NITROGEN FIXATION BY *ULEX EUROPAEUS* (GORSE) AND *CYTISUS SCOPARIUS* (BROOM)

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CONTENTS

SUMMARY

A series of glasshouse and laboratory experiments was carried out to enable comparison of two woody perennial legumes, gorse and broom, with other legumes, nodulated non-legumes and other biological nitrogen fixing systems.

Both species had distinct juvenile phases in which broom closely resembled herbaceous species in appearance, but adult plants of both species bore little resemblance to each other or to other legume species. Nodule development was similar to that of other legumes, but mature nodules exhibited structural adaptations to longevity - meristematic activity, a well developed vascular system and numerous cytoplasmic granules in cortical cells. Acetylene reduction and $^{15}N_2$ fixation continued for much longer following excision than has been observed in other legumes. In all experiments, broom nodules exhibited higher rates of acetylene reduction and nitrogen fixation than did gorse nodules.

The first detectable product of nitrogen fixation in excised nodules - ammonia - was rapidly incorporated into amide and α-amino groups and another unidentified fraction. The principle free amino acid in nodules and sap was asparagine. Its preponderance increased as plants aged.

Whole nodulated plants and excised nodules of both species exhibited a relatively low temperature optimum for growth and nitrogen fixation ($22^{o}C$). They were very sensitive to elevated temperatures. Results indicated that gorse and broom have relatively low light requirements.

When aeration was sufficient, combined nitrogen had little effect on growth of nodulated plants. Nodulation in both species was reduced by increasing amounts of combined nitrogen. High levels (100 mg/l) of nitrate and ammonia caused considerable inhibition of nitrogen function.

Both species showed large responses to phosphate, but were able to grow and fix nitrogen when supplied with low amounts of phosphate.

Boron deficiency reduced nitrogen fixation. Nodulation was increased to compensate for this.

Considerable amounts of nitrogen can be contributed to the ecosystem in gorse and broom litter. Direct transfer between gorse or broom and Pinus radiata is likely to be small and may be masked by competition for other nutrients.

These findings are discussed with respect to the use of gorse and broom to overcome nitrogen deficiency in reafforestation on the Moutere Gravels, in Nelson, N.Z.

CHAPTER I

INTRODUCTION

PROBLEM ANALYSIS

A band of soil formations, termed the "Moutere Gravels" occurs over an area of some 315,000 acres in Central Waimea County, Nelson Province, N.Z. At the time of European settlement, in the mid-nineteenth century, vegetation on these soils was mainly Leptospermum, the fern Pteridium, and grasses, with pockets of Nothofagus in gullies (Stone and Will, 1965; Appleton and Slow, 1966). After failure of farming and orcharding on many of these soils in the 1920's and 1930's large areas were planted in forests, mainly Pinus radiata.

Growth of the first rotation (15-30 years) on the best forest sites on the Moutere Gravels was considered to be similar to that on other good sites in New Zealand and poor sites were not considered to be extensive.

After clear felling of areas of forests, and hauling tree lengths over slash remaining after trimming, dense natural tree regeneration was allowed to occur. In many areas, growth of this regeneration has been shown to be poor (Stone and Will, 1965). By comparing these second rotation trees with stumps remaining from the first generation, Whyte (1966) was able to demonstrate that height growth stagnated longer in the second rotation on all but the best sites. Later growth was generally comparable with first crops.

This lag represents a loss of approximately three years in the early life of the tree. On very poor soils (for example on ridges), there was no sign of the later recovery of second crop growth, even twelve years after establishment. This and the lack of early thinning resulted, on these very poor sites, in 12 year old crops producing only 30% of the basal area of first rotation trees, and only 76% of the height. The decline was generally transitory lasting up to 5 - 8 years, though in the worst areas it was considerably more persistent (Whyte, 1966; Whyte et al, 1968).

Early indications were that this productivity decline was due to deficiency of nitrogen and phosphate (Stone and Will, 1965; Jackson, 1967). Appleton and Slow (1966) were able to obtain responses to nitrogen in yellowing Pinus radiata on these soils. This treatment, however, accentuated both phosphate deficiency and seasonal die back of young growing tips, the latter a characteristic of boron deficiency (Appleton and Slow, loc.cit.; Proctor, 1967).

