure 8.6. Relationship between shoot dry weight and physiological water use efficiency for plants across the Watered and Stressed treatments in the Kopu II subset.
8.3.2 Leaf water potential

Overall, the clover entry x watering treatment x date interaction for Ψ in the Kopu II subset was not significant, with changes over time appearing to be more affected by the ambient environmental conditions. However, the clover entry x watering treatment interaction was significant ($P=0.037$), and was the same at each date, showing that the effect of water stress on Ψ for Kopu II BC1 was larger than that for Kopu II BC2 ($P=0.039$) and Kopu II ($P=0.034$). Calculated over all measurement dates, water stress decreased the Ψ of Kopu II BC1 by 47%, compared with 28% for Kopu II BC2 and 31% for Kopu II (Figure 8.7). Mean Ψ at each measurement date is shown in Appendix 40.

![Figure 8.7. Mean overall water potential (±SEM) across time for Kopu II BC1, Kopu II BC2 and Kopu II in the Watered and Stressed treatments.]

8.3.3 Chlorophyll fluorescence

8.3.3.1 Midday fluorescence

As with Ψ, there was no clover entry x watering treatment x date interaction for mean midday chlorophyll fluorescence in the Kopu II subset, but the clover entry x watering treatment interaction was significant ($P=0.023$), and was the same at each date. Compared with Kopu II, the effect of water stress on midday chlorophyll fluorescence was smaller for Kopu II BC1 ($P=0.007$) and Kopu II BC2 ($P=0.020$), which did not differ to each other. Calculated over all measurement dates, the chlorophyll fluorescence yield of Kopu II BC1 increased by 0.8% and that of Kopu II BC2 decreased by 0.3%, compared with a 2.8% decrease for Kopu II (Figure 8.8). Mean midday chlorophyll fluorescence yield at each measurement date is shown in Appendix 41.
8.3.3.2 Pre-dawn fluorescence

There was no overall clover type effect for pre-dawn chlorophyll fluorescence yield in the full data set on 10 March 2010. On average, fluorescence yield was 0.843 for the BC1 generation, 0.841 for the BC2 generation and 0.840 for white clover. Overall, mean pre-dawn fluorescence yield was 1.7% higher ($P<0.001$) in the Stressed treatment (0.849) than the Watered treatment (0.834).

However, there was no clover type x watering treatment interaction, with mean fluorescence yield of all clover types being higher in the Stressed treatment than the Watered treatment (Figure 8.9), based on the 5% LSD. Within the Watered treatment there were no differences in fluorescence yield among clover types, but in the Stressed treatment $F_v/F_m$ of the BC1 generation was 0.7–0.8% higher than that of the BC2 generation and white clover (Figure 8.9), based on the 5% LSD.
Figure 8.9. Mean pre-dawn chlorophyll fluorescence yield ($F_v/F_m$) (±SEM) on 10 March 2010 for BC1, BC2 and white clover in the Watered and Stressed treatments.

In the Kopu II subset, there was an overall clover entry effect ($P=0.015$), with the mean pre-dawn fluorescence yield of Kopu II BC1 (0.850) being approximately 1.4% higher ($P<0.05$) than that of Kopu II BC2 (0.837) and Kopu II (0.838). Overall, mean fluorescence yield in the Stressed treatment (0.847) was higher than the Watered treatment (0.836) by 1.3% ($P=0.007$), but among the individual clover entries only Kopu II BC1 had higher fluorescence in the Stressed treatment than in the Watered treatment, by 2.5%, based on the 5% LSD (Figure 8.10). As with the total plant pool, there were no differences in mean pre-dawn fluorescence yield among clover entries in the Watered treatment, but Kopu II BC1 was 2.2–2.5% higher than Kopu II BC2 and Kopu II in the Stressed treatment (Figure 8.10), based on the 5% LSD.
Figure 8.10. Mean pre-dawn chlorophyll fluorescence yield ($F_v/F_m$) ($\pm$SEM) on 10 March 2010 for Kopu II BC1, Kopu II BC2 and Kopu II in the Watered and Stressed treatments.

8.3.4 Phenolic compounds

There was no overall clover type effect for quercetin glycoside concentration, but the overall watering treatment effect was significant ($P<0.001$), with 2.5 times more quercetin glycosides in the Stressed treatment (5.57 mg g$^{-1}$) than in the Watered treatment (2.24 mg g$^{-1}$). The quercetin glycoside concentration in all clover types increased with drought stress, based on the 5% LSD (Figure 8.11A), but there was no clover type x watering treatment interaction. Quercetin was 2.1 times higher in the Stressed treatment for BC1, and 2.8 times higher for BC2 and white clover. Based on the 5% LSD, there were no difference among clover types within each watering treatment (Figure 8.11A).

The overall clover type effect was significant ($P=0.020$) for kaempferol glycoside concentration, which was 28% lower ($P<0.05$) in the BC2 generation (1.95 mg g$^{-1}$) than in the BC1 generation (2.71 mg g$^{-1}$). White clover (2.34 mg g$^{-1}$) did not differ to either BC1 or BC2. This relationship was also present in the Watered treatment, where the kaempferol glycoside concentration of the BC1 generation was higher than that of the BC2 generation by 51%, based on the 5% LSD, but there were no differences among clover types in the Stressed treatment (Figure 8.11B). Overall, the watering treatment effect was also significant ($P=0.020$), with 24% more kaempferol glycosides in the Stressed treatment (2.67 mg g$^{-1}$) than in the Watered treatment (2.12 mg g$^{-1}$). However, there were no differences between watering treatments for
the individual clover types, based on the 5% LSD (Figure 8.11B), and no clover type x watering treatment interaction.

Overall, the clover type effect was also significant ($P=0.002$) for hydroxycinnamic acid concentration, which was higher ($P<0.05$) in the BC$_1$ generation (1.48 mg g$^{-1}$) than in the BC$_2$ generation (1.02 mg g$^{-1}$) and white clover (1.17 mg g$^{-1}$), by 45% and 27%, respectively. However, in the Watered treatment there were no differences among clover types, while in the Stressed treatment the hydroxycinnamic acid concentration of BC$_1$ was higher (by 50%) than BC$_2$ only, based on the 5% LSD (Figure 8.11C). The overall watering treatment effect was significant ($P<0.001$), with a 56% higher hydroxycinnamic acid concentration in the Stressed treatment (1.56 mg g$^{-1}$) than the Watered treatment (1.00 mg g$^{-1}$). However, LSD$_{0.05}$ comparisons showed that among individual clover types, only the BC$_1$ generation and white clover had higher hydroxycinnamic acid concentrations in the Stressed treatment than in the Watered treatment (Figure 8.11C) (by 51% and 81% respectively). There was no clover type x watering treatment interaction.

There was a trend towards an overall clover type effect ($P=0.091$) for quercetin:kaempferol ratio, with the ratio for the BC$_1$ generation (1.9) being 31% lower ($P<0.05$) than for the BC$_2$ generation (2.7). White clover (2.1) did not differ to either BC$_1$ or BC$_2$. Within each watering treatment there were no differences among clover types (Figure 8.11D). Overall, the quercetin:kaempferol ratio was higher ($P<0.001$) in the Stressed treatment (2.7) than in the Watered treatment (1.6). Among individual clover types, LSD$_{0.05}$ comparisons showed that only the BC$_2$ generation and white clover had higher quercetin:kaempferol ratios under water stress (Figure 8.11D) (by 53% and 82% respectively). There was no clover type x watering treatment interaction.

There was a trend towards an overall clover type effect ($P=0.091$) for quercetin:kaempferol ratio, with the ratio for the BC$_1$ generation (1.9) being 31% lower ($P<0.05$) than for the BC$_2$ generation (2.7). White clover (2.1) did not differ to either BC$_1$ or BC$_2$. Within each watering treatment there were no differences among clover types (Figure 8.11D). Overall, the quercetin:kaempferol ratio was higher ($P<0.001$) in the Stressed treatment (2.7) than in the Watered treatment (1.6). Among individual clover types, LSD$_{0.05}$ comparisons showed that only the BC$_2$ generation and white clover had higher quercetin:kaempferol ratios under water stress (Figure 8.11D) (by 53% and 82% respectively). There was no clover type x watering treatment interaction.

Variability of clover types

Within the Watered treatment, there were no differences in variability among clover types for quercetin glycoside, kaempferol glycoside or hydroxycinnamic acid concentrations (see Appendix 51). However, in the Stressed treatment, the BC$_2$ generation was more variable ($P=0.035$) than the BC$_1$ generation for quercetin glycosides, while BC$_1$ was more variable ($P=0.016$) than BC$_2$ for hydroxycinnamic acids. The quercetin:kaempferol ratio of the BC$_2$ generation was more variable than the BC$_1$ generation ($P=0.002$) and white clover ($P<0.001$) in the Watered treatment, but variability did not differ among clover types in the Stressed treatment (see Appendix 51).
Figure 8.11. Mean (±SEM) quercetin glycosides (A), kaempferol glycosides (B), hydroxycinnamic acids (C) and quercetin:kaempferol ratio (D) for BC1, BC2 and white clover in the Watered and Stressed treatments.
Correlations with shoot dry weight

There were significant correlations with shoot DW for quercetin glycoside \( (P<0.001) \), kaempferol glycoside \( (P=0.005) \) and hydroxycinnamic acid concentrations \( (P=0.006) \) when regressions were plotted for all clover types and watering treatments combined. However, within the Watered treatment alone, correlations were not significant, and in the Stressed treatment the relationship was only significant \( (P=0.006) \) for quercetin glycosides (Figure 8.12). In addition, in the Stressed treatment, only the BC\(_1\) generation showed a significant \( (P=0.036) \) correlation (negative) between quercetin glycoside levels and shoot DW, although several large quercetin glycoside values may have obscured this relationship for the BC\(_2\) generation and white clover (Appendix 42).

![Relationship between quercetin glycoside concentration and shoot dry weight across all plants in the Stressed treatment.](image)

**Figure 8.12.** Relationship between quercetin glycoside concentration and shoot dry weight across all plants in the Stressed treatment.

Kopu II subset

As with the total plant pool, there was no overall clover entry effect for quercetin glycoside concentration in the Kopu II subset and, based on the 5% LSD, also no differences among clover entries within each watering treatment (Figure 8.13A). Overall, the watering treatment effect was significant \( (P<0.001) \), with 2.7 times more quercetin glycoside accumulation in the Stressed treatment \( (6.09 \text{ mg g}^{-1}) \) than the Watered treatment \( (2.25 \text{ mg g}^{-1}) \). Among individual clover entries, only Kopu II BC\(_1\) and Kopu II BC\(_2\) had a higher quercetin glycoside concentration in the Stressed treatment than in the Watered treatment, based on the 5% LSD (by 2.3 and 2.9 times, respectively) (Figure 8.13A). There was no clover entry x watering treatment interaction.
Figure 8.13. Mean (±SEM) quercetin glycosides (A), kaempferol glycosides (B), hydroxycinnamic acids (C) and quercetin:kaempferol ratio (D) for Kopu II BC₁, Kopu II BC₂ and Kopu II in the Watered and Stressed treatments.
The overall clover entry effect in the Kopu II subset was significant for total kaempferol glycosides \((P<0.001)\). Kaempferol glycoside concentration in Kopu II BC\(_1\) (3.10 mg g\(^{-1}\)) was higher \((P<0.05)\) than in Kopu II BC\(_2\) (1.04 mg g\(^{-1}\)) and Kopu II (1.68 mg g\(^{-1}\)), by 3 and 1.8 times respectively. In turn, kaempferol glycoside concentration in Kopu II was 1.6 times higher \((P<0.05)\) than in Kopu II BC\(_2\). Based on the 5% LSD, the kaempferol glycoside concentration of Kopu II BC\(_1\) was also 1.5–4.4 times higher than that of Kopu II BC\(_2\) and Kopu II in both watering treatments, and in Kopu II it was 1.6 times higher than in Kopu II BC\(_2\) in the Stressed treatment (Figure 8.13B). Overall, the watering treatment effect was significant \((P=0.002)\), with 55% more kaempferol glycosides in the Stressed treatment (2.36 mg g\(^{-1}\)) than in the Watered treatment (1.52 mg g\(^{-1}\)). There was no clover entry x watering treatment interaction, but based on the 5% LSD, only Kopu II had a higher kaempferol glycoside concentration in the Stressed treatment than in the Watered treatment, by just over 2 times (Figure 8.13B). Correlation analyses showed that genotypes with higher kaempferol concentrations in the Watered treatment tended to have smaller \((P=0.056, R^2=0.2529)\) decreases in shoot DW under water stress (Appendix 43), and smaller increases in senescence \((P=0.005, R^2=0.4611)\).

Overall, the clover entry effect for hydroxycinnamic acid concentration in the Kopu II subset was not statistically significant, but when means were compared using the LSD\(_{0.05}\) then the concentration for Kopu II BC\(_1\) (1.80 mg g\(^{-1}\)) was 64% higher \((P<0.05)\) than that for Kopu II (1.10 mg g\(^{-1}\)). Hydroxycinnamic acid levels in Kopu II BC\(_2\) (1.52 mg g\(^{-1}\)) did not differ to either Kopu II BC\(_1\) or Kopu II. There were no differences among clover entries in the Watered treatment, but the hydroxycinnamic acid concentration of Kopu II BC\(_1\) was 69% higher than that of Kopu II in the Stressed treatment, based on the 5% LSD (Figure 8.13C). The overall watering treatment effect was significant \((P=0.008)\), with a 73% higher hydroxycinnamic acid concentration in the Stressed treatment than the Watered treatment. However, among individual clover entries, only Kopu II BC\(_1\) increased its hydroxycinnamic acid concentration with water stress (by just over 2 times), based on the 5% LSD (Figure 8.13C). There was no clover entry x watering treatment interaction.

There was an overall clover entry effect in the Kopu II subset for the quercetin:kaempferol ratio \((P=0.002)\), which was higher \((P<0.05)\) for Kopu II BC\(_2\) (5.39) than for both Kopu II BC\(_1\) (1.46) and Kopu II (1.89), by 3.7 and 2.9 times respectively. Within the Watered treatment, the quercetin:kaempferol ratio of Kopu II BC\(_2\) was 6.1 times higher than that of Kopu II BC\(_1\) and 3.4 times higher than that of Kopu II, based on the 5% LSD, but there were no differences among clover entries in the Stressed treatment (Figure 8.13D). Overall, there was no watering
treatment effect on the quercetin:kaempferol ratio, which was identical in both treatments (2.91), and none of the clover entries changed with water stress, based on the 5% LSD (Figure 8.13D).

**Variability of clover entries**
As with the total plant pool, there were no differences in variability among clover entries for quercetin glycoside, kaempferol glycoside or hydroxycinnamic acid concentrations in the Watered treatment (see Appendix 51). However, the hydroxycinnamic acid concentration of Kopu II BC2 tended to be more variable than that of Kopu II ($P=0.079$). There were more differences in variability in the Stressed treatment (see Appendix 51). The quercetin glycoside concentration of Kopu II BC2 was more variable ($P=0.012$) than that of Kopu II, and Kopu II BC1 also tended to be more variable ($P=0.068$) than that of Kopu II. Kopu II BC1 was also more variable ($P=0.032$) than Kopu II for hydroxycinnamic acid concentration. The quercetin:kaempferol ratio of Kopu II BC2 in both the Watered and Stressed treatments was more variable than that of Kopu II BC1 ($P=0.001$ and $P=0.037$ respectively) and Kopu II ($P=0.001$ and $P=0.030$ respectively).

### 8.3.5 $^{13}$C discrimination
Results from the $\delta^{13}$C analysis were used to calculate $\Delta$ for both the full data set and the Kopu II subset. Clover type means for $\delta^{13}$C in each treatment are presented in Appendix 44. Overall, there was no clover type effect for $\Delta$ in the full data set, but it was lower ($P<0.001$) in the Stressed treatment (18.4‰) than in the Watered treatment (20.6‰). A significant clover type x watering treatment interaction ($P=0.02$) occurred due to a smaller ($P<0.001$) decrease in $\Delta$ for the BC2 generation compared with the BC1 generation and white clover. Discrimination of the BC2 generation was lower than that of BC1 and white clover in the Watered treatment but, based on the 5% LSD, there were no differences among clover types in the Stressed treatment (Figure 8.14).
Figure 8.14. Mean $^{13}$C discrimination (±SEM) for BC$_1$, BC$_2$ and white clover in the Watered and Stressed treatments.

There was a significant ($P<0.001$), positive correlation between $\Delta$ and shoot DW for plants from both treatments combined in the full data set (Figure 8.15), but within each watering treatment there was no such relationship (data not shown).

Figure 8.15. Relationship between $^{13}$C discrimination and shoot dry weight for all plants in both treatments combined.
**Kopu II subset**

For the Kopu II subset there was again no overall clover entry effect, although $\Delta$ of the BC$_1$ generation (19.6‰) was higher ($P<0.05$) than the BC$_2$ generation (19.0‰) when compared using the LSD$_{0.05}$. As with the full data set, $\Delta$ in the Stressed treatment (18.3‰) was lower ($P<0.001$) than in the Watered treatment (20.4‰). However, while $\Delta$ decreased less for Kopu II BC$_2$ compared with Kopu II BC$_1$ and Kopu II, there was no clover entry x watering treatment interaction. Discrimination in Kopu II BC$_2$ was lower than in Kopu II BC$_1$ and Kopu II in the Watered treatment, based on the 5% LSD, but there were no differences among clover entries in the Stressed treatment (Figure 8.16).

