Identification of pasture mixtures that maximise dry matter yield

A.D. BLACK, S. ANDERSON and S.K. DALGETY
Field Research Centre, Department of Agricultural Sciences, PO Box 85084, Lincoln University, Lincoln 7647, New Zealand
alistair.black@lincoln.ac.nz

Abstract
A mixture experiment was conducted to identify an optimal pasture seed mixture that maximised dry matter (DM) yield under irrigated, sheep-grazed conditions in mid-Canterbury, New Zealand. Nineteen seed mixtures were created using a simplex design from a pool of four species: perennial ryegrass, plantain, white clover and red clover. Seed mixtures were drilled into plots in March 2015 and the yield from sowing to May 2017 was modelled. The model analysis defined the optimal seed mixture proportions as 0.25 ryegrass, 0.28 plantain and 0.47 red clover of total number of seeds per unit area, or 7.5, 6.3 and 8.2 kg/ha, respectively (22.0 kg total seed). This mixture was predicted to yield 39.81 t DM/ha after 2 years. Ryegrass and plantain interacted more strongly with red clover than white clover, leading to increased yields over monocultures (diversity effects). Additional yield benefits arose from the three-species mixture of ryegrass, plantain and red clover.

Keywords: diversity effect, pasture establishment, perennial ryegrass, plantain, red clover, seed mixtures, simplex design, white clover

Introduction
In New Zealand, seed mixtures of grasses, clovers and herbs are available to farmers wanting to renew their pastures. A key consideration in the formulation of mixtures is deciding on which species to include for particular conditions (Stewart et al. 2014). This is usually based on information about the performance of individual species from sources such as the New Zealand National Forage Variety Trial (NFVT) for ryegrasses. However, evaluation and analysis of pasture seed mixtures is necessary to identify the optimum mixture of the constituent species (Harris 1968, 2001).

In pasture mixtures, the initial expectation is for constituent species to contribute to the pasture response as much as their monoculture performance (identity effect) scaled by their relative proportion in the mixture. However, the performance of a mixture can differ from that expected from the individual species performances due to interspecific interactions. This difference between the actual performance of a mixture and the performance expected from the monocultures is the diversity effect. Interspecific interactions can be synergistic or antagonistic in their effect on pasture responses. Synergistic interactions are normally the result of differences in resource use among species, and facilitation, such as the way clovers can favour a companion grass or herb. Species may also interact antagonistically to have a negative effect on pasture responses, interactions may involve two or more species, and several positive and negative interactions may occur at the same time. The diversity effect is the net result of all of these. The strength of an interaction may also depend on the relative abundances of the species involved. These concepts have been applied to quantify the yield and weed suppression of multi-species grasslands (Connolly *et al*. 2009; Kirwan *et al*. 2009; Nyfeler *et al.* 2009; Finn *et al*. 2013), and to formulate optimal pasture (Harris 1968, 2001; Ryan-Salter & Black 2012) and turf grass (Friell *et al*. 2015) seed mixtures.

The objectives of this study were to 1) identify an optimal seed mixture that maximises pasture dry matter (DM) production, and 2) quantify the extent of identity and diversity effects operating in pasture mixtures, under irrigated and sheep-grazing conditions in mid-Canterbury, New Zealand. The initial work reported herein analysed annual DM yields for the first 2 years after sowing of an ongoing experiment.

Methods
Four pasture species were the constituents for a mixture experiment: perennial ryegrass (*Lolium perenne*; RG), plantain (*Plantago lanceolata*; P), white clover (*Trifolium repens*; WC) and red clover (*Trifolium pratense*; RC). A single, commercially available cultivar represented each species: Base, Tonic, Apex and Sensation, respectively. Selection of species and cultivars related to their ability to establish and produce high quality pasture under irrigated conditions in mid-Canterbury. Stewart *et al*. (2014) described their basic characteristics.