Use of nitrogen, phosphorus and boron fertilizers can overcome these deficiencies to a large extent, but in some soils, magnesium deficiency requires treatment. However, there are some drawbacks to the use of nitrogenous fertilizers in these forests:-

1. the stimulatory effect is only transitory and declines by the third year of treatment (Richardson, 1965; Will, 1966),
2. economics of the use of such fertilizers in New Zealand are doubtful.

Responses of forest trees to nitrogen fertilizers have been reported in other countries; in Corsican Pine in Scotland (Miller, 1966), in Red Pine seed production in Canada (Cayford and Jarvis, 1967) and in Kauri Pine in Australia (Richards and Bevege, 1968). Richards and Bevege (loc.cit.) also observed a depression in tree growth when phosphate was applied without nitrogen. Such a response was also noted by Appleton and Slow (1966) in Nelson forests. The use of fertilizers in forests was reviewed by Stewart and Swain (1965) who reported responses of Norway Spruce to nitrogen fertilizers in Finland and Florida, and by Baule and Fricker (1970) in Germany, who noted responses by several species to a variety of fertilizers. According to Hewitt (1966), macro-nutrient deficiencies are quite common in forest tree species and in nurseries, although it appears that the importance of these deficiencies (especially nitrogen, phosphorus and potassium) was only then being fully realized.

The widespread use of legumes as sources of nitrogen in New Zealand pastures, suggests the possibility of using suitable legume species to overcome nitrogen deficiencies in forests. Very little attention, however, has been paid to legumes other than those used in agriculture (see Norris, 1968)

and their possible use in forests has been largely ignored (see Richards and Bevege, 1967a). However, Baule and Fricker (1970), recommend introduction of legumes into forests where light conditions permit. Adequate phosphate, potassium, calcium and magnesium levels are necessary for this. The possibility of using gorse and broom in forests on the Moutere Gravels where gorse and to a lesser extent, broom, are most important weeds. (Bunn, 1964), was first suggested by improvement in tree colour noted by Appleton and Slow (1966) of phosphate and boron fertilized trees (but not those fertilized with boron alone) where gorse was present. This present study compares aspects of the nutritional and environmental responses of the two woody leguminous species, gorse and broom, with those of other nitrogen fixing systems, both of legumes and of nodule-bearing non-leguminous angiosperms, with particular regard to their use in overcoming nitrogen deficiency in second rotation Pinus radiata forests in the Nelson Conservancy.

References on the use of legumes in forests are not extensive, but use is made of legumes as nurse crops in some areas of the world. Glesinger (1959) recommends planting trees in strips 2-3 feet wide 6 - 10 feet apart cleaned through undergrowth of Indigofera and Desmodium in temperate Asia. Chinnamani et al (1965) observed a stimulation in growth of young black wattle and blue gum in India when planted with broom as a nurse crop. During the second year after tree planting, broom growth was removed. In the fourth year, broom had regenerated to form a dense undergrowth, and after six years, it had formed a dense understory. Only brief mention was made of the fact that broom was a legume, and most of the effect noted was attributed to the provision of shelter by broom in the early stages of tree growth. In an earlier ecological study of regenerated forest areas in the same region of India where broom and to a lesser extent gorse were major shrubs, Agrawal et al (1961), concluded that broom "..... has practically no economic value except that it is a poor man's fuel .....". Tarrant (1961, quoted in Richards and Bevege, 1968) produced evidence that red alder as a nurse crop greatly accelerated growth of douglas

fir, increasing nitrogen content in the foliage and soil. Alder was the most effective nurse crop for Abies alba (silver fir) among trees (pines, larches, birches) tested by Holmsgaard (1962). This effect was attributed to nitrogen fixation by alder, but could be attributed to differences in root or shoot competition among the nurse species used. In Germany, after a survey of manurial trials in forests, Weiderman (1932, quoted in Burns, 1935) concluded that intercultivation with legumes (perennial lupins on moister spruce soils, and yellow lupins on drier pine soils) was both more effective, and more certain than the use of inorganic fertilizers alone. Probably the best indication of the potential use of legumes as sources of nitrogen for associated forest species was obtained by Richards and Bevege (1967b), in Southern Queensland, who found over a 5 year period, that growing three perennial legume species, Lotononins bainesii, Desmodium uncinatum and Phaseolus atropurpureas in plantations markedly increased growth rate and nitrogen content of associated Australian native conifers Araucaria cunninghamii and Agathis robusta, but had no effect on the exotic conifers Pinus taeda, P.caribaea or P.elliottii.