![Figure 8.16](image)

**Figure 8.16.** Mean $^{13}$C discrimination (±SEM) for Kopu II BC$_1$, Kopu II BC$_2$ and Kopu II in the Watered and Stressed treatments.
**13C discrimination and physiological water use efficiency**

There was a significant ($P=0.002$), negative correlation between $\Delta$ and physiological WUE in plants from the Kopu II subset when both treatments were combined (Figure 8.17), but within each treatment there was no such relationship (data not shown).

![Figure 8.17. Relationship between $^{13}$C discrimination and physiological water use efficiency for all plants in the Kopu II subset, in both treatments combined.](image)

8.3.6 Feed quality

There were overall clover type effects in the full data set for all feed quality parameters, except %CHO (Table 8.1). However, analyses for %CHO were based on only three replicates. Clover type means are shown in Table 8.2.

<table>
<thead>
<tr>
<th>%OM</th>
<th>%ADF</th>
<th>%NDF</th>
<th>%DMD</th>
<th>%DOMD</th>
<th>%OMD</th>
<th>%CHO</th>
<th>%Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clover type</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>&lt;0.001</td>
<td>0.020</td>
<td>&lt;0.001</td>
<td>0.037</td>
<td>0.213</td>
</tr>
<tr>
<td>Watering treatment</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.093</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Type x Treatment</td>
<td>0.232</td>
<td>0.742</td>
<td>0.985</td>
<td>0.184</td>
<td>0.908</td>
<td>0.359</td>
<td>0.021</td>
</tr>
</tbody>
</table>
Table 8.2. Clover type means (±SEM) for feed quality parameters where there was no clover type x watering treatment interaction, in the full data set. OM = organic matter; ADF = acid detergent fibre; NDF = neutral detergent fibre; DMD = dry matter digestibility; DOMD = digestible organic matter in dry matter; OMD = organic matter digestibility.

<table>
<thead>
<tr>
<th>Clover type</th>
<th>%OM</th>
<th>%ADF</th>
<th>%NDF</th>
<th>%DMD</th>
<th>%DOMD</th>
<th>%OMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC1</td>
<td>88.3</td>
<td>16.8</td>
<td>19.0</td>
<td>81.5</td>
<td>77.6</td>
<td>87.2</td>
</tr>
<tr>
<td></td>
<td>±0.11</td>
<td>±0.10</td>
<td>±0.18</td>
<td>±0.13</td>
<td>±0.17</td>
<td>±0.15</td>
</tr>
<tr>
<td>BC2</td>
<td>88.5</td>
<td>16.8</td>
<td>20.1</td>
<td>81.3</td>
<td>77.6</td>
<td>87.0</td>
</tr>
<tr>
<td></td>
<td>±0.15</td>
<td>±0.14</td>
<td>±0.24</td>
<td>±0.17</td>
<td>±0.24</td>
<td>±0.20</td>
</tr>
<tr>
<td>White clover</td>
<td>88.7</td>
<td>16.4</td>
<td>19.8</td>
<td>81.7</td>
<td>78.2</td>
<td>87.4</td>
</tr>
<tr>
<td></td>
<td>±0.14</td>
<td>±0.12</td>
<td>±0.22</td>
<td>±0.15</td>
<td>±0.21</td>
<td>±0.18</td>
</tr>
<tr>
<td>LSD</td>
<td>0.370</td>
<td>0.333</td>
<td>0.602</td>
<td>0.420</td>
<td>0.579</td>
<td>0.493</td>
</tr>
</tbody>
</table>

While significant, differences in overall clover type means in the full data set, based on the 5% LSD, were very small, ranging from 0.4–1.1% of total DM. Overall differences for %OM and %ADF reflect clover type differences within the Stressed treatment, but there were no differences among clover types within the Watered treatment for these parameters (Appendix 45). The only parameters with significant clover type effects in the Watered treatment were %NDF and %Protein (Appendix 45 and Table 8.4).

Based on the 5% LSD, overall %ADF of both hybrids was greater than that of white clover, but these differences were small, and there were no differences among clover types in the Watered treatment. In contrast, overall %NDF was lower in the BC1 generation than in both BC2 and white clover (Table 8.2). However, within the individual watering treatments, %NDF of the BC1 generation differed to BC2 only (Appendix 45). Again, the overall differences in %NDF were small.

There were generally very few differences in digestibility among clover types, based on the 5% LSDs. %DOMD, overall, was lower in BC1 and BC2 than in white clover by very small amounts (0.63 and 0.67 respectively) (Table 8.2), but did not differ among clover types in the individual watering treatments (Appendix 45). Although the overall clover type effect was significant for %OMD, the clover type means did not differ using the LSD0.05 (Table 8.2), and differences among clover types within each watering treatment were also not significant (Appendix 45).
The overall watering treatment effect in the full data set was significant for all parameters, except %CHO which was assessed in three replicates only (Table 8.1). Treatment means are shown in Table 8.3. Based on the 5% LSDs, %ADF was higher in the Stressed treatment compared with the Watered treatment, by 2.14; while %OM, %NDF, %DMD, %DOMD, and %OMD all decreased under water stress. The largest decrease was for %DOMD (9.0), with smaller decreases for the remaining parameters (4.2–5.6).

Table 8.3. Watering treatment means (±SEM) for feed quality parameters in the full data set, where there were no clover type x watering treatment interactions. OM = organic matter; ADF = acid detergent fibre; NDF = neutral detergent fibre; DMD = dry matter digestibility; DOMD = digestible organic matter in dry matter; OMD = organic matter digestibility.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%OM (±SEM)</th>
<th>%ADF (±SEM)</th>
<th>%NDF (±SEM)</th>
<th>%DMD (±SEM)</th>
<th>%DOMD (±SEM)</th>
<th>%OMD (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Watered</td>
<td>90.9 ±0.10</td>
<td>15.7 ±0.10</td>
<td>21.4 ±0.16</td>
<td>83.8 ±0.11</td>
<td>81.8 ±0.16</td>
<td>89.2 ±0.13</td>
</tr>
<tr>
<td>Stressed</td>
<td>85.3 ±0.11</td>
<td>17.8 ±0.10</td>
<td>17.2 ±0.18</td>
<td>78.7 ±0.13</td>
<td>72.8 ±0.17</td>
<td>84.7 ±0.15</td>
</tr>
<tr>
<td>LSD</td>
<td>0.299</td>
<td>0.266</td>
<td>0.481</td>
<td>0.336</td>
<td>0.463</td>
<td>0.394</td>
</tr>
</tbody>
</table>

In the full data set, there were clover type x watering treatment interactions for carbohydrates and protein only (Table 8.1). Based on the 5% LSD, carbohydrate content decreased under water stress for the BC1 generation, but not for BC2 or white clover (Table 8.4). Thus, the watering treatment effect on %CHO was larger for BC1, compared with BC2 and white clover. The carbohydrate content did not differ among clover types in the Watered treatment using LSD0.05, but %CHO of BC1 was lower than in BC2 and white clover in the Stressed treatment (Table 8.4).

Based on the 5% LSDs, protein content decreased under water stress for all clover types, but the treatment difference was smaller for BC1 (8.5%) than for BC2 and white clover (10.6%) (Table 8.4). The %Protein of BC1 was lower than for BC2 and white clover in the Watered treatment, when compared using the 5% LSD, but there were no differences in protein content among clover types in the Stressed treatment (Table 8.4).
Table 8.4. Clover type means (±SEM) for %CHO (carbohydrate) and %Protein within the Watered and Stressed treatments in the full data set, where there was a significant clover type x watering treatment interaction.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>BC1</th>
<th>BC2</th>
<th>White clover</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>%CHO</td>
<td>Watered</td>
<td>15.9 ±0.44</td>
<td>15.2 ±0.58</td>
<td>15.6 ±0.52</td>
<td>1.487</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>13.9 ±0.44</td>
<td>15.5 ±0.61</td>
<td>16.1 ±0.58</td>
<td></td>
</tr>
<tr>
<td>%Protein</td>
<td>Watered</td>
<td>29.8 ±0.32</td>
<td>31.3 ±0.42</td>
<td>31.9 ±0.37</td>
<td>1.102</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>21.3 ±0.34</td>
<td>20.7 ±0.47</td>
<td>21.3 ±0.44</td>
<td></td>
</tr>
</tbody>
</table>

Kopu II subset

Clover entry effects (Appendices 46 and 47) for the Kopu II subset differed slightly to those in the full data set but watering treatment effects were very similar (Appendices 46 and 48). There was also a clover entry x watering treatment interaction for %Protein (Appendix 46).

The watering treatment difference for %Protein was smaller for Kopu II BC1 (5.7%) than for Kopu II BC2 (12%) and Kopu II (10.5%), based on the 5% LSD. As with the total plant pool, %Protein of Kopu II BC1 in the Watered treatment was lower than that of Kopu II BC2 and Kopu II (Table 8.5).

Table 8.5. Clover entry means (±SEM) for %Protein within the Watered and Stressed treatments in the Kopu II subset, where there was a significant clover entry x watering treatment interaction.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Kopu II BC1</th>
<th>Kopu II BC2</th>
<th>Kopu II</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Protein</td>
<td>Watered</td>
<td>29.1 ±0.96</td>
<td>31.8 ±0.66</td>
<td>32.0 ±0.56</td>
<td>2.336</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>23.4 ±0.48</td>
<td>19.8 ±0.80</td>
<td>21.5 ±1.31</td>
<td></td>
</tr>
</tbody>
</table>

Unlike the full data set, there was no clover entry x watering treatment interaction for %CHO in the Kopu II subset (Appendix 46). However, only Kopu II BC1 had a decrease in %CHO under water stress (Appendix 49), based on the 5% LSD.
8.4 Discussion

The findings from this study revealed a number of clover type- or entry-specific differences in physiological and biochemical drought responses. In accordance with the hypotheses outlined in the Introduction, the responses of the BC1 generation were different to those of white clover, and they also differed to the responses of the BC2 generation. In contrast, the latter were often similar to white clover. Traits in the BC1 generation are likely to reflect adaptations inherited from the *T. uniflorum* parent, which is native to the Mediterranean region and grows in dry environments (Tela Botanica, 2012; Zohary and Heller, 1984). *T. uniflorum* was not studied in the current experiment, as it propagates poorly from stolon cuttings, which were the basis of the clonal material used here.

8.4.1 Physiology

Net photosynthesis was reduced in the BC2 and white clover entries under water stress while, in contrast, the BC1 entry was able to maintain its photosynthetic rate in the Stressed treatment (Figure 8.1). This is likely to have contributed to the smaller reduction in biomass compared with the BC2 generation and white clover observed in Chapter 7. Photosynthesis and biomass production are reduced under drought by stomatal closure, which reduces C uptake (Chaves et al., 2002). The maintenance of photosynthesis was reflected by higher rates of transpiration for the BC1 entry under water stress (Figure 8.4). Although the results showed that stomatal conductance, and its response to water stress, did not differ among clover entries (Figure 8.2), transpiration differed due to the non-linear relationship between CO2 and water vapour (Schulze, 1986). Compared with BC2 and white clover, BC1 hybrids may, therefore, be viewed as “water spenders” (Levitt, 1980), which can maintain stomatal opening during drought through higher uptake of water from the soil. The hybrids may be able to afford this strategy by increasing the allocation of biomass to the root system (see Chapter 7). In contrast, “water savers” can avoid negative drought effects by closing stomata in order to limit water losses.

In addition to soil moisture stress, leaf water potential measurements (which are an indication of the water status of the plant) were affected by other ambient environmental conditions, as they varied from week to week (Appendix 40). Air temperature and wind conditions (through disturbance of the boundary layer), therefore, affected transpiration and leaf water potential. However, the clover entry x watering treatment interactions showed that, at all times, water stress affected leaf water potential significantly more for the BC1 entry, which is likely to be a consequence of higher transpiration rates as a result of stomatal opening for the maintenance
of photosynthesis. The ability to decrease shoot DW less at these levels, compared with larger decreases in shoot DW at higher water potentials for Kopu II BC₂ and Kopu II, suggests a greater tolerance to water stress in the Kopu II BC₁ hybrids. This could be due to increased levels of osmotic adjustment, thus decreasing the osmotic and overall water potential of Kopu II BC₁.

Increased WUE in plants is frequently attained by a decrease in water use, which in turn is achieved by plant responses that decrease yield, such as reduced leaf size, stomatal conductance and transpiration (Blum, 2005; Condon et al., 2002). This was observed in the current experiment, which showed a negative correlation between physiological WUE and shoot DW (Figure 8.6). Condon et al. (2002) concluded that while high WUE may improve yields of cereals under water stress, high inherent WUE will actually be a disadvantage in environments where water is not limiting. The ability to adjust WUE under water limiting conditions may therefore be important. This also raises a key question. What is the target under water stress – survival or growth? While high WUE may limit growth, minimisation of water loss will help plants to survive drought.

Water use efficiency frequently increases under drought, as plants adjust to minimise the amount of water lost per unit of C gained (Blum, 2005). This occurred for physiological WUE in the current experiment, as an overall treatment effect and also for both Kopu II BC₁ and Kopu II (Figure 8.5). The absence of a significant response for Kopu II BC₂ suggests this material may not have been able to adjust its water use under drought stress. The lack of differences in physiological WUE among clover entries in the individual treatments (Figure 8.5) may have been influenced by the variability in the hybrids for net photosynthesis and transpiration, which are used to calculate physiological WUE (Appendix 50). Barbour et al. (1996) also found no differences in WUE among ten white clover cultivars at a range of moisture levels, and suggested that genotypic variability within cultivars may be more important than differences between cultivars when breeding for drought tolerance. This could occur in the current data set, given the expected variability of interspecific hybrids, however there were no significant differences among clover entries in variability for physiological WUE, in either watering treatment. Again, this may be due to the variability of net photosynthesis and transpiration.

The relationship between WUE and $^{13}$C discrimination suggests that plants with a higher WUE will discriminate less against $^{13}$C (Farquhar et al., 1982; Farquhar and Richards, 1984). $^{13}$C discrimination may then be used as a tool to select for high WUE (Farquhar and Richards, 1984; Barbour et al., 1996) and also provides an integrated measure of stress over time. In the
current study it also enabled WUE to be assessed for the full data set, in addition to the physiological WUE measurements in the Kopu II subset. As expected, there was a significant negative correlation between $^{13}$C discrimination and physiological WUE across both watering treatments (Figure 8.17), but there was no relationship within the individual treatments. The same result was found by Barbour et al. (1996) for the means of a range of white clover cultivars, which was attributed to the absence of differences among cultivars for either parameter. Similar factors may be responsible in the current experiment, where there were few differences among clover types for $^{13}$C discrimination (Figure 8.14) and none for physiological WUE (Figure 8.5). Reflecting the general, negative relationship between physiological WUE and $^{13}$C discrimination, as well as the recorded increase in physiological WUE with water stress, $^{13}$C discrimination decreased in the Stressed treatment of the current experiment. The significant, positive correlation between $^{13}$C discrimination and shoot DW across both watering treatments (Figure 8.15), but not within treatments, was also reported by Barbour et al. (1996) for white clover cultivars under water stress. Again, this was attributed to the absence of differences in $^{13}$C discrimination among cultivars. The significant clover type x watering treatment interaction for $^{13}$C discrimination in the full data set suggests that WUE of second generation backcrosses differs to BC$_1$ and white clover (Figure 8.14). This is not consistent with the intermediate characteristics which may be expected for BC$_2$ hybrids (in between BC$_1$ and white clover). However, physiological WUE also had a different response to water stress for the BC$_2$ hybrid compared with the other clover entries (Figure 8.5).

Pre-dawn chlorophyll fluorescence confirmed that there was no permanent damage to the photochemistry under water stress in this experiment (Figures 8.9 and 8.10). Although the clover type and watering treatment differences were statistically significant, the mean values for F$_v$/F$_m$ were above 0.800 and, therefore, show no damage to photosystem II (PS II) (Chaves et al., 2002; Björkman and Demmig, 1987). Similar results have been found in other studies for white clover (Grieu et al., 1995; Hofmann et al., 2003). The results of midday fluorescence measurements showed that there were some intermittent effects of water stress on PS II (Appendix 41), but overall the photochemistry of the BC$_1$ and BC$_2$ hybrids measured here was affected significantly less than that of the white clover parent (Kopu II).

Photosynthesis consists of three key components – C supply, the C cycle, and light reactions. The lack of damage to PS II suggests the main effect of photosynthesis on biomass production of BC$_2$ and white clover under water stress may, therefore, be through CO$_2$ assimilation. Internal CO$_2$ concentration in the Stressed treatment was higher for Kopu II BC$_1$ than for
Kopu II (Figure 8.3). Differences in C supply may, therefore, have influenced the differences in net photosynthesis for these clover types. It is also possible that fixation of C was a limiting factor in the Kopu II BC2 and Kopu II plants as Rubisco, the main enzyme in the C cycle, is known to be affected by water stress (Parry et al., 2002; Medrano et al., 1997).