Nineteen seed mixtures varying in species richness from one to four species and in species relative abundance were created using a simplex centroid design (Cornell 2002). There were four monocultures, six two-species mixtures (½ of each of two species), four three-species mixtures (⅓ of each of three species), the four-species centroid mixture (¼ of each species) and four four-species mixtures dominated in turn by each species (⅝ of one species and ⅛ of each of the other

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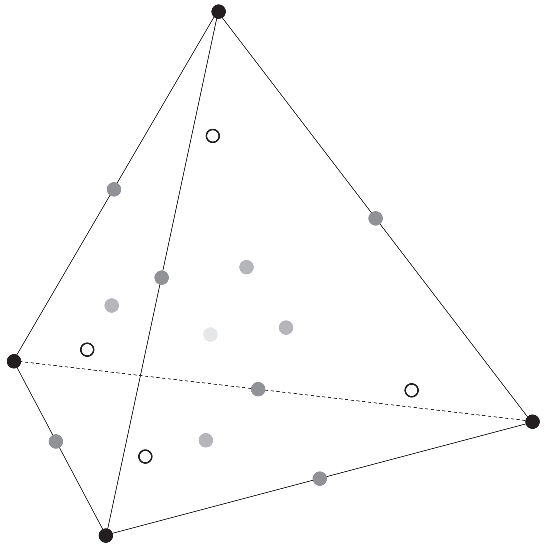

Figure 1 Four-species simplex centroid design with four monocultures (black circles), six two-species mixtures (½ of each of two species; dark grey), four three-species mixtures (1/3 of each of three species; medium grey), a four-species centroid mixture (¼ of each species; light grey) and four four-species mixtures dominated in turn by one species (5/8 of one species and 1/8 of each other species; white).

species) (Figure 1). The species proportions were based on number of seeds per unit area and the total sowing rate was fixed at 833 seeds/m^2. This was equivalent to 30 kg/ha of ryegrass. Average 1000-seed weights were 3.6, 2.7, 0.9 and 2.1 g and average germination percentages were 93, 99, 91 and 88% for ryegrass, plantain, white clover and red clover, respectively. White clover seed had AGRICOTE treatment (a lime-based coating with nutrients, insecticide and fungicide). The other species were not treated. Sowing rates were unadjusted for germination percentage.

The mixtures were evaluated at Lincoln University, Canterbury, New Zealand (43°38′53.1″ S, 172°27′11.7″ E and 10 m elevation) on a Templeton silt loam soil (Cox 1978). The site had been in lucerne (*Medicago sativa*) in 2011 and 2012, oilseed rape (*Brassica napus*) in 2013 and forage oats (*Avena sativa*) in 2014. The site was ploughed and tilled in November 2014, sprayed with Roundup ULTRA® MAX (570 g/litre glyphosate at 3 litres/ha) in February 2015, irrigated (60 mm) and tilled into a seedbed in March 2015. On 26 March 2015, mixtures were drilled into 2.1 x 6 m plots in a randomised complete block design with four replicates, using a Flexiseeder precision drill with 14 coulters spaced 0.15 m apart. The sowing depth was 10-15 mm.

At the first harvest on 16 August 2015, plots were cut to a 4-5 cm stubble to minimise plant damage. Subsequently, the plots were grazed by sheep on: 22-25 September, 27-30 October and 1-3 December in 2015; 7-11 January, 16-22 February, 1-5 April, 27-30 May, 2-5 August, 26-30 September, 1-7 November and 9-13 December in 2016; 16-19 January, 17-21 February, 31 March-7 April and 26-30 May in 2017. The average pre-grazing herbage mass ranged from 1000 kg DM/ha in August and May to 3500 kg DM/ha in January. The sheep removed most of the herbage within 3-7 days, with the residual trimmed to 4-5 cm.