In New Zealand, extensive use is made of perennial lupins (Lupinus arboreus) in sand-dune reclamation. After the establishment of marram grass (Ammophila arenaria) with, or without lupins, Pinus radiata seedlings are planted, either with lupin seed or after line cutting through lupin scrub.

An extensive literature review and problem analysis on the use of lupins to improve nitrogen status of Pinus radiata was carried out by Cadgil (1966) as part of a study at Forest Research Institute, New Zealand Forest Service, Rotorua, to determine the effect of lupins on nitrogen nutrition of P.radiata on Woodhill sand dunes (Cadgil, 1967, 1968, 1971 a,b,c; Cadgil and Mead, 1967). It was thought that exudation of nitrogen compounds by germinating lupin seeds (average density 800 seeds per square metre) under a two year old lupin stand at Woodhill might be a significant source of nitrogen for the early stages of P.radiata growth (Cadgil, 1968). This was confirmed in pot trials and laboratory experiments at Rotorua. Lupin seeds (150 per pot) germinating on the surface of potted Woodhill sand (of mean initial total nitrogen content 0.08 g/kg were

shown to increase significantly the nitrogen content of P.radiata seedlings planted after removal of lupin seedlings (Cadgil, 1971a). Lupin seeds raised the mean nitrogen content of sterile pads on which they were germinated (25 per pad) 16 fold from 0.17 mg to 3 mg. In another series of pots 50 g of lupin litter significantly raised the nitrogen content of P.radiata seedlings (Cadgil, 1971a).

However, in gorse and broom, with their smaller seeds and irregular germination, excretion of nitrogen by germinating seeds would probably be much less significant than in Cadgil's experiments with lupin.

The use of legumes in experiments in Nelson forests has been limited by poor germination and growth of the species tried (mainly clovers and lupins), partly because of competition from gorse (Bunn, 1963; Appleton and Slow, 1966). However, use of lupins in sand-dune areas has been shown to be beneficial to tree growth (Cadgil, 1966) as it raises the amount of available nitrogen in the ecosystem considerably over a 3–4 year period (Cadgil, 1971c).

In the forests in the Waimea County, gorse is a very prevalent weed (Bunn, 1964; Appleton and Slow, 1966). Consequently most reports on gorse in those forest areas have been concerned with its eradication, prior to afforestation and its control during establishment and growth (Bunn, 1964 ; Cornwell, 1969; Chavasse, 1969).

In addition to the nitrogen deficiency of these soils mentioned previously, the typical structure of the Moutere Gravels is in many cases, very poor. Kingston (1968) was able to develop a multiple correlation equation to explain 49% of the variation in chosen site index (tree height at 12 years) on the basis of soil physical properties alone. Of the factors measured by Kingston (loc.cit.), that with greatest effect on height was the distance of the measured tree from the adjacent ridge. The effect of many soil properties measured, especially this main one, could be partly or wholly explained in terms of soil nutrient levels which they reflect. Nevertheless, results of that study gave strong indication of the importance of the physical structure of these soils for tree growth.

Burns (1935), in a study of soil conditions and vegetation in Scotland, showed that both gorse and bracken fern (Pteridium) were beneficial to tree growth. Heavy nodulation was observed on gorse and mention was made of possible enhancement of tree growth by exudation of nitrogen compounds from gorse as had been suggested by Virtanen et al, (1932). Burns (loc.cit.) also observed the presence of a thick humus layer on the soil. An additional important effect of gorse dominance in a succession, was the breaking up of the pan and consequent improvement in soil physical condition, giving better drainage, aeration etc. In view of Kingston's (1968) results, it is possible that such an effect of gorse on structure could be of some importance on the Moutere Gravels.