8.4.2 Protective compounds

As well as possessing a greater ability to maintain growth under water stress, BC1 hybrids also produced more of some protective compounds, which may enable leaf and stolon tissues to be maintained. Phenolic compounds such as quercetin and kaempferol glycosides are produced in the epidermis and provide this protective function, primarily as antioxidants and sunscreens against UV damage (Agati et al., 2011). Hofmann et al. (2003) reported that quercetin glycoside accumulation was also involved in drought responses of white clover under controlled environment conditions, and this has also been found by Ballizany et al. (2012a; 2012b) in white clover crosses grown under outdoor conditions. However, in the current experiment, differences in quercetin and kaempferol glycosides did not appear to play a large role in drought resistance of T. repens x T. uniflorum hybrids, in general, but this may be of more importance in specific families. While quercetin glycoside concentrations increased in the Stressed treatment, this response did not differ between the hybrids and white clover, indicating it was a generic response to water stress (Figure 8.11A). However, in the Kopu II subset, quercetin increased under water stress for the BC1 and BC2 entries only (Figure 8.13A). The kaempferol glycoside concentrations of Kopu II BC1 were also higher than those of Kopu II BC2 and Kopu II in both watering treatments (Figure 8.13B), while higher constitutive levels of kaempferol were correlated with smaller decreases in shoot DW under water stress (Appendix 43).

In white clover cultivars and ecotypes under UV-B radiation, Hofmann et al. (2003) observed a negative correlation between biomass and quercetin glycoside accumulation. Disruption of this relationship would enable selection of high productivity genotypes with increased levels of protective quercetin glycosides. A negative correlation between quercetin glycosides and biomass was also observed in the current experiment under water stress – i.e. the highest levels of these stress protective compounds were present in the least productive plants (Figure 8.12). Hofmann et al. (2003) and Hofmann and Jahufer (2011) found no relationship between kaempferol glycosides and biomass in white clover, and this was also observed in the current experiment.
Hydroxycinnamic acids are also involved in responses to drought and UV stress, as antioxidant conjugates and sunscreens (Tattini et al., 2004). In contrast to quercetin and kaempferol glycosides, which are often present in the vacuoles of epidermal cells, soluble forms of hydroxycinnamic acids are found in the cytoplasm of all cells (Shahidi and Chandrasekara, 2010) and so may provide more general protection to the plant. In the current experiment, hydroxycinnamic acid concentrations did increase under water stress. Higher overall hydroxycinnamic acid concentrations in the BC1 generation than in both BC2 and white clover, and higher concentrations in Kopu II BC1 than in Kopu II suggest that hydroxycinnamic acid accumulation in the BC1 generation differs to that in BC2 and white clover. Furthermore, the hydroxycinnamic acid concentrations of both BC1 in general, and also Kopu II BC1, increased under water stress, as did those of white clover in general, but concentrations were higher in BC1 than in BC2 (Figure 8.11C), and in Kopu II BC1 than in Kopu II (Figure 8.13C). Accumulation of hydroxycinnamic acids may therefore provide more protection against drought to the BC1 generation than for the BC2 generation and white clover, and warrants further investigation of the role of these compounds in drought responses of T. repens x T. uniflorum hybrids. In the Stressed treatment, greater variability for hydroxycinnamic acid concentrations in Kopu II BC1 than in Kopu II, and in BC1 than BC2 in general, suggests there may be scope to select for this characteristic (Appendix 51).

Insoluble hydroxycinnamic acids also cross-link lignin and hemicellulose in cell walls (Ralph and Helm, 1993), which increases structural strength but has also been found to be negatively correlated with digestibility of plant material (Buxton and Russell, 1988; Lam et al., 2003; Riboulet et al., 2008). Most studies have focussed on monocotyledons such as grasses, maize and sugar cane (Lam et al., 2003; Riboulet et al., 2008; Siqueira et al., 2011) and little is known about the relationship between hydroxycinnamic acids and digestibility in forage legumes. However, reported levels of these compounds in dicotyledons are lower than those for monocotyledons (Ishii, 1997; Lozovaya et al., 1999; Hartley and Jones, 1977).

### 8.4.3 Feed quality

The scope of the current experiment did not involve studies into how the differences in hydroxycinnamic acid concentrations affected feed quality, but the differences in digestibility among clover types and entries were very small, and unlikely to have an impact on ruminant production (Appendices 45 and 49). However, only healthy leaves were sampled. Therefore, under grazing conditions, higher levels of senescence in the BC2 generation and white clover during water stress could decrease digestibility compared with the BC1 generation.
The differences among clover types and entries for the remaining parameters, in both the Watered and Stressed treatments, were also minor and unlikely to impact ruminant production. Differential effects of water stress among clover types and entries were observed only for %CHO and %Protein. This interaction merits further investigation of the effect of water stress on these parameters in *T. repens* x *T. uniflorum* hybrids, especially given the limited number of replicates used in the analysis for %CHO. Measurements showed that Kopu II BC₁ was the only clover entry in the measured subset in which the carbohydrate content decreased significantly under water stress (Appendix 49). Combined with the increases in root cross-sectional area and root DW for Kopu II BC₁ (Chapter 7), this may indicate that Kopu II BC₁ diverted carbohydrates from the shoots to the root system. The decrease in %CHO under water stress for the BC₁ generation in general, and its lower content compared with BC₂ and white clover in the Stressed treatment (Table 8.4) may indicate that an increase in biomass allocation to roots is an inherent response to water stress for the BC₁ generation, but further study is necessary to confirm this.

Protein content of BC₁ hybrids differed somewhat to that of BC₂ hybrids and white clover in the Watered treatment (Tables 8.4 and 8.5), but levels were still high relative to ruminant requirements. For temperate forages, protein concentrations greater than 20% are in excess of animal needs (Pacheco and Waghorn, 2008). While protein is essential for animal production, excess amounts can be a metabolic cost to the animal as it must be converted to urea before being excreted (Waghorn, 2007). However, the lower %Protein content of BC₁ hybrids in both the full data set and the Kopu II data set may indicate that there is potential to make some improvements in feed quality for N metabolism through breeding and selection of *T. repens* x *T. uniflorum* hybrids. Other studies on white clover hybrids with *T. ambiguum* and *T. nigrescens* (Marshall et al., 2003a; 2004) have also found decreased protein or %N, as well as increased levels of carbohydrates, compared with white clover. Similarly to the *T. repens* x *T. uniflorum* hybrids in the current experiment, these studies also found relatively small differences in digestibility (DMD) between white clover and its hybrids.

Results suggest that cell wall components, as indicated by %NDF, may be slightly lower in BC₁ hybrids (Table 8.2 and Appendix 47). In contrast, the %ADF data (Table 8.2 and Appendix 47) suggests that less digestible components may be slightly higher in both BC₁ and BC₂ hybrids. This could be indicative of anatomical adaptations to the drier native environment of *T. uniflorum*. But, again, these small differences in %NDF and %ADF are unlikely to affect ruminant production. The lower %NDF values in the Stressed treatment are
consistent with other studies which have found decreases in cell wall material under water stress (Wilson, 1982; Buxton and Casler, 1993).

8.5 Conclusions

- The hypotheses outlined in the Introduction were supported, showing that a variety of physiological and biochemical factors of relevance to drought resistance were present in *T. repens* x *T. uniflorum* hybrids.

- BC₁ hybrids were able to maintain net photosynthesis during water stress, which may have contributed to the smaller decreases in DM production and morphological characteristics reported in Chapter 7. In particular, this may have been facilitated by the influence of higher internal CO₂ concentrations on C supply.

- There were higher rates of transpiration and larger decreases in leaf water potential for BC₁ hybrids compared with white clover. BC₁ hybrids may therefore be more pronounced “water spenders”, able to maintain water uptake during drought by increasing the allocation of biomass to the root system.

- There were differences in (and variability for) hydroxycinnamic acid accumulation in the BC₁ generation, which may warrant further investigation into their contribution to drought resistance in this material. These compounds appear to be more widespread among hybrid families than quercetin and kaempferol glycosides, which differed more among the Kopu II subset (i.e. individual clover entries) than among the broad clover types. The lower senescence in BC₁ hybrids under water stress, reported in Chapter 7, may also be attributable to protection from these phenolic compounds.

- Differences in feed quality among clover types are unlikely to impact ruminant production. Lower protein content in the Watered treatment for the BC₁ generation may indicate potential for improvement in N metabolism through breeding and selection. Given the limited replication for carbohydrate content, further study of this trait is required. An observed decrease in shoot %CHO suggests that carbohydrates may be diverted to the root system of BC₁ hybrids under water stress.

- In addition to flowering (Chapter 7), differences in the effect of water stress on BC₂ hybrids, compared with the other clover types, may also have been mediated by differences in WUE.
Chapter 9
General discussion

9.1 Background
The objectives of this study sought to fill key knowledge gaps on the morphological and physiological characteristics of *T. repens* x *T. uniflorum* interspecific hybrids. Chapter 3 investigated the effects of hybridisation on key white clover traits, such as growth, stolon morphology, tap root life span, N fixation, growth habit, and flowering, in a field situation. In Chapter 4, root depth distribution of the BC₁ (backcross 1) hybrids was compared with the white clover and *T. uniflorum* parents over four harvests, and differences in growth were identified. Chapter 5 then investigated these growth effects more closely, over a range of applied nutrient concentrations. Root morphology and architecture were determined in Chapter 6, and related to the potential adaptation of *T. uniflorum* and *T. repens* x *T. uniflorum* BC₁ hybrids to particular environmental conditions. The morphological, physiological and biochemical responses of white clover and of two backcross hybrid generations to water stress were investigated in Chapters 7 and 8. This study has provided considerable new information on the traits of this novel plant material and at the same time, many new questions have arisen. This chapter brings together the main conclusions of the study, to provide an overall picture of the characteristics of *T. repens* x *T. uniflorum* interspecific hybrids, and identifies some key issues which remain to be addressed. Major findings of the study are shown in Figure 9.1. This illustrates key differences between white clover and BC₁ hybrids under control and stressed conditions, which indicate potential adaptation to contrasting levels of soil moisture and fertility. Inherent characteristics that provide further evidence for such adaptations are also shown, along with inherent and stress-induced characteristics for BC₁ hybrids which contribute to both yield differences and stress adaptation.

9.2 General effects of hybridisation
Hybridisation with *T. uniflorum* has affected many characteristics of white clover. While some newly introgressed traits were lost by a second generation of backcrossing, others were maintained. The general morphological type of BC₁ hybrids had a smaller leaf lamina area, shorter internodes and a more prostrate growth habit than white clover (Chapter 3 and 7). Under water stress the BC₁ generation was compact and dense compared with the more spreading, open habit of white clover and BC₂ (backcross 2) hybrids (Chapter 7). In this study, N fixation was not affected by hybridisation under what were most likely low N
conditions (Chapter 3). Tap root survival of *T. uniflorum* and the BC$_1$ generation was significantly higher than in white clover, although the length of additional life span was less than expected (Chapter 3). Targeted selection for tap root longevity could improve upon this through further breeding cycles. The inflorescence height of hybrid generations was lower than that of white clover, due to shorter peduncle lengths and heights and, in particular, a lower peduncle angle and shorter length of the peduncle relative to the supporting petiole. While this has potential commercial implications for seed harvesting, work by Naeem (in prep.) indicates that it should be possible to select for superior inflorescence height in some families.

Figure 9.1. Summary of the major findings of the study, and proposed relationships. ↓ = drought-induced decrease; ↑ = drought-induced increase; SLA = specific leaf area.
9.3 Growth

As expected, dry matter (DM) production in the key traits experiment (Chapter 3) was reduced in the hybrids compared with white clover, but this may also be overcome by phenotypic selection. However, in some cases, growth of BC₁ hybrids was considerably higher than that of white clover (Chapter 4 and 5) or was less affected by water stress (Chapter 7) (Figure 9.1).

The relative performances of BC₁, BC₂, and white clover in the drought experiment showed some comparable results (Chapter 7) to those reported in the key traits experiment (Chapter 3). For example, in the Watered treatment – which is comparable to the ambient conditions of the key traits experiment – the effect of hybridisation on stolon morphological characteristics of the BC₁ generation, compared with white clover, was the same as reported in Chapter 3. Leaf lamina area, specific leaf area (SLA) and internode length in the BC₁ generation were all significantly smaller than in the BC₂ generation and white clover. As mentioned in Chapter 7, some differences in clover type comparisons between Chapter 3 and Chapter 7 were observed for growth related results (lateral spread, stolon morphology and DM scores/shoot DW). These may reflect differing abilities of clover types to tolerate grazing and/or grass competition, or differences in seasonal growth or phenology.

Differences in DM production among clover types in this study may also reflect differences in growth rate arising from possible adaptation to different fertility environments (Chapin, 1980) (Figure 9.1). Tissue density is indicative of both soil fertility and growth rate, and the high root tissue density of T. uniflorum may reflect these factors (Chapter 6) (Wahl and Ryser, 2000; Arredondo and Johnson, 2011). In this study, specific leaf mass (SLM) was used as a proxy for leaf thickness, however Garnier and Laurent (1994) found SLM was not correlated with thickness, but was correlated with tissue density. Using SLA as the inverse of SLM suggests that leaf thickness/lamina tissue density of T. uniflorum would be greater than the hybrids and white clover (Chapter 3). Specific leaf area and SLM results also suggest potential trends in tissue density and growth rate with increasing proportions of T. uniflorum genes (Chapter 3 and 7). Greater tissue densities may result in greater root and shoot life spans in T. uniflorum and hybrid clovers, which is also characteristic of low fertility environments (van der Krift and Berendse, 2002; Ryser, 1996). This may produce a trade-off between greater life span and slower growth rates. Further study of the shoot anatomy of T. uniflorum, T. repens x T. uniflorum hybrids, and white clover would confirm these apparent differences in tissue density, and would also allow quantative measurements of leaf thickness.
9.4 Adaptation to low soil resources

One of the key findings of this study (Figure 9.1) is the potential tolerance of *T. repens* x *T. uniflorum* interspecific hybrids to low soil fertility and moisture (Chapters 4, 5, 7 and 8). Both these adaptations have significant practical implications. Results from this study suggest that the hybrids may have inherited the ability to tolerate low soil fertility from *T. uniflorum* (Chapters 4 and 5), although little information is published on its native habitat. Analyses of shoot mineral concentrations indicate that P, and possibly N, were limiting for white clover growth in those experimental systems, although low N was probably a symptom of P deficiency. Differences in P physiology may be responsible for the growth effects observed. Lower P and Pi concentrations in some hybrid families suggest that they may tolerate low P and sequester less Pi in their vacuoles than white clover. In addition, P and Pi use efficiencies of some hybrids were higher than for white clover, and also higher than other hybrid families.

New Zealand soils have a low natural fertility (McLaren and Cameron, 1990), which has been increased by decades of fertiliser application, and fertiliser is one of the largest on-farm expenses. For example, in the 2011 MAF national sheep and beef budget model, fertiliser was the major expense ($45,557), while in the national dairy budget model it was the third highest cost ($69,297) behind labour and feed ($95,179 and $183,624) (Ministry of Agriculture and Forestry, 2011a, 2011b). Exact and relative costs vary depending on region and farming systems. White clover has a requirement for high soil fertility, particularly P, and higher levels of applied P are needed to grow white clover in association with grasses than in monoculture (Jackman and Mouat, 1970). Any improvements in white clover which reduce fertiliser use must have considerable economic and environmental benefits. Furthermore, where soil P levels are low, such as in New Zealand hill country areas, productivity is limited by low legume content (Caradus and Williams, 1981). Improvements which enable the use of white clover in previously limiting environments, such as those demonstrated by the hybrids in this study, may therefore have positive effects on farm productivity.

White clover is also highly sensitive to low soil moisture, which restricts its use in dryland areas of New Zealand (Aparicio-Tejo *et al.*, 1980; Knowles *et al.*, 2003; Barbour *et al.*, 1996). Eastern parts of the country, where these dryland areas predominantly occur, are forecast to become drier in the future (Salinger, 2003). The findings from this study illustrate that *T. repens* x *T. uniflorum* BC1 hybrids have potential for improved productivity and survival, compared with white clover, under dryland and/or drought conditions. As hypothesized, BC1 hybrids were found to be more drought resistant than white clover, due to various physiological, morphological and biochemical factors (Chapters 7 and 8). Principally, DM
production of the BC₁ generation was less affected by water stress than that of white clover. This may have been influenced by the maintenance of photosynthesis under water stress. Smaller effects of water stress on stolon morphological characteristics of the BC₁ hybrids, compared with white clover, are also likely to have been a major contributor. In particular, the effect on traits related to lamina area suggests the BC₁ hybrids maintained cell turgor and growth better than white clover. The smaller effect on production in the BC₁ generation, compared with the BC₂ generation and white clover, is also likely to have been affected by lower senescence and higher stolon density. Differences in flavonol accumulation may also play some role.