Soil analysis (4 May 2015) was pH 5.7, Olsen P 13 mg/litre, Ca 7.3 me/100 g, Mg 0.84 me/100 g, K 0.32 me/100 g, Na 0.17 me/100 g and sulphate S 13 mg/kg. Superphosphate (9% P, 11% S) was applied on 30 September 2015 (500 kg/ha) and 12 October 2016 (480 kg/ha). Irrigation occurred every 3-5 weeks from 30 October 2015 to 6 April 2016 (total, 360 mm) and from 9 November 2016 to 15 February 2017 (total, 260 mm) to maintain soil moisture above a critical limit of 24% (Black & Murdoch 2013). T-Max^{TM} (30 g/litre aminopyralid at 2 litres/ha) was used to remove volunteer white clover from the ryegrass plots (10 March 2017) and the plantain and ryegrass-plantain plots on 5 May 2017. Dew^{TM} 600 (600 g/litre diazinon at 4 litres/ha) was sprayed for grass grub (*Costelytra zealandica*) on 10 May 2017.

Before each harvest, one 0.3 m^2 quadrat (1 m of two adjacent drill rows) was clipped to 1-2 cm for each plot. A subsample of the clippings was separated into sown species and weeds, before drying (70°C for 48 hours) with the rest of the clippings, and the dry weights were used to calculate the herbage mass of the sown species. After each harvest, a 0.2 m^2 quadrat per monoculture and centroid plot was clipped and dried (as above) and residual herbage mass for each plot was calculated. Yield was calculated as the pre-harvest herbage mass less the previous residual herbage mass, and summed for Year 1 (sowing to 27 May 2016) and Year 2 (30 May 2016 to 26 May 2017).

Yield was analysed using the mixture regression method in Minitab® 18 statistical software whereby a special cubic model was fitted to the data for each year. This model can quantify separate two-species (pairwise) and multi-species interactions in the mixtures. It had the general form:

$$\hat{y} = \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_4x_4 + \beta_{12}x_1x_2 + \beta_{13}x_1x_3 + \beta_{14}x_1x_4 + \beta_{23}x_2x_3 + \beta_{24}x_2x_4 + \beta_{34}x_3x_4 + \beta_{123}x_1x_2x_3 + \beta_{124}x_1x_2x_4 + \beta_{134}x_1x_3x_4 + \beta_{234}x_2x_3x_4 + \beta_{1234}x_1x_2x_3x_4 + \varepsilon$$

(Model 1)

where $\hat{y}$ is the predicted yield response from a mixture; x_1, x_2, x_3 and x_4 are the sown proportions of ryegrass, plantain, white clover and red clover, respectively; β_1 to β_4 are estimates of the response of the monocultures; β_{12} to β_{34} represent the interaction effects for the combination of two species; β_{123} to β_{234} are the additional interaction effects for the combination of three species; β_{1234} is the additional interaction effect for the combination of four species; and ε is a random error term, assumed to be normally and independently distributed with mean zero and constant variance.

The analysis of variance tested if the estimates were significantly different (P<0.05) from zero or not. There were no t-tests and P values for the first four estimates - the predicted yields of the monocultures - because the model does not have an intercept term. The response optimisation procedure in Minitab® 18 searched the entire tetrahedral design space (Figure 1) to define the optimal seed mixture for maximum yield for each year.