SPECIES STUDIED

The two species used in this present study were Ulex europaeus L.em Rothm commonly known in New Zealand as gorse, but also known in the British Isles as whin or furze, and Cytisus scoparius Link (= Saromanthus scoparius Koch) commonly called broom. For convenience they will be referred to by their common names throughout this work. Both species belong to the family Papilionaceae of the Leguminoseae, and both are native to the British Isles and Western Europe extending from Spain and Portugal to Southern Scandinavia. The first references to these plants in New Zealand were by Darwin in 1835 in the Bay of Islands, and Armstrong in 1872 in Canterbury (Allen, 1940).

According to Clapham et al (1962) there are 20 species of the genus Ulex in Western Europe and Northern and Western Africa. However, only three are of any importance in the British Isles:- U.europaeus L.em Rothm (common or European gorse, furze or whin)

U. gallii Planch (Western furze)

U.minor Rothm (= U.manus Forst) (dwarf furze)

Of these U.europaeus is the commonest and is the only one occurring to any extent in New Zealand. Egunjobi (1967) states that this species may be sub-

divided into 2 sub-species - U.europaeus borealis (2n= 96), and U.europaeus latebracheatus (2n = 64) - with the more common former sub-species being further subdivided into three varieties - var.strictus, var.prostratus, and var.flove pleno (= var.plenus scheid). However, in view of the extreme variability in growth forms reported for this species (Skipper, 1922; Millener, 1961, 1962), such a division seems difficult to justify.

U.gallii has been recorded in a restricted area around Tauranga (Allen, 1940; Miller, 1970) and Taylor (1969) includes U.minor in the list of noxious weeds in New Zealand. Gorse was extensively used for farm hedges in some areas of New Zealand, especially the Canterbury Plains, and this probably contributed to its wide spread throughout the countryside.

There are four major species of the genus Cytisus growing in New Zealand. C.monospessulanis L (= C.candicans Oc.) and C.multifloris Sweet(=C.albus Link) are established in limited areas around Canterbury, C.proliferus L.Tagasate (tree lucerne) grows in areas of the North Island, and C.scoparius is widespread throughout New Zealand. Mention was also made of the occurrence in New Zealand of C.capensis and C.stenopetalus Christ (= C.maderensis Mast)by Allen (1940).

The economic importance of these species as weeds is emphasised by the introduction into New Zealand in 1926 of a gorse seed weevil, Apion ulicus Forst, as a method of biological control of gorse (Miller, 1970), and by the inclusion of both species on the noxious weeds list (Taylor, 1969).

LITERATURE REVIEW

Very few studies have been made on nutritional requirements of gorse and broom. MacConnell and Bond (1957a) included gorse as a legume for comparison with two woody nodulated non-legumes, Alnus glutinosa and Myrica gale in a study on the effect of combined nitrogen on nodulation. These authors, in another paper (MacConnell and Bond, 1957b) showed that 75 of 78 Rhizobium isolates from gorse nodules from areas of Scotland, formed fully effective nodules according to their definition, and three, nodules of intermediate

effectiveness.

The ecology and morphology of Ulex spp were studied by Skipper (1922), who described three main growth forms and two sub-divisions within these forms of common gorse growing on Hindhead Common, Herts., England. Later, this great variability of gorse was noted by Millener (1961, 1962) in a study of gorse seedling development. He showed that seedling development, as measured by the juvenile-adult transition was greatly affected by day length and by latitude of origin of the parent plants.

Egunjobi (1967), made an extensive review of the literature on this species. He intensively studied primary productivity of gorse on a natural low fertility soil in New Zealand. He showed (Egunjobi ,1967, 1969, 1971) that dry matter and nitrogen accumulation rates of stands of gorse (12-15 000 kg/ha/ha and 230 kg/ha/a respectively in the first five years), were much higher than in adjacent pasture plots (5 900 and 164 kg/ha/a respectively). However, response of gorse to applied fertilizer - urea (1 121 kg/ha)serpentine molybdenized superphosphate (560 kg/ha), lime (1 120 kg/ha) - was less than in pastures. In gorse on these soils there was a very high rate of litter fall and nutrient turnover.

As mentioned previously, Burns (1935) included gorse dominated areas in his study of soil conditions and vegetation in North East Scotland. Although some areas contained broom as a dominant species, his study was confined entirely to gorse.

Pate (1958) described biennial nodules on gorse and broom in Ireland and later (Pate,1961a) produced evidence to show that these nodules were in fact perennial.