Recovery from drought was not investigated in this study, however it may be hypothesised that this will be faster in *T. repens* x *T. uniflorum* BC₁ hybrids than in BC₂ hybrids or white clover. Engin and Sprent (1973) concluded that meristematic clover nodules recover more rapidly upon re-watering after drought than spherical nodules, such as those found in soybean. The higher proportions of coralloid nodules observed in *T. uniflorum* and BC₁ hybrids (Chapter 4) may suggest that they would resume N-fixing activity more rapidly than white clover following water stress. Greater stolon density in BC₁ hybrids during drought (Chapter 7) may also result in better recovery compared with white clover.

To a certain extent, the results of this study reflect the experimental conditions imposed. There was a convergence of DM scores between BC₁ hybrids and white clover after two months without irrigation (Chapter 7). It is therefore possible that if the experiment had continued, the yield of the hybrids may even have exceeded that of white clover. Larger differences in survival may also have become apparent. The study also started at a relatively high soil moisture content, which was close to field capacity. In drier environments, growth and physiological effects may occur more rapidly than seen here, leading to larger differences in the same time frame. The timing of drought in relation to the developmental stage of the plants may also affect the relative performance of the hybrids versus white clover. For example, hybrid clovers may be better adapted to spring droughts, which can have major effects on the survival of small, clonal white clover plants arising from spring fragmentation (Brock and Hay, 1996). Such events may also affect establishment and survival of seedlings.

The interaction of rainfall with soil type should also be taken into consideration, as the amount of water stored in the soil depends on soil texture, structure, organic matter, depth, profile layering and stone content (McLaren and Cameron, 1990). Available soil water depends on several factors, but is essentially dictated by the difference between the field capacity and permanent wilting point of the particular soil type (McLaren and Cameron,
Differences in soil type could, therefore, influence the relative performance of the hybrids and white clover, even where rainfall is similar. In Chapter 3, the soil type (Wakanui silt loam) may have prevented any advantage to hybrid DM production, despite annual rainfall for 2009 being below the levels suggested by Brock et al. (2003) for optimal white clover growth (750 mm). Moot et al. (2008) showed that water extraction of perennial ryegrass and lucerne (*Medicago sativa* L.) in a deep Wakanui silt loam, with a high water storage capacity, was greater than in a Lismore very stony loam and Lismore stony loam.

### 9.5 Interaction of soil fertility and soil moisture

The observed tolerance to low nutrient supply may also contribute to drought resistance in *T. repens* x *T. uniflorum* interspecific hybrids, due to the effects of soil moisture on nutrient availability and uptake. These include decreases in ion mobility, transpiration, and soil concentrations of some nutrients in dry soil (Tinker and Nye, 2000; Chapin, 1991; Sardans et al., 2008). Chapin (1991) suggested that the indirect effect of low soil moisture on plant growth, via nutrient availability, may be almost as important as the direct effect of soil moisture on growth. Nutrient mobility can be affected at soil moisture levels which have no effect in themselves on plant water relations (Nye and Tinker, 1977). If *T. repens* x *T. uniflorum* hybrids have a lower nutrient requirement than white clover, this may convey an additional growth advantage under decreased soil moisture. The combination of drought and low fertility tolerance, plus their potential interaction, may mean that *T. repens* x *T. uniflorum* hybrids are particularly suited to environments where both soil moisture and fertility are limiting.

In particular, the effects of soil P in alleviating the effects of water stress have been reported in several species (Rodrigues et al., 1996; Garg et al., 2004; Jin et al., 2006). Given that P appears to be a key nutrient in the growth of *T. repens* x *T. uniflorum* hybrids under low nutrient supply, it may also contribute to alleviation of water stress in this material. In white clover, plants growing in high P conditions have been found to show fewer symptoms of water stress, and recover faster after re-watering, than those growing in low P conditions (Singh et al., 1997). Singh and Sale (1998) also found that white clover plants supplied with high P in dry soil had similar or higher shoot growth, as well as a higher water and P uptake, compared with plants in wet soil. Singh and Sale (1998) and Singh et al. (2000) subsequently attributed these responses to greater osmotic adjustment and leaf expansion, and increased hydraulic conductance. Osmotic adjustment was not investigated in the current study, but high shoot concentrations of Na, and possibly K (Chapters 4 and 5), do suggest this could be greater in BC₁ hybrids. Smaller reductions in leaf lamina area for the BC₁ hybrids are also
indicative of greater leaf expansion (Chapter 7). In the studies by Singh and Sale, white clover
plants in high P, dry soil had a larger coarse root diameter (Singh and Sale, 1998, 2000), as
well as a greater mass and density of coarse roots (Singh and Sale, 2000). Increased hydraulic
conductance was attributed to the greater number and diameter of xylem vessels in these roots
(Singh and Sale, 2000). The thicker roots of *T. uniflorum* may thus improve drought tolerance
through greater hydraulic conductance, as well as through other structural features. However,
the root anatomy of *T. uniflorum* and *T. repens* x *T. uniflorum* hybrids has not been studied.

Phosphorus status has also been shown to affect photosynthesis in some species (Foyer and
Spencer, 1986; Dietz and Foyer, 1986). In subterranean clover, Bouma (1967) found that
photosynthesis was decreased by P deficiency but could be increased by additional P. The
potential effect of tolerance to low P on photosynthesis under drought in *T. repens* x *T.
uniflorum* hybrids is unknown.

**9.6 Root morphology and architecture**

Differences in root morphology and architecture are likely to have influenced the relative
performance of *T. uniflorum*, *T. repens* x *T. uniflorum* hybrids and white clover in this study
(Figure 9.1). Positive correlations have been found between quantitative trait loci (QTLs) for
root traits and QTLs for productivity in a number of species, including under drought
conditions (Tuberosa *et al.*, 2002; Babu *et al.*, 2003). While there were no differences in the
root diameter of the hybrids and white clover parents in Chapter 6, neither water nor nutrients
were limiting in that experiment. However, in the Stressed treatment in Chapter 7, the cross-
sectional area of the thickest root of Kopu II BC1 was larger than that of the Kopu II parent
(Figure 9.1). Caradus and Woodfield (1998) and Caradus (1977) described thick nodal roots
of white clover as “vertically penetrating”, which would facilitate access to deeper soil water.
Hussain *et al.* (2012) have also noted a higher proportion of coarse roots in *T. repens* x *T.
uniflorum* BC1 hybrids compared with white clover. The degree to which these observations
reflect differences in xylem number and diameter, and thus hydraulic conductance, among
BC1 hybrids and white clover are unknown. Results of this study (Chapter 8) also suggest BC1
hybrids may gain some drought resistance from increased allocation to root DW under water
stress (Figure 9.1), although the effects on root:shoot ratio could not be determined. This
would enable them to maintain a higher rate of water uptake than their BC2 and white clover
relatives under water stress.

In rice, Ekanayake *et al.* (1985) found significant correlations between root characteristics
(such as thickness, length and density), and drought stress symptoms and recovery. However,
Annicchiarico and Piano (2004) found no correlation between root features and drought tolerance in white clover, and suggested that this was due to its poor physiological adaptation to water stress. Improved physiological tolerance of *T. repens* x *T. uniflorum* hybrids may enable the beneficial effects of root characteristics on drought resistance to be expressed. Annicchiarico and Piano (2004) also speculated on the importance of fine roots during drought, and it is likely that both thick and fine roots do play a role in drought resistance. As with nutrient acquisition, fine roots will increase water interception, but thicker roots are also important for factors such as physical resistance, hydraulic conductance and penetration of dry soil. Further investigation of root morphology under control and drought conditions is necessary to determine the contribution of constitutive and adaptive root characteristics to drought resistance in this material.

In addition to the potential impact of root structure and size on drought, the root architecture and topology of *T. uniflorum* and some hybrid families appears to be adaptive for low soil fertility, based on models and data in the literature. Long link lengths and more herringbone-like branching patterns provide higher exploitation efficiency, and may also contribute to water acquisition. Based on the conflicting findings of Fitter (1987) and Nielsen *et al.* (1994), it is not clear whether this root system structure is better for the capture of immobile or mobile nutrients. However, ion diffusion was not an issue in the current study, due to the hydroponic growth medium used. The use of a soil medium in future work would enable the effects of root architecture and topology on uptake of immobile versus mobile nutrients to be determined. This would also assist with determining the plasticity of root responses in this material to variations in soil resources.

Regardless of the influence of root architecture and topology, the thicker roots of *T. uniflorum*, while having some advantages for drought tolerance, are not beneficial for nutrient acquisition due to a lower root surface area:soil volume ratio. Another strategy employed by plants to increase the uptake of nutrients, particularly P, is the production of root hairs (Jungk, 2001; Gahoonia and Nielsen, 1998), which increase the absorption area of roots at a relatively minimal cost (Clarkson, 1991). Gahoonia *et al.* (1997) reported that root hairs increased the root surface area of wheat and barley cultivars by 95–341%, and noted strong correlations between root hair length and P depletion of the soil. In white clover, Caradus (1979) found that selection for root hair length increased the surface area of root hairs of the cultivar Tamar by 10%, and increased the volume of soil explored by root hairs by 11%. The length and number of root hairs of white clover genotypes selected for P-responsiveness have also been found to be higher than in non-responsive genotypes (Care and Caradus, 1998).
Characterisation of root hairs was not part of this study, nor was the response of root hair production to varying nutrient (including P) concentrations. However, during the experimental harvest in Chapter 4, sand was noted to adhere to the roots of *T. uniflorum* plants (Plate 9.1) and, to a lesser extent, to those of some hybrid plants. This may have been due to the presence of long and/or dense root hairs.

Root mass distribution could also influence the ability of *T. uniflorum*, and that of some hybrids, to intercept nutrients and water (Figure 9.1). In Chapter 4, root system shape and root depth distribution of the hybrids was similar to that of *T. uniflorum*, with both having more root mass in the upper part of the profile than white clover. This may reflect the soil moisture and fertility of the natural environment of *T. uniflorum*.

Plate 9.1. Sand adhering to the roots of a *T. uniflorum* plant from the root depth distribution experiment in Chapter 4 (harvest 2, 119 days). This could indicate the presence of long and/or dense root hairs.

### 9.7 Genotypic variation and selection

It was expected that variability of traits would be greater in the hybrids than in white clover, and greater in the BC$_1$ generation than the BC$_2$ generation, due to recombination of genes and variations in the composition of the *T. uniflorum* part of the genome. However, in Chapter 3 and the Watered treatment of Chapter 7, the variability for DM scores and shoot DW of the BC$_1$ hybrids did not differ to the BC$_2$ hybrids and white clover, and variability for a range of morphological characteristics was actually lower than white clover. This may reflect the
influence of the genotype x environment interaction in field situations, where variable environmental effects may obscure genetic variability. In contrast, variability for shoot DW was higher for BC1 hybrids in Chapter 4, where the standardised environment may have enabled the genetic variation of the hybrids to be expressed more.

While DM scores of BC1 hybrids in Chapter 3 were, on average, lower than white clover, the difference was not as large as expected and may be overcome by phenotypic selection. Variation among individual BC1 families, for DM score relative to their parental cultivars, suggests selection based on high performing family groups may improve upon the DM yields found in the current study. Differences among hybrid families were seen throughout the study. Other characteristics, such as stolon density, lateral spread, and growth habit, also showed variation among hybrid families in their performance relative to parental cultivars (Chapter 3).

There were also differences among the hybrids in their responses to changing nutrient levels, with growth of some families being less affected than that of other families and their white clover parents (Chapter 5). Kopu II x 900-4, in particular, may be able to tolerate lower nutrient concentrations than other hybrids. The root architecture and topology of T. uniflorum may have been inherited by some hybrid families, such as Kopu II x 900-4, which also showed a more herringbone-like topology than white clover and Kopu II x 80-2. In contrast, both Kopu II x 80-2 and Crusader x 80-2 appeared to exhibit a higher frequency of root branching than their white clover parents. These differences in root architecture may reflect adaptation to different soil conditions among hybrid families. Results in Chapter 4 also suggested differences among hybrid families in DW and some root characteristics, based on T. uniflorum parentage. Finally, while BC1 hybrids in general did not accumulate more of the protective quercetin and kaempferol glycosides under water stress compared with white clover, examination of the Kopu II subset suggests that some individual hybrid families may accumulate more of these secondary metabolites than their respective parents (Chapter 8).

These family differences suggest that when comparing characteristics of T. repens x T. uniflorum hybrids and white clover, screening of an increased number of hybrid families, from a wide range of genetic backgrounds, should be carried out. For some traits, plant improvement may be possible through selection of high performing families and development of segregating populations may also be possible. Transgressive segregation is common in interspecific hybrids of many genera (Rieseberg et al., 1999), and there was also evidence of this in the current study. Much of the material used in the two field experiments in this study was not derived from elite white clover cultivars and, in all cases, a limited number of T. uniflorum accessions were available for use in F1 crosses. Targeted selection of parental
material is therefore likely to further improve the characteristics of \( T. \text{repens} \times T. \text{uniflorum} \) hybrids in the future.

### 9.8 Future work

#### 9.8.1 Major points of investigation

Future work could investigate the following areas:

- Further study of the P physiology of the hybrids, including P response curves, critical internal P concentrations, P efflux, and P\(_i\) sequestration.

- Effects of soil moisture, and contrasting and heterogeneous nutrient supply, on root architecture and morphology, and subsequent effects on growth and nutrient uptake.

- Effects of differences in timing and severity of soil moisture limitation on growth of hybrids compared with white clover.

- Wider screening of hybrid families, in order to capture the full genetic variation of the material, enable selection of elite families, and identify segregating populations.

#### 9.8.2 Other areas of interest

- To gain more understanding of the natural edaphic conditions of \( T. \text{uniflorum} \), and the implications of this for traits and adaptations in the hybrids.

- Interaction of soil fertility (particularly P) and soil moisture, and the effects on shoot and root growth.

- Recovery from drought of hybrids compared with white clover (growth, N fixation and photosynthesis).

- Comparison of root hair length and density among \( T. \text{uniflorum} \), \( T. \text{repens} \times T. \text{uniflorum} \), and white clover, and the potential implications for P uptake.

- The role of hydroxycinnamic acids in drought resistance of the hybrids, and screening for drought-induced quercetin and kaempferol accumulation among hybrid families.

- Investigation of shoot and root anatomy, and the implications for growth rate and stress resistance of the hybrids.

- Measurement of osmotic adjustment in BC\(_1\) and BC\(_2\) hybrids compared with white clover, and the effect of this on resistance to water stress.
References


Anderson JA, Taylor NL, Williams EG (1991b) Cytology and fertility of the interspecific hybrid *Trifolium ambiguum* x *T. repens* and backcross populations. Crop Science 31: 683-687


Caradus J (1994a) Selection for improved adaptation of white clover to low phosphorus and acid soils. Euphytica 77: 243-250


Caradus JR (1979) Selection for root hair length in white clover (Trifolium repens L.). Euphytica 28: 489-494


Chen C-C, Gibson PB (1970b) Meiosis in two species of *Trifolium* and their hybrids. Crop Science 10: 188-189
Chen C-C, Gibson PB (1971) Seed development following the mating of *Trifolium repens* x *T. uniflorum*. Crop Science 11: 667-672
Chen C-C, Gibson PB (1972a) Barriers to hybridization of *Trifolium repens* with related species. Canadian Journal of Genetics and Cytology 14: 381-389
Chen C-C, Gibson PB (1972b) Chromosome relationships of *Trifolium uniflorum* to *T. repens* and *T. occidentale*. Canadian Journal of Genetics and Cytology 14: 591-595
Chou M-c, Gibson PB (1968) Cross-compatibility of *Trifolium nigrescens* with diploid and tetraploid *Trifolium occidentale*. Crop Science 8: 266-267
Cocks PS (2001) Ecology of herbaceous perennial legumes: a review of characteristics that may provide management options for the control of salinity and waterlogging in dryland cropping systems. Australian Journal of Agricultural Research 52: 137-151

Daday H (1954) Gene frequencies in wild populations of Trifolium repens. I. Distribution by latitude. Heredity 8: 61-78


Evans AM (1962b) Species hybridization in Trifolium. II. Investigating the pre-fertilization barriers to compatibility. Euphytica 11: 256-262

Evans PS (1977) Root distribution and water-withdrawal patterns of some crop and pasture species. New Zealand Department of Scientific and Industrial Research Information Series 126: 186-190


Gahoonia TS, Care D, Nielsen NE (1997) Root hairs and phosphorus acquisition of wheat and barley cultivars. Plant and Soil 191: 181-188
Gibson PB, Chen C-C (1973) Success in hybridizing and selfing *Trifolium repens* at different temperatures. Crop Science 13: 728-730

Gibson PB, Chen C-C (1975) Registration of SC-2 and SC-3 clover germplasms. Crop Science 15: 605-606


Hart AL, Jessop D (1983) Phosphorus fractions in trifoliate leaves of white clover and lotus at various levels of phosphorus supply. New Zealand Journal of Agricultural Research 26: 357-361


Hofmann RW, Jahufer MZZ (2011) Tradeoff between biomass and flavonoid accumulation in white clover reflects contrasting plant strategies. PLoS ONE 6: e18949


Hovin AW (1962b) Species compatibility in subsection Euamoria of Trifolium. Crop Science 2: 527-530


Karsten HD, MacAdam JW (2001) Effect of drought on growth, carbohydrates, and soil water use by perennial ryegrass, tall fescue, and white clover. Crop Science 41: 156-166


Kazimierski T, Kazimierska EM, Strzyzewska C (1972) Species crossing in the genus Trifolium L. Genetica Polonica 13: 11-32


Lozovaya VV, Gorshkova TA, Yablokova EV, Rumyantseva NI, Valieva A, Ulanov A, Widholm JM (1999) Cold alkali can extract phenolic acids that are ether linked to cell wall components in dicotyledonous plants (buckwheat, soybean and flax). Phytochemistry 50: 395-400


generation backcross hybrids in the field – Reproductive and agronomic traits among BC3 hybrids of *T. repens* x *T. nigrescens*. Euphytica 126: 195-201


**McNaught KJ** (1970) Diagnosis of mineral deficiencies in grass-legume pastures by plant analysis


**Moot DJ, Scott WR, Roy AM, Nicholls AC** (2000) Base temperature and thermal time requirements for germination and emergence of temperate pasture species. New Zealand Journal of Agricultural Research 43: 15-25

**Morton JD, Smith LC** (2000) Chemical analyses of pasture for measuring nutrient status and requirements. In LD Currie, P Loganathan, eds. Soil research, a knowledge industry

Naeem M (in prep.) Improvement in seed production of interspecific hybrids between T. repens and T. uniflorum. PhD thesis. Massey University, Palmerston North


Pollock KM, Mead DJ, McKenzie BA (2009) Soil moisture and water used by pastures and silvopastures in a sub-humid temperate climate in New Zealand. Agroforestry Systems 75: 223-238


Sardans J, Peñuelas J, Ogaya R (2008) Drought’s impact on Ca, Fe, Mg, Mo and S concentration and accumulation patterns in the plants and soil of a Mediterranean evergreen *Quercus ilex* forest. Biogeochemistry 87: 49-69


Sutherland ORW (1979) Invertebrate-plant relationships and breeding pest-resistant plants. In TK Crosby, RP Pottinger, eds. 2nd Australasian Conference on Grassland Invertebrate Ecology, pp 84-88


### Appendix A

**Key traits**

**A.1 Full description of hybrid families**

Appendix 1. Full descriptions of BC₁ families used in the key traits (Chapter 3) and water stress (Chapters 7 and 8) experiments.