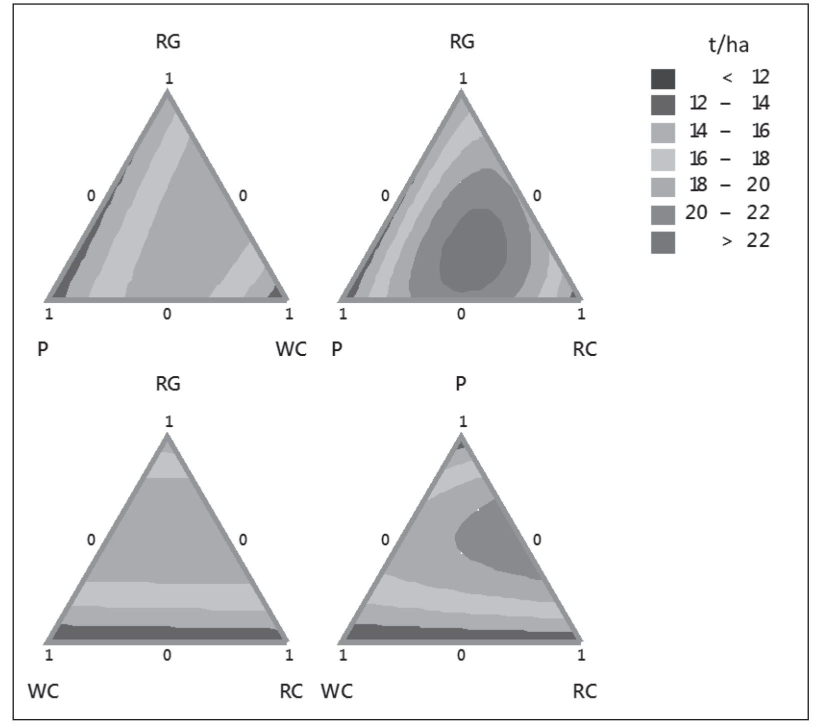

Figure 2 Contour plots of dry matter yield (t/ha) for Year 1 predicted from Model 2 as a function of sown proportions of perennial ryegrass (RG), plantain (P), white clover (WC) and red clover (RC).

The site received 200 mm of rainfall from 1 March to 30 June 2015, 493 mm from 1 July 2015 to 30 June 2016 and 489 mm from 1 July 2016 to 26 May 2017. Average monthly air temperature ranged between 4°C in July and 16°C in January.

Results and Discussion

A mixture of ryegrass, plantain and red clover resulted in the greatest yield for Year 1, as indicated by the location of the peak region in the top right contour plot in Figure 2. The more comprehensive search of the entire tetrahedral space (Figure 1) defined that optimal seed mixture depicted in the contour plot as 0.23 ryegrass, 0.33 plantain and 0.44 red clover of total number of seeds per unit area. These proportions were equivalent to seed rates of 6.9 kg ryegrass, 7.4 kg plantain and 7.7 kg red clover (22.0 kg total seed)/ha, and the mixture was predicted to produce a yield of 23.0 t DM/ha.

The fitted model that generated these contour plots was:

$$\text{Yield (t DM/ha)} = 14.27x_1 + 12.46x_2 + 11.80x_3 + 12.45x_4 + 26.60x_1x_3 + 25.18x_1x_4 + 26.67x_2x_3 + 36.25x_2x_4 + 69.56x_1x_2x_4$$

(Model 2)

The model was an adequate fit to the data (R^2=73.13 and adjusted R^2=69.92). The other two quadratic terms (RG*P, WC*RC), the three special cubic terms (RG*P*WC, RG*WC*RC, P*WC*RC) and the RG*P*WC*RC term were not significant (P>0.1), so they were removed from the final model.

The contour plots and estimated coefficients from Model 2 quantified the identity and diversity effects on yield, and predicted the yields for any seed mixture created from the pool of four species. The corners of the contour plots (Figure 2) and the coefficients for the linear terms (Table 1) indicated that the monoculture yields of the four species were similar (P=0.27) for Year 1. From the curvature along the edges of the contour plots and the coefficients for the quadratic terms, there also appeared to be differences between the four pairwise interaction effects. The synergism of plantain with red clover was stronger than plantain with white clover, and stronger than ryegrass with either white clover or red clover, as indicated by the higher curvature on the plantain-red clover axis and the larger coefficient of 36.25. This coefficient represents the potential of plantain and red clover to interact at the ½-½ point, and can be used to quantify the diversity effect (the difference between the actual yield of the