The only studies found which centred on broom were in India (Agrawal et al, 1961; Chinnamani et al, 1965). These were mentioned earlier.

The general field of nitrogen fixation has been extensively reviewed since publication of two early excellent books (Fred et al, 1932; Wilson, 1940). Allen and Allen (1958); Raggio and Raggio (1962); Burris (1966); Stewart, (1966),

and papers in McElroy and Glass (1956); Hallsworth (1958), and McKee (1962), review most aspects of legume and non-legume nitrogen fixing symbioses. Bond (1958, 1959, 1963) reviewed non-leguminous nodule bearing plants, Fogg and Stewart (1965) and Stewart (1969), blue-green algae, Nutman (1956) and Vincent (1966), respectively, structure and development and specificity of legume symbioses. Biochemistry and chemistry of nitrogen fixation has been excellently reviewed by Hardy and Burns (1968), Hardy and Knight(1969), Bergersen (1971 a,b); Hardy et al (1971 a,b); Postgate (1971) and Chatt and Leigh (1972). A series of papers presented at a meeting of the Royal Society, London, (Chatt and Fogg, 1969) covers many historical, chemical, biochemical, ecological and genetic aspects of legume and non-legume symbiosis, free-living micro-organisms and blue-green algae.

CHAPTER II

MATERIALS AND METHODS

PLANT PROPOGATION

Plants used in growth studies were propagated in a glass house at Lincoln College, Canterbury, under natural light.

(a) Broom

Seeds were shaken vigorously for 20-30 minutes while dry, and then surface sterilized in 100 vol. (30%) hydrogen peroxide for ½ hour. They were then germinated on dampened sterile germination pads in sterile petri dishes. As seeds germinated (commencing after approximately one week), they were transferred to sterile perlite in 2" plastic pots or in flamed metal trays and watered with ¼ strength nutrient solution (see below) containing nitrogen (100 mg/l). for several weeks before being given full strength nutrient or distilled water.

(b) Gorse

Gorse seed germination by this method was irregular and unreliable, possibly because the smaller size of gorse seed limited the removal of the strophiolar plug during dry shaking. Plants were, therefore propagated from 2 to 3 cm long cuttings, dipped in "Seradex" rooting hormone. These were placed in autoclaved sand and water with distilled water. About one month after setting out, well rooted cuttings (about 90%) were transplanted into sterile perlite in two-inch plastic pots or into sterile sand: perlite (1:1) in four-inch plastic pots and watered with nutrient solution containing nitrogen, or with distilled water.

Plants were later transplanted from these trays or pots as required. Innoculation was effected by standing plants in well grown cultures of

rhizobia isolated from nodules of gorse and broom and previously shown to be effective on re-inoculation. Suspensions of cultures were also poured, 5-10 ml per pot,on to the surface of the rooting medium.

NUTRIENT SOLUTIONS

The basic nutrient solution used throughout this work contained;-

Nutrient element	Compound used	Concentration of nutrient(mg/l)
K	KCl	20
Mg	$MgSO_4.7H_2O$	10
Ca	$CaCl_2$	80
S	Na_2SO_4+$MgSO_4.7H_2O$	40
P	$NaH_2PO_4.2H_2O$	25
Fe	Fe^{III} EDTA	15.2
Mo	$H_2MoO_4.2H_2O$ or Na_2MoO_4	0.022
Zn	$ZnCl_2$	0.12
B	H_3BO_3	0.5
Mn	$MnCl_2.4H_2O$	1.0
Cu	$CuCl_2$	0.04
Co	$CoCl_2.2H_2O$	0.026

plus nitrogen as $NaNO_3$ or $(NH_4)_2SO_4$ as required for various experiments. Phosphorus concentration was varied in two experiments by altering the amount of $NaH_2PO_4.2H_2O$ (Ch.VII). Boron-free solutions were made by omitting H_3BO_3 (Ch.IX). Laboratory grade chemicals were used throughout, except in the experiment on the effect of boron deficiency (Ch.IX) in which 'Analar' grade was used. Trace elements were used at half strength for that experiment, conducted in solution culture.

Concentrated stock solutions of each nutrient were made up in distilled water and stored in a refrigerator. These were diluted,5 ml of each to 1 l with distilled water as required.