<table>
<thead>
<tr>
<th>Entry number</th>
<th>White clover parent (female)</th>
<th>F₁ parent (male)</th>
<th>F₁ parentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Aran NC18 R1-8</td>
<td>900-3</td>
<td>(Kopu II-2 x AZ4383-11)-2</td>
</tr>
<tr>
<td>4</td>
<td>Barblanca NC 24-R3-5</td>
<td>82-3</td>
<td>(Sustain-1 x T66-3)</td>
</tr>
<tr>
<td>5</td>
<td>Crusader-5</td>
<td>80-2</td>
<td>(Kopu II-2 x T66-6)-2</td>
</tr>
<tr>
<td>6</td>
<td>Crusader-5</td>
<td>900-4</td>
<td>(Kopu II-2 x AZ4383-11)-3</td>
</tr>
<tr>
<td>7</td>
<td>Crusader-5</td>
<td>902-11</td>
<td>(Sustain-1 x AZ4437-3)-11</td>
</tr>
<tr>
<td>8</td>
<td>Kopu II NC51-R3-3</td>
<td>900-4</td>
<td>(Kopu II-2 x AZ4383-11)-3</td>
</tr>
<tr>
<td>9</td>
<td>Kopu II-7</td>
<td>80-2</td>
<td>(Kopu II-2 x T66-6)-2</td>
</tr>
<tr>
<td>10</td>
<td>Sustain</td>
<td>82-3</td>
<td>(Sustain-1 x T66-3)</td>
</tr>
<tr>
<td>11</td>
<td>Tribute</td>
<td>900-4</td>
<td>(Kopu II-2 x AZ4383-11)-3</td>
</tr>
<tr>
<td>12</td>
<td>Trophy NC8-R1-1</td>
<td>902-6</td>
<td>(Sustain-1 x AZ4437-3)-6</td>
</tr>
</tbody>
</table>

Note: T66-3 and T66-6 = specific genotypes of AZ4382.

Appendix 2. Full descriptions of the BC₂ families used in the key traits (Chapter 3) and water stress (Chapters 7 and 8) experiments. OP=open pollinated.

<table>
<thead>
<tr>
<th>Entry number</th>
<th>Female parent</th>
<th>Male parent</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Crusader 10-2</td>
<td>(Crusader-203 x 900-5)-5</td>
</tr>
<tr>
<td>14</td>
<td>Kopu II NC51-R3-3</td>
<td>(Kopu II-4 x 901-1)-3</td>
</tr>
<tr>
<td>15</td>
<td>902-1-OP-4</td>
<td>Trophy NC8-R2-9</td>
</tr>
<tr>
<td>16</td>
<td>(Crusader-10 x 902-1)-5</td>
<td>Open pollinated</td>
</tr>
<tr>
<td>17</td>
<td>Durana</td>
<td>(Crusader-10 x 902-1)-3</td>
</tr>
<tr>
<td>18</td>
<td>Durana</td>
<td>(Kopu II-2 x 902-4)-1</td>
</tr>
</tbody>
</table>
**A.2 Clover entry means**

Appendix 3. Clover entry means for growth parameters. Unless units are given, all means are score values. Means for dry matter score and lateral spread are averaged over all sampling dates; stolon density, growth habit and fungal disease were measured in May 2009 and virus infection in November 2009.

<table>
<thead>
<tr>
<th>Entry number</th>
<th>Clover type</th>
<th>Dry matter score</th>
<th>Lateral spread (cm)</th>
<th>Stolon density</th>
<th>Growth habit</th>
<th>Fungal disease</th>
<th>Virus infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>T. uniflorum</em></td>
<td>1.5</td>
<td>8.6</td>
<td>3.3</td>
<td>2.1</td>
<td>0.2</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td><em>T. uniflorum</em></td>
<td>1.6</td>
<td>8.4</td>
<td>3.8</td>
<td>1.7</td>
<td>0.4</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>BC1</td>
<td>4.0</td>
<td>27.8</td>
<td>3.2</td>
<td>2.7</td>
<td>2.3</td>
<td>0.20</td>
</tr>
<tr>
<td>4</td>
<td>BC1</td>
<td>4.2</td>
<td>30.0</td>
<td>3.0</td>
<td>2.6</td>
<td>2.5</td>
<td>0.14</td>
</tr>
<tr>
<td>5</td>
<td>BC1</td>
<td>4.5</td>
<td>36.9</td>
<td>2.8</td>
<td>2.6</td>
<td>2.6</td>
<td>0.07</td>
</tr>
<tr>
<td>6</td>
<td>BC1</td>
<td>4.4</td>
<td>41.9</td>
<td>2.7</td>
<td>2.5</td>
<td>3.4</td>
<td>0.28</td>
</tr>
<tr>
<td>7</td>
<td>BC1</td>
<td>4.1</td>
<td>33.3</td>
<td>2.8</td>
<td>2.8</td>
<td>2.1</td>
<td>0.07</td>
</tr>
<tr>
<td>8</td>
<td>BC1</td>
<td>4.3</td>
<td>26.5</td>
<td>3.1</td>
<td>3.0</td>
<td>2.1</td>
<td>0.17</td>
</tr>
<tr>
<td>9</td>
<td>BC1</td>
<td>4.4</td>
<td>31.1</td>
<td>2.9</td>
<td>2.6</td>
<td>2.3</td>
<td>0.00</td>
</tr>
<tr>
<td>10</td>
<td>BC1</td>
<td>4.5</td>
<td>33.0</td>
<td>3.4</td>
<td>2.7</td>
<td>2.0</td>
<td>0.21</td>
</tr>
<tr>
<td>11</td>
<td>BC1</td>
<td>4.6</td>
<td>30.7</td>
<td>4.2</td>
<td>3.2</td>
<td>1.4</td>
<td>0.14</td>
</tr>
<tr>
<td>12</td>
<td>BC1</td>
<td>4.5</td>
<td>39.5</td>
<td>3.1</td>
<td>2.3</td>
<td>1.8</td>
<td>0.06</td>
</tr>
<tr>
<td>13</td>
<td>BC2</td>
<td>4.4</td>
<td>28.2</td>
<td>2.8</td>
<td>2.4</td>
<td>2.0</td>
<td>0.00</td>
</tr>
<tr>
<td>14</td>
<td>BC2</td>
<td>4.3</td>
<td>27.4</td>
<td>2.9</td>
<td>3.2</td>
<td>1.8</td>
<td>0.06</td>
</tr>
<tr>
<td>15</td>
<td>BC2</td>
<td>4.8</td>
<td>34.4</td>
<td>2.9</td>
<td>3.2</td>
<td>1.8</td>
<td>0.29</td>
</tr>
<tr>
<td>16</td>
<td>BC2</td>
<td>3.8</td>
<td>29.8</td>
<td>3.0</td>
<td>2.7</td>
<td>1.2</td>
<td>0.28</td>
</tr>
<tr>
<td>17</td>
<td>BC2</td>
<td>4.5</td>
<td>43.5</td>
<td>2.9</td>
<td>2.4</td>
<td>2.1</td>
<td>0.18</td>
</tr>
<tr>
<td>18</td>
<td>BC2</td>
<td>4.4</td>
<td>36.5</td>
<td>2.6</td>
<td>2.2</td>
<td>2.1</td>
<td>0.07</td>
</tr>
<tr>
<td>19</td>
<td>White clover</td>
<td>5.7</td>
<td>51.7</td>
<td>2.9</td>
<td>2.8</td>
<td>1.9</td>
<td>0.30</td>
</tr>
<tr>
<td>20</td>
<td>White clover</td>
<td>4.8</td>
<td>27.2</td>
<td>2.8</td>
<td>3.0</td>
<td>1.8</td>
<td>0.21</td>
</tr>
<tr>
<td>21</td>
<td>White clover</td>
<td>5.1</td>
<td>35.8</td>
<td>3.1</td>
<td>2.9</td>
<td>2.1</td>
<td>0.30</td>
</tr>
<tr>
<td>22</td>
<td>White clover</td>
<td>4.9</td>
<td>36.2</td>
<td>3.1</td>
<td>2.1</td>
<td>2.6</td>
<td>0.11</td>
</tr>
<tr>
<td>23</td>
<td>White clover</td>
<td>5.6</td>
<td>45.4</td>
<td>2.9</td>
<td>2.8</td>
<td>1.5</td>
<td>0.40</td>
</tr>
<tr>
<td>24</td>
<td>White clover</td>
<td>5.5</td>
<td>45.0</td>
<td>3.1</td>
<td>3.3</td>
<td>2.1</td>
<td>0.17</td>
</tr>
<tr>
<td>25</td>
<td>White clover</td>
<td>5.3</td>
<td>33.2</td>
<td>2.9</td>
<td>3.3</td>
<td>1.5</td>
<td>0.18</td>
</tr>
<tr>
<td>26</td>
<td>White clover</td>
<td>5.9</td>
<td>38.9</td>
<td>3.8</td>
<td>3.4</td>
<td>1.3</td>
<td>0.67</td>
</tr>
<tr>
<td>27</td>
<td>Red clover</td>
<td>4.4</td>
<td>15.9</td>
<td>3.0</td>
<td>2.7</td>
<td>0.1</td>
<td>0.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Entry number</th>
<th>Clover type</th>
<th>Peduncle height (mm)</th>
<th>Peduncle length (mm)</th>
<th>Peduncle:petiole</th>
<th>Peduncle length:height</th>
<th>Flower height (score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>BC1</td>
<td>38.5</td>
<td>53.2</td>
<td>1.19</td>
<td>0.710</td>
<td>1.8</td>
</tr>
<tr>
<td>4</td>
<td>BC1</td>
<td>27.7</td>
<td>42.5</td>
<td>1.45</td>
<td>0.657</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>BC1</td>
<td>36.6</td>
<td>53.6</td>
<td>1.42</td>
<td>0.678</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>BC1</td>
<td>25.7</td>
<td>34.5</td>
<td>1.07</td>
<td>0.709</td>
<td>2.3</td>
</tr>
<tr>
<td>7</td>
<td>BC1</td>
<td>27.5</td>
<td>36.1</td>
<td>1.19</td>
<td>0.773</td>
<td>1.8</td>
</tr>
<tr>
<td>8</td>
<td>BC1</td>
<td>33.6</td>
<td>46.4</td>
<td>1.32</td>
<td>0.752</td>
<td>1.6</td>
</tr>
<tr>
<td>9</td>
<td>BC1</td>
<td>29.9</td>
<td>41.8</td>
<td>0.94</td>
<td>0.773</td>
<td>1.6</td>
</tr>
<tr>
<td>10</td>
<td>BC1</td>
<td>25.9</td>
<td>43.0</td>
<td>1.42</td>
<td>0.571</td>
<td>1.9</td>
</tr>
<tr>
<td>11</td>
<td>BC1</td>
<td>57.8</td>
<td>72.0</td>
<td>1.22</td>
<td>0.785</td>
<td>2.0</td>
</tr>
<tr>
<td>12</td>
<td>BC1</td>
<td>21.7</td>
<td>34.6</td>
<td>1.24</td>
<td>0.660</td>
<td>1.6</td>
</tr>
<tr>
<td>13</td>
<td>BC2</td>
<td>46.4</td>
<td>63.3</td>
<td>1.84</td>
<td>0.702</td>
<td>2.9</td>
</tr>
<tr>
<td>14</td>
<td>BC2</td>
<td>48.4</td>
<td>59.1</td>
<td>1.45</td>
<td>0.826</td>
<td>2.2</td>
</tr>
<tr>
<td>15</td>
<td>BC2</td>
<td>32.0</td>
<td>46.3</td>
<td>1.90</td>
<td>0.693</td>
<td>2.4</td>
</tr>
<tr>
<td>16</td>
<td>BC2</td>
<td>49.0</td>
<td>71.2</td>
<td>2.02</td>
<td>0.677</td>
<td>2.4</td>
</tr>
<tr>
<td>17</td>
<td>BC2</td>
<td>54.8</td>
<td>70.3</td>
<td>1.49</td>
<td>0.732</td>
<td>2.8</td>
</tr>
<tr>
<td>18</td>
<td>BC2</td>
<td>64.8</td>
<td>78.8</td>
<td>2.06</td>
<td>0.807</td>
<td>3.0</td>
</tr>
<tr>
<td>19</td>
<td>White clover</td>
<td>69.4</td>
<td>90.1</td>
<td>2.51</td>
<td>0.773</td>
<td>3.6</td>
</tr>
<tr>
<td>20</td>
<td>White clover</td>
<td>83.5</td>
<td>101.8</td>
<td>1.81</td>
<td>0.817</td>
<td>3.6</td>
</tr>
<tr>
<td>21</td>
<td>White clover</td>
<td>66.4</td>
<td>85.0</td>
<td>2.13</td>
<td>0.817</td>
<td>3.7</td>
</tr>
<tr>
<td>22</td>
<td>White clover</td>
<td>63.9</td>
<td>81.4</td>
<td>3.25</td>
<td>0.779</td>
<td>3.8</td>
</tr>
<tr>
<td>23</td>
<td>White clover</td>
<td>95.4</td>
<td>109.4</td>
<td>2.19</td>
<td>0.896</td>
<td>4.0</td>
</tr>
<tr>
<td>24</td>
<td>White clover</td>
<td>55.1</td>
<td>84.1</td>
<td>2.35</td>
<td>0.654</td>
<td>3.4</td>
</tr>
<tr>
<td>25</td>
<td>White clover</td>
<td>114.7</td>
<td>133.3</td>
<td>1.87</td>
<td>0.857</td>
<td>3.9</td>
</tr>
<tr>
<td>26</td>
<td>White clover</td>
<td>72.5</td>
<td>91.6</td>
<td>2.13</td>
<td>0.782</td>
<td>3.8</td>
</tr>
</tbody>
</table>
Appendix 5. Relative growth parameters for hybrid families, compared with their white clover parental cultivars. Values for dry matter scores are averaged over all harvests; stolon density, growth habit and fungal disease were scored in May 2009.