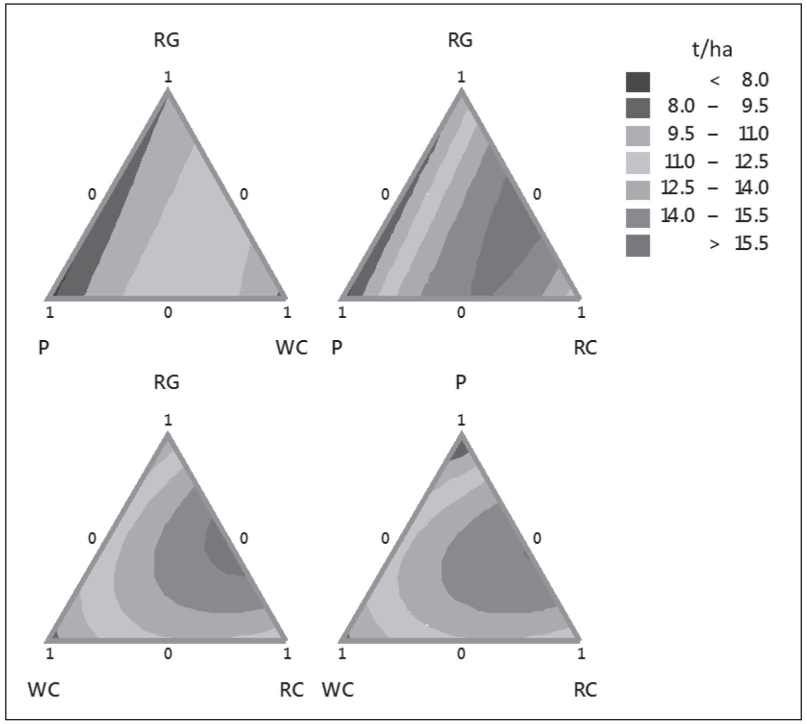

Figure 3 Contour plots of dry matter yield (t/ha) for Year 2 predicted from Model 3 as a function of sown proportions of perennial ryegrass (RG), plantain (P), white clover (WC) and red clover (RC).

mixture and the yield expected from the monocultures) for the even plantain-red clover mixture as 36.25 × ¼ = 9.06 t DM/ha. The additional synergism produced by mixing ryegrass, plantain and red clover was evident as the large coefficient of 69.56. This value equated to an additional yield increase for the combination of those three species at the ⅓-⅓-⅓ point of 69.56 × 1/27 = 2.58 t DM/ha.

At the optimal seed mixture (0.23 ryegrass, 0.33 plantain and 0.44 red clover), the average identity effect, which assumes no species interactions, can be calculated from Model 2 as the species' monoculture yields scaled by their relative proportions in the mixture, i.e. 14.27 × 0.23 + 12.46 × 0.33 + 12.45 × 0.44 = 12.87 t DM/ha. The diversity effect for the optimal mixture is quantified from the same model as 25.18 × 0.23 × 0.44 + 36.25 × 0.33 × 0.44 + 69.56 × 0.23 × 0.33 × 0.44 = 10.13 t DM/ha. This extra yield achieved simply by growing ryegrass, plantain and red clover together from sown proportions that optimised the species' individual contributions and the diversity effect. The reasons for the diversity effect were not confirmed in this study, but possible explanations include a more complete use of resources by the mixture through niche partitioning, and N fixation by the red clover that also favoured the ryegrass and plantain (Kirwan *et al*. 2009; Nyfeler *et al*. 2009). This resulted in the actual yield of the mixture being almost twice that expected from the monocultures.

For Year 2, a strong upward curve on the ryegrass-red clover axis and an equally strong upward curve

Table 1 Estimated coefficients from Model 2 fitted to dry matter yield (t/ha) for Year 1.

Term	Estimate	Standard error	P value
RG	14.27	0.96	-
P	12.46	0.96	-
WC	11.80	0.95	-
RC	12.45	0.96	-
RG*WC	26.60	4.61	<0.001
RG*RC	25.18	4.89	<0.001
P*WC	26.67	4.61	<0.001
P*RC	36.25	4.89	<0.001
RG*P*RC	69.56	32.23	0.035

Species are perennial ryegrass (RG), plantain (P), white clover (WC) and red clover (RC).

Table 2 Estimated coefficients from Model 3 fitted to dry matter yield (t/ha) for Year 2.