PLANT ANALYSES

(a) Dry weight

Plant parts to be analysed were dried for at least 48 hours at 80-110°C in a forced draught oven and cooled in a desiccator over silica gel before weighing.

(b) Nitrogen content

Nitrogen content of ground plant tissues was analysed by a modified Kjeldahl method:- approximately 0.2 g of dried, ground plant material was digested in a 55 ml calibrated Kjeldahl flask with approximately 5 g K_2SO_4 $CuSO_4.5H_2O$ (1:1 w/w) (both A.R. grade) and 5 ml concentrated sulphuric acid (A.R.grade). After preliminary digestion at 240° - 250°C for several hours, final digestion was carried out at 370° - 380°C for ½ hour after clearing. After cooling, the digest was dissolved in water and diluted to 55 ml. A 10 ml aliquot was taken and ammonia recovered by steam distillation from alkaline solution.

Ammonia distillate was trapped in 10 ml of water and titrated with approximately 0.005M H_2SO_4. Recoveries of nitrogen from glycine by this method were 97 - 99%.

CHAPTER III

MORPHOLOGY AND DEVELOPMENT OF GORSE AND BROOM PLANTS

INTRODUCTION

Although it is outside the scope of this work to present a detailed description of the morphology and growth patterns of Ulex europaeus and Cytisus scoparius, a brief account will be given here of observations made during the course of experiments carried out in this project. A more detailed examination was made of the structure and development of root nodules of these two species. Their development followed the course already well known in Trifolium spp. Pisum sativum, Glycine max, Sesbeana grandiflora, Caragana arboroscens, Medicago sativa, Vicia hursuta and others. (Chen and Thornton, 1940; Harris et al, 1949; Allen et al, 1955 and Nutman 1956, 1959a).

(a) Shoots

Millener (1961, 1962) studied the juvenile-adult transition in gorse and the effects of day length and ecotypic variations upon it. The transition stage is illustrated in Plate 3.1a. There was a very clear juvenile phase with trifoliate leaves and no lateral growth. The adult phase was characterised by very reduced leaves or bracts subtending the lateral spines, which were modified shoots. Later in adult growth, many lateral branches were formed so that mature plants are divaricate woody shrubs.

Skipper (1922) described two types of shoot tips on mature gorse plants - those with terminal spines which had ceased growth, and those with soft bushy, actively growing tips. Both types of shoots were observed during this work. New shoot growth on cuttings was of the second type. When shoots developed from cuttings taken from mature wood, they appeared to go through a juvenile-like phase (Plate 3.1b).

The flowers on mature gorse plants develop singly or in pairs in the

axils of bracts, with the spines. These often form an inflorescence at the tips of stems (Plate 3.1c).

No mention was found in the literature of ecological or morphological studies on broom analogous to those mentioned above for gorse. It was observed during this work, that broom also has a juvenile growth phase, albeit less well distinguished from the adult phase than in gorse. In this juvenile phase, leaves are palmately trifoliate, each leaflet having an entire margin. Thus, at this stage, and for some time, broom plants closely resemble herbaceous legumes in appearance (Plate 3.2a). On adult plants, most leaves are small and simple and the stems ribbed. Leaves carried on axillary buds are often trifoliate with elliptical leaflets. Younger mature stems are green and ribbed (Plate 3.2b,c). Skipper (1922) fully described this type of structure for gorse. In both species, both ribbing and green colour are largely lost as stems age (Plate 3.2b). Broom flowers are borne singly in the axils of adult single leaves (Plate 3.2c).

In the transition from juvenile to adult foliage of broom, trifoliate leaflets become more pointed and side leaflets reduced until only the centre leaflet remains, (Plate 3.2b,c).

Leaves on adult plants of both species are thus very much reduced in size compared with juvenile plants. It must be assumed that in adult plants the younger stems (and spines in gorse) carry out the normal photosynthetic functions of leaves.

Egunjobi (1967, 1969, 1971) showed a large annual litter fall of 10 000 kg/ha/a in a stand of gorse. A similar, although smaller litter fall was observed for broom in this present study (see Ch.X). In broom, this litter was made up of considerable leaf fall and twigs.

(b) Roots

Two glass-sided boxes similar to those used by Butler et al (1959) were filled with a mixture of sand: soil: perlite (ca 1:1:1). Three gorse plants were transplanted into one box and three broom plants into the other.