<table>
<thead>
<tr>
<th>Entry number</th>
<th>Clover type</th>
<th>Dry matter score (%)</th>
<th>Lateral spread (%)</th>
<th>Density (%)</th>
<th>Growth habit (%)</th>
<th>Disease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>May 2009</td>
<td>July 2009</td>
<td>August 2009</td>
<td>November 2009</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>BC1</td>
<td>76</td>
<td>74</td>
<td>74</td>
<td>89</td>
<td>99</td>
</tr>
<tr>
<td>4</td>
<td>BC1</td>
<td>71</td>
<td>77</td>
<td>67</td>
<td>82</td>
<td>83</td>
</tr>
<tr>
<td>5</td>
<td>BC1</td>
<td>80</td>
<td>71</td>
<td>63</td>
<td>76</td>
<td>77</td>
</tr>
<tr>
<td>6</td>
<td>BC1</td>
<td>78</td>
<td>82</td>
<td>73</td>
<td>103</td>
<td>68</td>
</tr>
<tr>
<td>7</td>
<td>BC1</td>
<td>71</td>
<td>70</td>
<td>56</td>
<td>72</td>
<td>61</td>
</tr>
<tr>
<td>8</td>
<td>BC1</td>
<td>90</td>
<td>101</td>
<td>95</td>
<td>106</td>
<td>84</td>
</tr>
<tr>
<td>9</td>
<td>BC1</td>
<td>92</td>
<td>108</td>
<td>99</td>
<td>104</td>
<td>139</td>
</tr>
<tr>
<td>10</td>
<td>BC1</td>
<td>88</td>
<td>97</td>
<td>90</td>
<td>98</td>
<td>90</td>
</tr>
<tr>
<td>11</td>
<td>BC1</td>
<td>83</td>
<td>76</td>
<td>68</td>
<td>73</td>
<td>64</td>
</tr>
<tr>
<td>12</td>
<td>BC1</td>
<td>83</td>
<td>87</td>
<td>77</td>
<td>101</td>
<td>83</td>
</tr>
<tr>
<td>13</td>
<td>BC2</td>
<td>78</td>
<td>51</td>
<td>51</td>
<td>55</td>
<td>67</td>
</tr>
<tr>
<td>14</td>
<td>BC2</td>
<td>89</td>
<td>97</td>
<td>88</td>
<td>103</td>
<td>109</td>
</tr>
</tbody>
</table>

A.3 Flowering score and height – variability of clover types

Appendix 6. Standard deviations for flowering scores of the five clover types in January 2009, and inflorescence height relative to the canopy in January 2010. Clover types with the same letter show no significant differences in variability at the 5% level.

<table>
<thead>
<tr>
<th>Clover type</th>
<th>Flowering score</th>
<th>Height relative to the canopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. uniflorum</td>
<td>1.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>BC1</td>
<td>1.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.96&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC2</td>
<td>1.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>White clover</td>
<td>1.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Red clover</td>
<td>1.09&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>NA</td>
</tr>
</tbody>
</table>
A.4 Above-ground fragmentation

There were no differences in the above-ground fragmentation of BC₁, BC₂, and white clover (Appendix 7), although there was a trend towards less fragmentation in BC₁ compared with BC₂ at 13 months old ($P=0.071$). *T. uniflorum* was more intact ($P<0.001$) than the hybrids and white clover at 16 months old, and also had a trend towards being more intact than BC₁ ($P<0.001$) and BC₂ and white clover ($P=0.059$) at 19–20 months old. Fragmentation of the hybrids and white clover increased from the first sampling date, whereas that of *T. uniflorum* stayed relatively constant until the final harvest.

Appendix 7. Mean scores for above-ground fragmentation (±SEM) of the five clover types, in 13 (October 2009), 16 (January 2010) and 19–20 month old plants (April/May 2010) (see Table 3.3 for details of the scoring system). Means with the same letter within sampling times show no significant differences at the 5% level.
A.5 Tap root measurements

Red clover had the greatest mean dry weight (DW) for intact tap roots (condition scores of 1) of the five clover types, which was 2.9 times larger ($P=0.033$) than that of white clover (Appendix 8). There were also trends towards greater tap root DW in red clover (2.2 times larger) compared with BC$_2$ ($P=0.093$) and _T. uniflorum_ ($P=0.066$), but there were no differences among the hybrids and parents.

Appendix 8. Mean tap root dry weight (top 100 mm, including laterals) (±SEM) of the five clover types, in 13 month old plants (October 2009). Means with the same letters show no significant differences at the 5% level.

The mean diameter of lateral roots (>1 mm in diameter) arising from the top 20 mm of the tap root was 26–41% higher ($P<0.05$) in red clover (Appendix 9A), but its lateral root numbers were the same as the other clover types (Appendix 9B). Lateral roots of _T. uniflorum_ and BC$_1$ were slightly thicker than those of BC$_2$ and white clover but there were no significant differences between the hybrids and parents. However, BC$_1$ had 47% more ($P=0.015$) lateral roots than the _T. uniflorum_ parent and 67% more ($P=0.016$) than the white clover parent (Appendix 9B).
Appendix 9. Back-transformed mean diameter (± estimated SEM) (A) and mean number (±SEM) (B) of lateral roots larger than 1 mm in diameter, arising from the top 20 mm of intact healthy tap roots for the five clover types, in 13 month old plants (October 2009). Means with the same letter show no significant differences at the 5% level.

A.6 Nitrogen fixation – variability of clover types

Variability in the proportion of N from fixation was higher ($P<0.05$) in BC$_1$ than in BC$_2$ and white clover in all months (Appendix 10). All other differences were not significant, but red clover tended to be more variable than BC$_2$ ($P=0.08$) in October 2009.

For %N content, the only difference in variability in October 2009 was between white clover and BC$_2$ ($P=0.043$) (Appendix 11), and there were no differences among clover types in November 2009. Variability for %N content did not differ among the hybrids and white
clover in January 2010, but all three clover types were less variable than *T. uniflorum* (*P*<0.004). There were also trends towards lower variability of %N for BC2 (*P*=0.07) and white clover (*P*=0.097) compared with red clover in January.

**Appendix 10. Standard deviations for the proportion of N from fixation in shoots of the five clover types, sampled in October 2009, November 2009 and January 2010. Clover types with the same letter within sampling dates show no significant differences in variability at the 5% level.**

<table>
<thead>
<tr>
<th>Clover type</th>
<th>October (%)</th>
<th>November (%)</th>
<th>January (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. uniflorum</em></td>
<td>6.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.72&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.90&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC1</td>
<td>7.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.22&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC2</td>
<td>4.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>White clover</td>
<td>4.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Red clover</td>
<td>7.57&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.03&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Appendix 11. Standard deviations for %N content of shoots of the five clover types, sampled in October 2009, November 2009 and January 2010. Clover types with the same letter within sampling dates show no significant differences in variability at the 5% level.**

<table>
<thead>
<tr>
<th>Clover type</th>
<th>October 2009 (%N)</th>
<th>November 2009 (%N)</th>
<th>January 2010 (%N)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. uniflorum</em></td>
<td>0.521&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.614&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.109&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC1</td>
<td>0.460&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.490&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.283&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC2</td>
<td>0.352&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.469&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.302&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>White clover</td>
<td>0.522&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.399&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.328&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Red clover</td>
<td>0.483&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.673&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.668&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
A.7 Changes in lateral spread

The lateral spread of all clover types decreased from May to July 2009 (Appendix 12), particularly for red clover (-88 mm), where the decrease was larger than that of *T. uniflorum* (-7 mm) (*P*=0.016), BC1 (-32 mm) (*P*=0.054) and BC2 (-20 mm) (*P*=0.02). From July to August 2009 the spread of most clover types increased considerably, with the change in white clover (+116 mm) being greater than that of *T. uniflorum* (+3 mm) (*P*<0.001), BC1 (+66 mm) (*P*=0.001), BC2 (+67 mm) (*P*=0.004) and red clover (+33 mm) (*P*=0.018) (Appendix 12). Between August and November 2009, the increase in the lateral spread of white clover (+71 mm) was greater (*P*<0.001) than the increase of BC2 (+8 mm) (*P*=0.014) and the decrease of BC1 (-42 mm) (Appendix 12). The decreasing spread of BC1 at this time, also differed to the slight increase of BC2 (*P*=0.054).

Appendix 12. Change in maximum lateral spread of the five clover types between measuring intervals (±SEM). Means with the same letter within time intervals show no significant differences at the 5% level.
A.8 Chlorophyll index measurements

Chlorophyll content did not differ among the five clover types in March 2009, based on the mean SPAD values (Appendix 13). However, in November 2009 the mean SPAD value for *T. uniflorum* was lower than those for BC1 (*P*=0.003), BC2 (*P*=0.035) and white clover (*P*=0.003). Standard deviations calculated on all combined data also showed that the variability in leaf greenness for *T. uniflorum* was higher (*P*<0.001) than that of the latter three clover types (Appendix 14).

![Bar chart showing mean SPAD measurements for different clover types in March and November 2009.](chart.png)

**Appendix 13.** Mean SPAD values (±SEM) of the five clover types measured in March and November 2009. Means with the same letter within sampling dates show no significant differences at the 5% level.

**Appendix 14.** Overall standard deviations for SPAD values of the five clover types, measured. Clover types with the same letter show no significant differences in variability at the 5% level.
Appendix B
Nutrient solution recipes

B.1 Low ionic strength solution

Appendix 15. Composition of the low ionic strength nutrient solution (Edmeades et al., 1985; Blamey et al., 1991).

<table>
<thead>
<tr>
<th></th>
<th>Stock solution g l(^{-1})</th>
<th>ml stock solution for 135 l nutrient solution</th>
<th>ml stock solution for 45 l nutrient solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH(_4)NO(_3)</td>
<td>54</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>136.48</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>MgSO(_4).7H(_2)O</td>
<td>110.88</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>NH(_4)H(_2)PO(_4)</td>
<td>4.2</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>NaCl</td>
<td>52.2</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td><strong>Micronutrients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe-EDTA</td>
<td>0.367</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Dilute to 200 ml l(^{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trace elements</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H(_3)BO(_3)</td>
<td>1.668</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>ZnSO(_4).7H(_2)O</td>
<td>1.294</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnSO(_4).4H(_2)O</td>
<td>1.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuSO(_4).5H(_2)O</td>
<td>0.220</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NH(_4))(_6)Mo(_7)O(_24).4H(_2)O</td>
<td>0.020</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaSO(_4).2H(_2)O</td>
<td>–</td>
<td>10.32 g</td>
<td>3.44 g</td>
</tr>
</tbody>
</table>
Appendix 16. Concentration of ions in the low ionic strength nutrient solution (Care, 1999).

<table>
<thead>
<tr>
<th>Ions</th>
<th>Concentration in basal nutrient solution (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$</td>
<td>500</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>100</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>10</td>
</tr>
<tr>
<td>K$^+$</td>
<td>300</td>
</tr>
<tr>
<td>NO$_3$-N</td>
<td>450</td>
</tr>
<tr>
<td>NH$_4$-N</td>
<td>150</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>600</td>
</tr>
<tr>
<td>PO$_4^{3-}$</td>
<td>3</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>5</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>6</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>1</td>
</tr>
<tr>
<td>BO$_4^{3-}$</td>
<td>1</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>0.1</td>
</tr>
</tbody>
</table>
B.2 Complete nutrient solution


<table>
<thead>
<tr>
<th></th>
<th>Stock solution g l(^{-1})</th>
<th>Nutrient solution ml stock solution l(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>40.4</td>
<td>2</td>
</tr>
<tr>
<td>Ca(NO(_3))(_2)</td>
<td>65.6</td>
<td>2</td>
</tr>
<tr>
<td>MgSO(_4).7H(_2)O</td>
<td>36.8</td>
<td>2</td>
</tr>
<tr>
<td>NaH(_2)PO(_4).2H(_2)O</td>
<td>20.8</td>
<td>1</td>
</tr>
<tr>
<td>NaNO(_3)</td>
<td>68.0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Micronutrients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA FeNa</td>
<td>1.84</td>
<td>0.5</td>
</tr>
<tr>
<td>Trace elements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnSO(_4).4H(_2)O</td>
<td>0.223</td>
<td>0.1</td>
</tr>
<tr>
<td>CuSO(_4).5H(_2)O</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>ZnSO(_4).7H(_2)O</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>H(_3)BO(_3)</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>(NH(_4))(_6)Mo(_7)O(_24).4H(_2)O</td>
<td>0.0088</td>
<td></td>
</tr>
</tbody>
</table>
Appendix C
Root depth distribution and associated traits

C.1 Differences among families - change in root length density and specific root length

As with the six broad clover entries, root length density (RLD) decreased with depth in both families, but the proportion at 400–500 mm was higher (by 2.7 times) for the 900-4 family (Appendix 18). There were no differences between the families in the decrease of specific root length (SRL) with depth (Appendix 18).

Appendix 18. Proportion of root length density and specific root length (±SEM), with P values, at 400–500 mm compared with 50–100 mm for the 80-2 and 900-4 families at harvest 3.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>80-2</th>
<th>900-4</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root length density (%)</td>
<td>1.57 ±0.757</td>
<td>4.28 ±0.718</td>
<td>0.019</td>
</tr>
<tr>
<td>Specific root length (%)</td>
<td>68.6 ±12.62</td>
<td>88.2 ±11.98</td>
<td>0.274</td>
</tr>
</tbody>
</table>
Appendix D

Nutrient effects on growth

D.1 Dry weight back-transformed means


<table>
<thead>
<tr>
<th>Clover entry</th>
<th>Shoot dry weight (g)</th>
<th>Root dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complete</td>
<td>LIS</td>
</tr>
<tr>
<td>Kopu II</td>
<td>2.850</td>
<td>0.256</td>
</tr>
<tr>
<td>Kopu II x 487-9</td>
<td>2.280</td>
<td>0.785</td>
</tr>
<tr>
<td>Kopu II x 900-4</td>
<td>2.853</td>
<td>1.360</td>
</tr>
<tr>
<td>Crusader x 900-4</td>
<td>1.958</td>
<td>0.352</td>
</tr>
</tbody>
</table>

D.2 Phosphorus and inorganic phosphorus

Appendix 20. Back-transformed means for P use-efficiency and P\textsubscript{i} use-efficiency (dry weight per unit P and P\textsubscript{i}) for clover entries in the subset of plants sampled for shoot elemental analyses in the Complete, LIS and ½ LIS nutrient treatments (note different dry weight units to logged values in Figure 5.10). LIS = low ionic strength.

<table>
<thead>
<tr>
<th>Clover entry</th>
<th>mg DW mg\textsuperscript{-1} kg\textsuperscript{-1} P</th>
<th>mg DW mg\textsuperscript{-1} kg\textsuperscript{-1} P\textsubscript{i}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complete</td>
<td>LIS</td>
</tr>
<tr>
<td>Kopu II</td>
<td>2.48</td>
<td>0.24</td>
</tr>
<tr>
<td>Kopu II x 487-9</td>
<td>1.38</td>
<td>0.55</td>
</tr>
<tr>
<td>Kopu II x 900-4</td>
<td>2.46</td>
<td>1.29</td>
</tr>
<tr>
<td>Crusader x 900-4</td>
<td>1.36</td>
<td>0.37</td>
</tr>
</tbody>
</table>
Appendix 21. Standard deviations for P related data in the Complete, LIS and ½ LIS treatments, for clover entries in the subset of plants sampled for shoot elemental analyses. LIS = low ionic strength. Lower case letters indicate differences among Kopu II related clover entries; upper case letters indicate differences between Kopu II x 900-4 and Crusader x 900-4. Clover entries with the same letter show no significant differences in variability at the 5% level. \( P_i \) = leaf inorganic P; Fraction \( P_i = P_i/\text{Total P} \); †mg DW mg\(^{-1}\) kg\(^{-1}\), note different dry weight units to logged means for P use-efficiency and \( P_i \) use-efficiency (Figure 5.10).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Parameter</th>
<th>Kopu II</th>
<th>Kopu II x 487-9</th>
<th>Kopu II x 900-4</th>
<th>Crusader x 900-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>( P ) (mg kg(^{-1}))</td>
<td>195.7(^{ab})</td>
<td>326.7(^{a})</td>
<td>148.3(^{b,A})</td>
<td>141.3(^{A})</td>
</tr>
<tr>
<td></td>
<td>( P_i ) (mg kg(^{-1}))</td>
<td>145.2(^{a})</td>
<td>125.7(^{a})</td>
<td>126.0(^{a,A})</td>
<td>142.4(^{A})</td>
</tr>
<tr>
<td></td>
<td>Fraction ( P_i ) (%)</td>
<td>7.3(^{a})</td>
<td>9.9(^{a})</td>
<td>10.7(^{a,A})</td>
<td>10.0(^{A})</td>
</tr>
<tr>
<td></td>
<td>( P ) use efficiency†</td>
<td>1.81(^{a})</td>
<td>1.21(^{a})</td>
<td>1.80(^{a,A})</td>
<td>0.99(^{A})</td>
</tr>
<tr>
<td></td>
<td>( P_i ) use efficiency†</td>
<td>3.58(^{a})</td>
<td>2.05(^{a})</td>
<td>7.95(^{b,A})</td>
<td>1.77(^{B})</td>
</tr>
<tr>
<td>LIS</td>
<td>( P ) (mg kg(^{-1}))</td>
<td>327.1(^{a})</td>
<td>183.3(^{ab})</td>
<td>154.0(^{b,A})</td>
<td>218.6(^{A})</td>
</tr>
<tr>
<td></td>
<td>( P_i ) (mg kg(^{-1}))</td>
<td>150.1(^{a})</td>
<td>129.3(^{a})</td>
<td>125.4(^{a,A})</td>
<td>199.4(^{A})</td>
</tr>
<tr>
<td></td>
<td>Fraction ( P_i ) (%)</td>
<td>19.5(^{a})</td>
<td>6.3(^{b})</td>
<td>10.9(^{a,A})</td>
<td>22.7(^{B})</td>
</tr>
<tr>
<td></td>
<td>( P ) use efficiency†</td>
<td>0.17(^{a})</td>
<td>0.46(^{b})</td>
<td>1.44(^{c,A})</td>
<td>0.24(^{B})</td>
</tr>
<tr>
<td></td>
<td>( P_i ) use efficiency†</td>
<td>0.19(^{a})</td>
<td>1.05(^{b})</td>
<td>2.82(^{2,A})</td>
<td>0.53(^{B})</td>
</tr>
<tr>
<td>½ LIS</td>
<td>( P ) (mg kg(^{-1}))</td>
<td>174.4(^{a})</td>
<td>191.0(^{a})</td>
<td>110.4(^{a,A})</td>
<td>240.5(^{B})</td>
</tr>
<tr>
<td></td>
<td>( P_i ) (mg kg(^{-1}))</td>
<td>161.6(^{a})</td>
<td>187.9(^{a})</td>
<td>116.6(^{a,A})</td>
<td>135.3(^{a,A})</td>
</tr>
<tr>
<td></td>
<td>Fraction ( P_i ) (%)</td>
<td>15.1(^{a})</td>
<td>13.1(^{a})</td>
<td>11.8(^{a,A})</td>
<td>10.6(^{A})</td>
</tr>
<tr>
<td></td>
<td>( P ) use efficiency†</td>
<td>0.43(^{a})</td>
<td>0.37(^{a})</td>
<td>1.22(^{B,A})</td>
<td>1.05(^{A})</td>
</tr>
<tr>
<td></td>
<td>( P_i ) use efficiency†</td>
<td>0.86(^{a})</td>
<td>0.65(^{a})</td>
<td>3.74(^{2,A})</td>
<td>1.86(^{A})</td>
</tr>
</tbody>
</table>
Appendix E
Root system structure

E.1 Dry weight data

Appendix 22. Mean root dry weight, shoot dry weight and root:shoot ratio for clover entries in Kopu II and Crusader based groups, at week 6. Means with the same letter, within clover groups, show no significant differences at the 5% level.