Term	Estimate	Standard error	P value
RG	9.39	0.76	-
P	7.46	0.76	-
WC	8.95	0.84	-
RC	11.30	0.84	-
RG*WC	9.45	3.70	0.013
RG*RC	22.46	3.70	<0.001
P*WC	14.63	3.70	<0.001
P*RC	23.99	3.70	<0.001
WC*RC	8.86	3.70	0.020

Species are perennial ryegrass (RG), plantain (P), white clover (WC) and red clover (RC).

on the plantain-red clover axis (Figure 3) suggested that a seed mixture of either ryegrass and red clover, plantain and red clover, or a combination of ryegrass, plantain and red clover, resulted in the greatest yield. The computer-aided analysis of the complete design defined the optimal seed mixture as 0.46 ryegrass and 0.54 red clover (13.8 and 9.5 kg/ha, respectively) and this mixture was predicted to yield 16.0 t DM/ha.

The fitted model that generated the contour plots for Year 2 was:

$$\text{Yield (t DM/ha)} = 9.39x_1 + 7.46x_2 + 8.95x_3 + 11.30x_4 + 9.45x_1x_3 + 22.46x_1x_4 + 14.63x_2x_3 + 23.99x_2x_4 + 8.86x_3x_4$$

(Model 3)

The R^2=68.08 and adjusted R^2=64.27. The quadratic term RG*P, the four three-species terms and the four-species term were not significant (P>0.08) so they were removed.

The contour plots (Figure 3) and coefficients (Table 2) for Year 2 revealed that the monoculture yields were greatest for red clover and least for plantain: 11.30 red clover > 9.39 ryegrass > 8.95 white clover > 7.46 plantain, t DM/ha (P<0.05). There were also differences between the pairwise interaction effects. Both ryegrass and plantain interacted more strongly with red clover than white clover, as indicated by the higher surfaces and coefficients. The diversity effect produced by the optimal ryegrass-red clover seed mixture was 22.46 × 0.46 × 0.54 = 5.58 t DM/ha. In comparison, the diversity effect at the ½-½ point for ryegrass-white clover was 9.45 × ¼ = 2.36 t DM/ha. White clover also seemed to interact positively with red clover. This was unexpected, but possible mechanisms that may combine to produce such an effect include differences in shoots and light capture (Black *et al*. 2009) and in roots and access to water and nutrients (Thomas 2003) between the two clovers.

The different optimal seed mixtures identified in Years 1 and 2 did not have a major impact on the predicted yields. In fact, a pasture sown with the optimal seed mixture that maximised yield in Year 1 would have only sacrificed 0.6 t DM/ha of the maximum yield in Year 2, based on Model 3. This was due to the strong and consistent interactions among ryegrass, plantain and red clover in both years (Tables 1, 2; Figures 2, 3). However, these differences warranted a combined analysis of the yields summed over both years:

$$\text{Yield (t DM/ha)} = 23.44x_1 + 19.71x_2 + 21.62x_3 + 24.75x_4 + 35.74x_1x_3 + 44.46x_1x_4 + 41.00x_2x_3 + 57.05x_2x_4 + 123.60x_1x_2x_4$$

(Model 4)

(R^2 = 79.71 and adjusted R^2 = 77.29)

This model defined an optimal seed mixture that maximised yield over both years (39.81 t DM/ha) as 0.25 ryegrass, 0.28 plantain and 0.47 red clover, which was equivalent to 7.5 kg ryegrass, 6.3 kg plantain and 8.2 kg red clover (22.0 kg total seed)/ha. By comparison, a seed rate of 20 kg ryegrass and 5 kg white clover/ha, which provided the same number of seed of the two species per unit area, was predicted to yield 31.47 t DM/ha. The monoculture yields after 2 years remained greatest for red clover and least for plantain: 24.75 red clover > 23.44 ryegrass > 21.62 white clover > 19.71 plantain t DM/ha (P=0.054; Table 3). The combined analysis confirmed the strong interactions for ryegrass and plantain with red clover, and the additional yield benefit produced by mixing ryegrass, plantain and red clover.