<table>
<thead>
<tr>
<th></th>
<th>Root dry weight (mg)</th>
<th>Shoot dry weight (mg)</th>
<th>Root:shoot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kopu II</td>
<td>39.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>104.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.404&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kopu II x 80-2</td>
<td>30.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.356&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kopu II x 900-4</td>
<td>25.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3418&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>T. uniflorum</em></td>
<td>8.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3189&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P</em> value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.121</td>
</tr>
<tr>
<td>Crusader</td>
<td>36.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>101.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.3645&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Crusader x 80-2</td>
<td>29.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3643&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Crusader x 900-4</td>
<td>36.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>107.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3451&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>T. uniflorum</em></td>
<td>8.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3189&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P</em> value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.121</td>
</tr>
</tbody>
</table>
Appendix 23. Mean total root length (A and B), total surface area (C and D), and number of root tips (E and F) for clover entries in Kopu II and Crusader based groups, from week 0 to week 6. Statistical comparisons were made at week 4 and 6. Means with the same letter, within times, show no significant differences at the 5% level.
Appendix 24. Mean number of links (A and B), altitude (C and D), external path length (E and F) and %EI links (G and H) for clover entries in Kopu II (A, C, E, G) and Crusader (B, D, F, H) based groups, from week 0 to week 4. Statistical comparisons were made at week 3 and 4. Means with the same letter, within times, show no significant differences at the 5% level.
Appendix 25. Mean (±SEM) length (A and B), surface area (C and D) and average diameter (E and F) of base links (BL) for clover entries the Kopu II (A, C, E) and Crusader (B, D, F) based groups at week 4. Means with the same letter show no significant differences at the 5% level.
Appendix 26. Mean (±SEM) length (A and B), surface area (C and D) and average diameter (E and F) of external-external (EE) links for clover entries in Kopu II (A, C, E) and Crusader (B, D, F) based groups at week 4. Means with the same letter show no significant differences at the 5% level.
Appendix 27. Mean (±SEM) length (A and B), surface area (C and D) and average diameter (E and F) of external-internal (EI) links for clover entries in Kopu II (A, C, E) and Crusader (B, D, F) based groups at week 4. Means with the same letter show no significant differences at the 5% level.
Appendix 28. Mean (±SEM) length (A and B), surface area (C and D), and average link diameter (E and F) of axis order 0 (tap root) for clover entries in Kopu II (A, C, E) and Crusader (B, D, F) groups, at week 4. Means with the same letter show no significant differences at the 5% level.
Appendix 29. Mean (±SEM) length (A and B), surface area (C and D), average link diameter (E and F), and number (G and H) of 1st order (primary) lateral roots for clover entries in Kopu II (A, C, E, G) and Crusader (B, D, F, H) groups, at week 4. Means with the same letter show no significant differences at the 5% level.
Appendix 30. Mean (±SEM) length (A and B), surface area (C and D), average link diameter (E and F), and number (G and H) of 2nd order (secondary) lateral roots for clover entries in Kopu II (A, C, E, G) and Crusader (B, D, F, H) groups, at week 4. Means with the same letter show no significant differences at the 5% level.
Appendix 31. Mean (±SEM) length (A and B), surface area (C and D), average link diameter (E and F), and number (G and H) of 3rd order (tertiary) lateral roots for clover entries in Kopu II (A, C, E, G) and Crusader (B, D, F, H) groups, at week 4. Means with the same letter show no significant differences at the 5% level.
Appendix F

Morphological and growth responses to water stress

F.1 Senescence score examples

Appendix 32. Examples of senescence scores from 1 (minimal) to 10 (whole plant dead)
F.2 Total shoot dry weight – variability of clover types

There were no differences in variability for shoot DW among clover types in either watering treatment (Appendix 33), but there were trends towards lower variability for the BC₁ generation than for the BC₂ generation in the Watered treatment \((P=0.064)\), and higher variability in the Stressed treatment \((P=0.092)\).

**Appendix 33. Standard deviations for shoot dry weight of BC₁, BC₂ and white clover in the Watered and Stressed treatments. Clover types with the same letter, within watering treatments, show no significant differences in variability at the 5% level.**

<table>
<thead>
<tr>
<th>Clover type</th>
<th>Watered (g)</th>
<th>Stressed (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC₁</td>
<td>119.0(^a)</td>
<td>66.9 (^a)</td>
</tr>
<tr>
<td>BC₂</td>
<td>165.9 (^a)</td>
<td>48.0 (^a)</td>
</tr>
<tr>
<td>White clover</td>
<td>128.6 (^a)</td>
<td>58.5 (^a)</td>
</tr>
</tbody>
</table>

In the Kopu II subset, the variability for shoot DW of Kopu II BC₁ was lower than Kopu II BC₂ in the Watered treatment, and lower than Kopu II in the Stressed treatment (Appendix 34). The variability of Kopu II and Kopu II BC₂ did not differ in either treatment.

**Appendix 34. Standard deviations for shoot dry weight of Kopu II BC₁, Kopu II BC₂ and Kopu II in the Watered and Stressed treatments. Clover entries with the same letter, within watering treatments, show no significant differences in variability at the 5% level.**

<table>
<thead>
<tr>
<th>Clover entry</th>
<th>Watered (g)</th>
<th>Stressed (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kopu II BC₁</td>
<td>67.9 (^a)</td>
<td>27.1 (^a)</td>
</tr>
<tr>
<td>Kopu II BC₂</td>
<td>202.5 (^b)</td>
<td>32.3 (^{ab})</td>
</tr>
<tr>
<td>Kopu II</td>
<td>119.3 (^{ab})</td>
<td>76.9 (^b)</td>
</tr>
</tbody>
</table>
Appendix 35. Watering treatment effects and clover type x watering treatment interactions for mean shoot dry matter scores (±SEM) of BC1, BC2 and white clover in the Watered and Stressed treatments on 10 November 2009 (A), 14 December 2009 (B), 9 January 2010 (C), 1 February 2010 (D), 8 March 2010 (E) and 23 March 2010 (F).
F.4 Correlations with changes in shoot DW

Appendix 36. Relationship between internode length in the Watered treatment and changes in shoot dry weight under water stress, in the Kopu II subset.

Appendix 37. Relationship between the mean number of totally deflexed inflorescences in the Watered treatment and changes in shoot dry weight under water stress, in the Kopu II subset.
Appendix 38. Relationship between the mean number of inflorescences in flowering category 3 in the Watered treatment and changes in shoot dry weight under water stress, in the Kopu II subset.

F.5 Senescence – variability of clover types

The senescence scores of the BC₁ generation were less variable than the BC₂ generation in the Watered treatment, and less variable than both BC₂ and white clover in the Stressed treatment (Appendix 39).

Appendix 39. Standard deviations for senescence scores of BC₁, BC₂ and white clover in the Watered and Stressed treatments on 23 March 2010. Clover types with the same letter, within watering treatments, show no significant differences in variability at the 5% level.

<table>
<thead>
<tr>
<th>Clover type</th>
<th>Watered</th>
<th>Stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC₁</td>
<td>0.55ᵃ</td>
<td>1.31ᵃ</td>
</tr>
<tr>
<td>BC₂</td>
<td>2.12ᵇ</td>
<td>2.32ᵇ</td>
</tr>
<tr>
<td>White clover</td>
<td>0.37ᶜ</td>
<td>2.33ᵇ</td>
</tr>
</tbody>
</table>
Appendix G
Physiological and biochemical responses to water stress

G.1 Water potential

Appendix 40. Mean water potential (±SEM) of Kopu II BC₁, Kopu II BC₂ and Kopu II in the Watered and Stressed treatments, on 10 February (A), 25 February (B), 4 March (C), 12 March (D) and 19 March 2010 (E).

The difference in Ψ between Kopu II BC₁ and Kopu II tended towards significance on 4 March in the Stressed treatment (P=0.056) (Appendix 40C), otherwise there were no differences among clover entries until the end of the experiment. On 19 March, the mean Ψ of Kopu II BC₁ in the Stressed treatment (-23.3 MPa) was lower than that of Kopu II BC₂ (-20.7...
MPa) \((P=0.027)\) and Kopu II (-18.7 MPa) \((P<0.001)\). Mean Ψ of Kopu II BC₁ in the Watered treatment was also lower (-14.8 MPa) than Kopu II (-12.6 MPa) \((P=0.051)\) at that time (Appendix 40E).

### G.2 Chlorophyll fluorescence

#### A.

![Graph A](image.png)

#### B.

![Graph B](image.png)

#### C.

![Graph C](image.png)

#### D.

![Graph D](image.png)

#### E.

![Graph E](image.png)

**Appendix 41.** Mean midday, dark-adapted chlorophyll fluorescence yield \((F_v/F_m)\) \((±SEM)\) of Kopu II BC₁, Kopu II BC₂, and Kopu II in the Watered and Stressed treatments, on 11 February (A), 27 February (B), 3 March (C), 10 March (D) and 18 March 2010 (E).

Midday chlorophyll fluorescence yield of Kopu II was lower in the Stressed treatment (0.814) than the Watered treatment (0.833) on 11 February \((P=0.037)\) (Appendix 41A), and also on 27 February \((P=0.006)\) (0.716 v 0.755) (Appendix 41B). Both Kopu II BC₁ \((P=0.002)\) and Kopu II BC₂ \((P=0.007)\) had higher mean fluorescence yields in the Stressed treatment compared with the Watered treatment on 3 March (Appendix 41C). The Stressed treatment
means were 0.824 (Kopu II BC₁) and 0.818 (Kopu II BC₂), while the Watered treatment means were 0.789 (Kopu II BC₁) and 0.786 (Kopu II BC₂). At the end of the experiment (10 and 18 March) there were no treatment differences for any of the three clover entries (Appendices 41D and 41E).

G.3 Correlation between phenolics and biomass

Appendix 42. Relationship between quercetin glycoside concentration and shoot dry weight for BC₁ (A), BC₂ (B) and white clover (C) plants in the Stressed treatment.
Appendix 43. Relationship between mean kaempferol concentration in the Watered treatment and changes in shoot DW under water stress, in the Kopu II subset.

G.4 δ\textsuperscript{13}C

Overall, there were no differences among clover types for δ\textsuperscript{13}C, but it was higher (P<0.001) in the Stressed treatment (-25.9‰) than in the Watered treatment (-28.0‰). The clover type x watering treatment interaction was also significant (P=0.020), with a smaller increase in δ\textsuperscript{13}C for the BC\textsubscript{2} generation than for the BC\textsubscript{1} generation and white clover (Appendix 44). Variability of δ\textsuperscript{13}C did not differ among clover types for either treatment (see Appendix 51).

Appendix 44. Mean δ\textsuperscript{13}C (±SEM) of BC\textsubscript{1}, BC\textsubscript{2} and white clover in the Watered and Stressed treatments, for the full data set and the Kopu II subset.

<table>
<thead>
<tr>
<th></th>
<th>BC\textsubscript{1}</th>
<th>BC\textsubscript{2}</th>
<th>White clover</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ\textsuperscript{13}C ( ‰ )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>all plants</td>
<td>Watered</td>
<td>-28.08</td>
<td>-27.74</td>
<td>-28.08</td>
</tr>
<tr>
<td></td>
<td>±0.101</td>
<td>±0.135</td>
<td>±0.118</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>-25.88</td>
<td>-26.13</td>
<td>-25.8</td>
</tr>
<tr>
<td></td>
<td>±0.102</td>
<td>±0.138</td>
<td>±0.128</td>
<td></td>
</tr>
<tr>
<td>δ\textsuperscript{13}C ( ‰ )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kopu II subset</td>
<td>Watered</td>
<td>-27.98</td>
<td>-27.29</td>
<td>-28.08</td>
</tr>
<tr>
<td></td>
<td>±0.219</td>
<td>±0.214</td>
<td>±0.185</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>-26.05</td>
<td>-25.7</td>
<td>-25.58</td>
</tr>
<tr>
<td></td>
<td>±0.355</td>
<td>±0.457</td>
<td>±0.398</td>
<td></td>
</tr>
</tbody>
</table>

Like the total plant pool, there was no overall clover entry effect for δ\textsuperscript{13}C in the Kopu II subset, but when means were compared using the LSD\textsubscript{0.05} then δ\textsuperscript{13}C was lower in Kopu II BC\textsubscript{1} (-27.02‰) than in Kopu II BC\textsubscript{2} (-26.5‰). Neither hybrid differed to white clover (-26.83‰).
There was also an overall watering treatment effect, in the Kopu II subset, for δ\(^{13}\)C (\(P<0.001\)), which was higher in the Stressed treatment (-25.8‰) than the Watered treatment (-27.8‰), but the clover entry x watering treatment interaction was not significant (Appendix 44). Within both treatments, there were no differences among clover entries for δ\(^{13}\)C. The variability of δ\(^{13}\)C also did not differ among clover entries in either watering treatment (see Appendix 51).
### G.5 Feed quality

Appendix 45. Means (±SEM) for feed quality parameters within watering treatments for BC₁, BC₂ and white clover. OM = organic matter; ADF = acid detergent fibre; NDF = neutral detergent fibre; DMD = dry matter digestibility; DOMD = digestible organic matter in dry matter; OMD = organic matter digestibility.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>BC₁</th>
<th>BC₂</th>
<th>White clover</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>%OM</td>
<td>Watered</td>
<td>90.9 ± 0.15</td>
<td>90.8 ± 0.20</td>
<td>91.0 ± 0.17</td>
<td>0.528</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>85.0 ± 0.16</td>
<td>85.4 ± 0.23</td>
<td>85.7 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>%ADF</td>
<td>Watered</td>
<td>15.8 ± 0.14</td>
<td>15.8 ± 0.18</td>
<td>15.5 ± 0.16</td>
<td>0.474</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>18.0 ± 0.14</td>
<td>18.0 ± 0.20</td>
<td>17.5 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>%NDF</td>
<td>Watered</td>
<td>20.9 ± 0.25</td>
<td>21.9 ± 0.33</td>
<td>21.6 ± 0.29</td>
<td>0.857</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>16.7 ± 0.26</td>
<td>17.7 ± 0.37</td>
<td>17.5 ± 0.34</td>
<td></td>
</tr>
<tr>
<td>%DMD</td>
<td>Watered</td>
<td>83.6 ± 0.17</td>
<td>83.7 ± 0.23</td>
<td>84.1 ± 0.20</td>
<td>0.598</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>78.9 ± 0.18</td>
<td>78.4 ± 0.26</td>
<td>78.8 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>%DOMD</td>
<td>Watered</td>
<td>81.6 ± 0.24</td>
<td>81.7 ± 0.31</td>
<td>82.2 ± 0.28</td>
<td>0.824</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>72.5 ± 0.25</td>
<td>72.5 ± 0.36</td>
<td>73.3 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>%OMD</td>
<td>Watered</td>
<td>89.0 ± 0.20</td>
<td>89.1 ± 0.27</td>
<td>89.5 ± 0.23</td>
<td>0.702</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>84.9 ± 0.21</td>
<td>84.3 ± 0.30</td>
<td>84.9 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>%Ash</td>
<td>Watered</td>
<td>9.1 ± 0.15</td>
<td>9.20 ± 0.20</td>
<td>9.0 ± 0.17</td>
<td>0.528</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>15.0 ± 0.16</td>
<td>14.6 ± 0.23</td>
<td>14.3 ± 0.21</td>
<td></td>
</tr>
</tbody>
</table>
### Appendix 46. Main effects ($P$ values) for feed quality parameters in the Kopu II subset.