The absence of white clover in the optimal seed mixture suggests that it had little influence on mixture yield over the first 2 years. This was probably because white clover was slower to establish than the other three species (Stewart *et al*. 2014) and, therefore, did not facilitate the growth of ryegrass and plantain as much as red clover (Kirwan *et al*. 2009). However, pasture mixtures are not stable over time (Harris 2001) and white clover may eventually increase its contribution in the mixtures through its stoloniferous growth, which could justify its inclusion in the seed mixture. It is also possible that plantain and red clover will decrease in abundance, as they are both less persistent than ryegrass (Stewart *et al*. 2014), and weeds will invade any bare ground. In this study, the proportion of weeds in the total yields averaged 0.12 for the monocultures and 0.03 for the mixtures (data not shown). Such changes in species' relative abundance in the long-term are difficult to predict from this study. However, it is possible to predict what might happen after 2 years if the proportions of the species in the seed mixture were changed. For example, replacing half of the number

Table 3 Estimated coefficients from Model 4 fitted to dry matter yield (t/ha) summed over Years 1 and 2.

Term	Estimate	Standard error	P value
RG	23.44	1.29	-
P	19.71	1.29	-
WC	21.62	1.28	-
RC	24.75	1.29	-
RG*WC	35.74	6.22	<0.001
RG*RC	44.46	6.61	<0.001
P*WC	41.00	6.22	<0.001
P*RC	57.05	6.61	<0.001
RG*P*RC	123.60	43.50	0.006

Species are perennial ryegrass (RG), plantain (P), white clover (WC) and red clover (RC).

of red clover seeds in the optimal mixture with white clover (i.e. 7.5 kg ryegrass, 6.3 kg plantain, 1.8 kg white clover and 4.1 kg red clover/ha) would sacrifice 4.3 t DM/ha of the maximum yield over the first 2 years, based on Model 4.

Overall, the species identity effects and the pairwise interaction effects produced by combining either ryegrass or plantain with either white clover or red clover were the main contributors to mixture yield (Tables 1-3; Figures 2, 3). This is consistent with previous mixture experiments that used two-species grass-legume or grass-grass combinations in replacement series designs (Harris 1968; Parry *et al*. 1994; Stevens & Hickey 2000). The additional contribution produced by the ryegrass-plantain-red clover mixture was less important than the pairwise clover-non-clover interactions, and there were no additional yield benefits from the other mixtures with three or four species. Some other pasture mixture studies have reported that yield can increase with increasing numbers of species compared with ryegrass-white clover pasture (Johnson *et al*. 1994; Daly *et al*. 1996; Goh & Bruce 2005; Nobilly *et al*. 2013; Woodward *et al*. 2013; Cranston *et al*. 2015). However, such studies seldom include all possible monocultures and pairwise combinations of the constituent species used in the mixtures, making the results difficult to interpret with respect to diversity effects (Connolly *et al*. 2009; Kirwan *et al*. 2009; Nyfeler *et al.* 2009; Finn *et al*. 2013).

The results also show that the application of simplex designs and diversity-interaction modelling (Kirwan *et al*. 2009) to seed mixture experiments has the potential to identify seed mixtures that maximise response variables such as DM yield. The evaluation of pasture seed mixtures has traditionally used methods that provide little or no predictive ability to evaluate seed mixtures beyond those included in the experimental design. The approach used in this study and others like it (e.g. Harris 1968; Connolly *et al*. 2009; Kirwan *et al*. 2009; Nyfeler *et al.* 2009; Ryan-Salter & Black 2012; Finn *et al*. 2013; Friell *et al*. 2015) could be applied in the seed industry in New Zealand, particularly where pre-prepared seed blends are promoted and sold. Further research is needed to validate the optimal pasture seed mixtures identified in this study, and to explain the identity and diversity effects operating in the mixtures.

Conclusion

This study revealed strong and different pairwise interactions between species in pasture mixtures that resulted in increased annual yields (diversity effects) during the first 2 years after sowing. Both ryegrass and plantain interacted more strongly with red clover than white clover, with additional yield benefits achieved from the combination of ryegrass, plantain and red clover. The optimal seed mixture that maximised yield over the first 2 years was identified as 0.25 ryegrass, 0.28 plantain and 0.47 red clover, which was equivalent to seed rates of 7.5 kg ryegrass, 6.3 kg plantain and 8.2 kg red clover/ha.

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