OM = organic matter; ADF = acid detergent fibre; NDF = neutral detergent fibre; DMD = dry matter digestibility; DOMD = digestible organic matter in dry matter; OMD = organic matter digestibility; CHO = carbohydrate.

<table>
<thead>
<tr>
<th></th>
<th>%OM</th>
<th>%ADF</th>
<th>%NDF</th>
<th>%DMD</th>
<th>%DOMD</th>
<th>%OMD</th>
<th>%CHO</th>
<th>%Protein</th>
<th>%Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clover type</td>
<td>0.239</td>
<td>0.315</td>
<td>0.001</td>
<td>0.063</td>
<td>0.008</td>
<td>0.042</td>
<td>0.915</td>
<td>0.514</td>
<td>0.239</td>
</tr>
<tr>
<td>Watering treatment</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.113</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Type x Treatment</td>
<td>0.484</td>
<td>0.484</td>
<td>0.303</td>
<td>0.104</td>
<td>0.878</td>
<td>0.177</td>
<td>0.125</td>
<td>0.002</td>
<td>0.484</td>
</tr>
</tbody>
</table>

### Appendix 47. Clover entry means (±SEM) for feed quality parameters in the Kopu II subset, where there were no clover entry x watering treatment interactions.

OM = organic matter; ADF = acid detergent fibre; NDF = neutral detergent fibre; DMD = dry matter digestibility; DOMD = digestible organic matter in dry matter; OMD = organic matter digestibility; CHO = carbohydrate.

<table>
<thead>
<tr>
<th>Clover entry</th>
<th>%OM</th>
<th>%ADF</th>
<th>%NDF</th>
<th>%DMD</th>
<th>%DOMD</th>
<th>%OMD</th>
<th>%CHO</th>
<th>%Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kopu II BC₁</td>
<td>88.0</td>
<td>16.4</td>
<td>17.5</td>
<td>81.2</td>
<td>76.8</td>
<td>86.9</td>
<td>15.7</td>
<td>12.0</td>
</tr>
<tr>
<td>±0.97</td>
<td>±0.40</td>
<td>±0.85</td>
<td>±0.78</td>
<td>±1.55</td>
<td>±0.74</td>
<td>±0.785</td>
<td>±0.97</td>
<td></td>
</tr>
<tr>
<td>Kopu II BC₂</td>
<td>88.3</td>
<td>16.8</td>
<td>19.9</td>
<td>81.3</td>
<td>77.7</td>
<td>87.0</td>
<td>16.1</td>
<td>11.7</td>
</tr>
<tr>
<td>±0.84</td>
<td>±0.49</td>
<td>±0.71</td>
<td>±0.95</td>
<td>±1.51</td>
<td>±0.90</td>
<td>±0.785</td>
<td>±0.84</td>
<td></td>
</tr>
<tr>
<td>Kopu II</td>
<td>88.5</td>
<td>16.3</td>
<td>19.3</td>
<td>82.0</td>
<td>78.5</td>
<td>87.9</td>
<td>16.1</td>
<td>11.5</td>
</tr>
<tr>
<td>±0.81</td>
<td>±0.39</td>
<td>±0.83</td>
<td>±0.97</td>
<td>±1.47</td>
<td>±0.90</td>
<td>±0.785</td>
<td>±0.81</td>
<td></td>
</tr>
<tr>
<td>LSD</td>
<td>0.546</td>
<td>0.727</td>
<td>1.197</td>
<td>0.711</td>
<td>1.029</td>
<td>0.807</td>
<td>2.51</td>
<td>0.543</td>
</tr>
</tbody>
</table>

### Appendix 48. Watering treatment means (±SEM) for feed quality parameters in the Kopu II subset, where there were no clover entry x watering treatment interactions.

OM = organic matter; ADF = acid detergent fibre; NDF = neutral detergent fibre; DMD = dry matter digestibility; DOMD = digestible organic matter in dry matter; OMD = organic matter digestibility; CHO = carbohydrate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%OM</th>
<th>%ADF</th>
<th>%NDF</th>
<th>%DMD</th>
<th>%DOMD</th>
<th>%OMD</th>
<th>%CHO</th>
<th>%Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Watered</td>
<td>90.9</td>
<td>15.4</td>
<td>21.0</td>
<td>84.1</td>
<td>82.3</td>
<td>89.7</td>
<td>16.8</td>
<td>9.1</td>
</tr>
<tr>
<td>±0.07</td>
<td>±0.20</td>
<td>±0.39</td>
<td>±0.30</td>
<td>±0.33</td>
<td>±0.32</td>
<td>±0.641</td>
<td>±0.07</td>
<td></td>
</tr>
<tr>
<td>Stressed</td>
<td>85.6</td>
<td>17.6</td>
<td>16.8</td>
<td>78.9</td>
<td>73.0</td>
<td>84.9</td>
<td>15.2</td>
<td>14.4</td>
</tr>
<tr>
<td>±0.28</td>
<td>±0.25</td>
<td>±0.51</td>
<td>±0.32</td>
<td>±0.52</td>
<td>±0.37</td>
<td>±0.641</td>
<td>±0.28</td>
<td></td>
</tr>
<tr>
<td>LSD</td>
<td>0.446</td>
<td>0.594</td>
<td>0.977</td>
<td>0.584</td>
<td>0.840</td>
<td>0.698</td>
<td>2.050</td>
<td>0.446</td>
</tr>
</tbody>
</table>
Appendix 49. Clover entry means (±SEM) for feed quality parameters within watering treatments for Kopu II BC1, Kopu II BC2 and Kopu II. OM = organic matter; ADF = acid detergent fibre; NDF = neutral detergent fibre; DMD = dry matter digestibility; DOMD = digestible organic matter in dry matter; OMD = organic matter digestibility; CHO = carbohydrate.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Kopu II BC1</th>
<th>Kopu II BC2</th>
<th>Kopu II</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>%OM</td>
<td>Watered</td>
<td>90.9 ±0.20</td>
<td>90.9 ±0.06</td>
<td>91.0 ±0.10</td>
<td>0.772</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>85.2 ±0.73</td>
<td>85.7 ±0.37</td>
<td>85.9 ±0.36</td>
<td></td>
</tr>
<tr>
<td>%ADF</td>
<td>Watered</td>
<td>15.5 ±0.39</td>
<td>15.5 ±0.37</td>
<td>15.2 ±0.32</td>
<td>1.029</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>17.3 ±0.48</td>
<td>18.1 ±0.41</td>
<td>17.4 ±0.16</td>
<td></td>
</tr>
<tr>
<td>%NDF</td>
<td>Watered</td>
<td>20.1 ±0.49</td>
<td>21.6 ±0.66</td>
<td>21.2 ±0.78</td>
<td>1.693</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>14.9 ±0.49</td>
<td>18.1 ±0.60</td>
<td>17.4 ±0.90</td>
<td></td>
</tr>
<tr>
<td>%DMD</td>
<td>Watered</td>
<td>83.4 ±0.66</td>
<td>84.2 ±0.32</td>
<td>84.8 ±0.43</td>
<td>1.006</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>79.0 ±0.55</td>
<td>78.4 ±0.55</td>
<td>79.2 ±0.62</td>
<td></td>
</tr>
<tr>
<td>%DOMD</td>
<td>Watered</td>
<td>81.5 ±0.74</td>
<td>82.4 ±0.36</td>
<td>83.0 ±0.40</td>
<td>1.455</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>72.1 ±1.07</td>
<td>72.9 ±0.57</td>
<td>74.0 ±0.59</td>
<td></td>
</tr>
<tr>
<td>%OMD</td>
<td>Watered</td>
<td>88.9 ±0.69</td>
<td>89.7 ±0.31</td>
<td>90.4 ±0.53</td>
<td>1.142</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>84.9 ±0.61</td>
<td>84.3 ±0.69</td>
<td>85.4 ±0.72</td>
<td></td>
</tr>
<tr>
<td>%CHO</td>
<td>Watered</td>
<td>17.9 ±2.01</td>
<td>16.6 ±0.59</td>
<td>15.8 ±0.43</td>
<td>3.551</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>13.5 ±0.80</td>
<td>15.6 ±1.25</td>
<td>16.4 ±0.05</td>
<td></td>
</tr>
<tr>
<td>%Ash</td>
<td>Watered</td>
<td>9.1 ±0.20</td>
<td>9.1 ±0.06</td>
<td>9.0 ±0.10</td>
<td>0.772</td>
</tr>
<tr>
<td></td>
<td>Stressed</td>
<td>14.8 ±0.73</td>
<td>14.3 ±0.37</td>
<td>14.1 ±0.36</td>
<td></td>
</tr>
</tbody>
</table>
### G.6 Variability of clover types and entries

Appendix 50. Standard deviations, within watering treatments, for physiological parameters that were measured in the Kopu II subset only. Clover entries with the same letter, within watering treatments, show no differences in variability at the 5% level.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Clover entry</th>
<th>Watered</th>
<th>Stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water potential 10/02/10</td>
<td>Kopu II BC₁</td>
<td>1.79a</td>
<td>4.38a</td>
</tr>
<tr>
<td>(Ψ, MPa)</td>
<td>Kopu II BC₂</td>
<td>2.62a</td>
<td>4.37a</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>2.62a</td>
<td>3.14a</td>
</tr>
<tr>
<td>Water potential 25/02/10</td>
<td>Kopu II BC₁</td>
<td>3.88a</td>
<td>1.74a</td>
</tr>
<tr>
<td>(Ψ, MPa)</td>
<td>Kopu II BC₂</td>
<td>2.56a</td>
<td>5.89b</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>3.03a</td>
<td>3.18ab</td>
</tr>
<tr>
<td>Water potential 4/03/10</td>
<td>Kopu II BC₁</td>
<td>1.74a</td>
<td>2.34a</td>
</tr>
<tr>
<td>(Ψ, MPa)</td>
<td>Kopu II BC₂</td>
<td>2.28a</td>
<td>4.02a</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>2.09a</td>
<td>3.03a</td>
</tr>
<tr>
<td>Water potential 12/03/10</td>
<td>Kopu II BC₁</td>
<td>0.66a</td>
<td>2.50a</td>
</tr>
<tr>
<td>(Ψ, MPa)</td>
<td>Kopu II BC₂</td>
<td>1.36a</td>
<td>3.83a</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>0.66a</td>
<td>2.70a</td>
</tr>
<tr>
<td>Water potential 19/03/10</td>
<td>Kopu II BC₁</td>
<td>2.38a</td>
<td>1.866</td>
</tr>
<tr>
<td>(Ψ, MPa)</td>
<td>Kopu II BC₂</td>
<td>2.50a</td>
<td>2.61a</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>2.40a</td>
<td>3.74a</td>
</tr>
<tr>
<td>Midday Fᵥ/Fₘ 11/02/10</td>
<td>Kopu II BC₁</td>
<td>16.03a</td>
<td>14.42a</td>
</tr>
<tr>
<td></td>
<td>Kopu II BC₂</td>
<td>30.05a</td>
<td>14.79a</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>17.27a</td>
<td>20.67a</td>
</tr>
<tr>
<td>Midday Fᵥ/Fₘ 27/02/10</td>
<td>Kopu II BC₁</td>
<td>17.90a</td>
<td>33.78a</td>
</tr>
<tr>
<td></td>
<td>Kopu II BC₂</td>
<td>47.03a</td>
<td>32.81a</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>22.93a</td>
<td>65.72a</td>
</tr>
<tr>
<td>Midday Fᵥ/Fₘ 3/03/10</td>
<td>Kopu II BC₁</td>
<td>43.37a</td>
<td>12.28a</td>
</tr>
<tr>
<td></td>
<td>Kopu II BC₂</td>
<td>37.58a</td>
<td>20.35a</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>10.00b</td>
<td>22.41a</td>
</tr>
<tr>
<td>Midday Fᵥ/Fₘ 10/03/10</td>
<td>Kopu II BC₁</td>
<td>45.17a</td>
<td>37.30a</td>
</tr>
<tr>
<td></td>
<td>Kopu II BC₂</td>
<td>47.72a</td>
<td>58.12a</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>29.93a</td>
<td>36.22a</td>
</tr>
<tr>
<td>Net photosynthesis (Pᵥ, μmol m⁻² s⁻¹)</td>
<td>Kopu II BC₁</td>
<td>7.7a</td>
<td>9.4a</td>
</tr>
<tr>
<td></td>
<td>Kopu II BC₂</td>
<td>7.3a</td>
<td>5.2ab</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>1.8b</td>
<td>3.1b</td>
</tr>
<tr>
<td>Stomatal conductance (g, mol m⁻² s⁻¹)</td>
<td>Kopu II BC₁</td>
<td>0.278a</td>
<td>0.215a</td>
</tr>
<tr>
<td></td>
<td>Kopu II BC₂</td>
<td>0.165a</td>
<td>0.060a</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>0.121a</td>
<td>0.033a</td>
</tr>
<tr>
<td>Internal CO₂ concentration (Cᵥ, μmol mol⁻¹)</td>
<td>Kopu II BC₁</td>
<td>17.2a</td>
<td>36.5a</td>
</tr>
<tr>
<td></td>
<td>Kopu II BC₂</td>
<td>14.1a</td>
<td>17.5a</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>19.2a</td>
<td>14.7a</td>
</tr>
<tr>
<td>Transpiration (E, mmol m⁻² s⁻¹)</td>
<td>Kopu II BC₁</td>
<td>2.31a</td>
<td>2.71a</td>
</tr>
<tr>
<td></td>
<td>Kopu II BC₂</td>
<td>1.50a</td>
<td>0.31b</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>0.51b</td>
<td>0.50b</td>
</tr>
<tr>
<td>Physiological WUE (mmol mol⁻¹)</td>
<td>Kopu II BC₁</td>
<td>1.30a</td>
<td>1.96a</td>
</tr>
<tr>
<td></td>
<td>Kopu II BC₂</td>
<td>1.20a</td>
<td>1.84a</td>
</tr>
<tr>
<td></td>
<td>Kopu II</td>
<td>0.87a</td>
<td>2.26a</td>
</tr>
</tbody>
</table>
Appendix 51. Standard deviations, within watering treatments, for physiological and $^{13}$C parameters that were measured in the full data set, plus the Kopu II data set where relevant. Clover types or entries with the same letter, within watering treatments, show no differences in variability at the 5% level.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Clover type or entry</th>
<th>Full data set</th>
<th>Kopu II subset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Watered</td>
<td>Stressed</td>
</tr>
<tr>
<td>Pre-dawn $F_v/F_m$</td>
<td>BC$_1$</td>
<td>13.10$^a$</td>
<td>7.84$^a$</td>
</tr>
<tr>
<td></td>
<td>BC$_2$</td>
<td>11.08$^a$</td>
<td>11.43$^b$</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>13.04$^a$</td>
<td>8.22$^{ab}$</td>
</tr>
<tr>
<td>Quercetin glycosides (mg g$^{-1}$)</td>
<td>BC$_1$</td>
<td>1.23$^a$</td>
<td>2.38$^a$</td>
</tr>
<tr>
<td></td>
<td>BC$_2$</td>
<td>0.88$^a$</td>
<td>3.66$^b$</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>1.01$^a$</td>
<td>2.89$^{ab}$</td>
</tr>
<tr>
<td>Kaempferol glycosides (mg g$^{-1}$)</td>
<td>BC$_1$</td>
<td>1.34$^a$</td>
<td>1.51$^a$</td>
</tr>
<tr>
<td></td>
<td>BC$_2$</td>
<td>1.19$^a$</td>
<td>1.29$^a$</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>1.12$^a$</td>
<td>1.40$^a$</td>
</tr>
<tr>
<td>Hydroxycinnamic acids (mg g$^{-1}$)</td>
<td>BC$_1$</td>
<td>0.63$^a$</td>
<td>1.09$^a$</td>
</tr>
<tr>
<td></td>
<td>BC$_2$</td>
<td>0.56$^a$</td>
<td>0.62$^b$</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>0.46$^a$</td>
<td>0.81$^{ab}$</td>
</tr>
<tr>
<td>Quercetin:kaempferol</td>
<td>BC$_1$</td>
<td>1.62$^a$</td>
<td>1.94$^a$</td>
</tr>
<tr>
<td></td>
<td>BC$_2$</td>
<td>3.01$^b$</td>
<td>2.34$^a$</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>1.31$^a$</td>
<td>1.74$^a$</td>
</tr>
<tr>
<td>$\delta^{13}$C (‰)</td>
<td>BC$_1$</td>
<td>0.62$^a$</td>
<td>0.75$^a$</td>
</tr>
<tr>
<td></td>
<td>BC$_2$</td>
<td>0.61$^a$</td>
<td>0.66$^a$</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>0.45$^a$</td>
<td>0.77$^a$</td>
</tr>
<tr>
<td>$^{13}$C discrimination ($\Delta$) (%)</td>
<td>BC$_1$</td>
<td>0.65$^a$</td>
<td>0.79$^a$</td>
</tr>
<tr>
<td></td>
<td>BC$_2$</td>
<td>0.64$^a$</td>
<td>0.69$^a$</td>
</tr>
<tr>
<td></td>
<td>White clover</td>
<td>0.48$^a$</td>
<td>0.80$^a$</td>
</tr>
</tbody>
</table>