The boxes were set up in February in a heated glasshouse, and the experiment continued over winter for 6 months.

Observations were made on root-systems at regular intervals by:-

(i) photographing root systems behind the glass plate,

(ii) following changes in nodules marked by white tape on the glass plate. These patches and others recording date and plant identification are shown in the following plates.

It has been anticipated that changes in nodulation would be recorded in the photographs. This, however, proved impossible because of poor definition.

The root systems of some of these plants are shown in Plates 3.3 a,b.

Plate 3.3a shows a typical root system of broom eight weeks after transplanting. Typical roots of gorse plants are similar to those illustrated for broom. The arrows mark the positions of root tips two weeks previously. It is evident that these root systems were characterised by relatively rapid growth of only a few major roots.

When the experiment was dismantled, extensive deep root development was evident for all plants (Plate 3.3b).

The amount of nodule senescence and decay observed during this experiment was very minor in gorse, but in broom considerable nodule turnover occurred between 6 and 10 weeks (Table 3.1).

Interesting comparisons can be drawn between the morphology and development of roots in this experiment and that observed in preliminary experiments carried out in solution culture (Plate 3.3c). Roots of plants grown in soil were characteristically fine and long with a few fine, but well developed laterals (Plates 3.3a,b). In contrast, plants grown in solution had much thicker, fleshier roots with shortened, thickened laterals.(Plate 3.3c)

A fuller discussion on the use of solution culture for studying growth and nitrogen fixation follows in Chapter VIII.

(c) Nodules

The normal legume nodule development process (viz:- curling of root hairs, infection thread formation and subsequent bacteroid formation) is well documented (Chen and Thornton, 1940; Bond, 1948; Harris et al, 1949; Allen et al, 1955; Nutman, 1956, 1959; Bergersen and Briggs, 1958; Raggio and Raggio, 1962; Jordan, 1962; Dart and Mercer, 1963, a, b, 1964, a, b, 1965 b, c; Dixon, 1964; Goodchild and Bergersen, 1966; Bergersen, 1968; Pankhurst et al, 1972).

Observations on nodule development were made on broom plants grown in soil/perlite mixture in 4 inch plastic pots, and on rooted gorse cuttings grown in sand. The plants were watered daily and given nutrient solution regularly. Inoculation was effected by pouring a suspension of crushed field-grown nodules over the growth medium. The plants were then watered well.

Samples of 6 broom and 7 gorse plants were taken at random 4 days after inoculation and then at intervals over the next 6 weeks. Root systems were carefully washed out and the nodules counted. They were then fixed in glacial acetic acid/ethanol (1:3) for 3 hours and stored in 700 ml/l aqueous ethanol. Root samples taken before the appearance of the first nodule were preserved directly in 700 ml/l ethanol.

Typical nodules were chosen from each stage and after embedding in wax and sectioning, were stained according to the procedure of Allen et al(1955). Selected root systems from the inoculation period and early in the subsequent nodule formation phase were stained with Loefflers Alkaline Methylene Blue (Purchase, 1958), and examined under a microscope. According to Purchase (loc.cit.), this differentially stains root hairs and infection threads. Careful examination of root systems using this technique, failed to reveal any infection thread formation. However, as the number of root hairs which become infected is a very small proportion of the total number(Yao and Vincent, 1969), it is unlikely that infection threads would be observed by this technique (Lowther, pers.comm.).

The mode of infection in Arachis hypogea is through points of emergence of lateral roots. As a result, all nodules are subtended by a root (Nutman, 1956). Such roots were rarely observed in gorse and broom. Thus it must be assumed that the mode of infection in these species is the normal mechanism of root hair curling and infection thread formation.

Photomicrographs of nodule sections are shown in Plates 3.4 - 3.11a. As the structure and development of gorse and broom nodules appeared identical, the following discussion will not distinguish between the two species.

Plate 3.4 shows a section through a very young nodule, 12 days after inoculation. At this stage nodules were visible macroscopically as small waxy white swellings on the roots. Intensified nuclear staining is clearly visible within the nodule by comparison with the few normal nucleii remaining in cells on the opposite side of the stele. This intensified nuclear staining presumably reflects changes in nuclear structure following bacterial invasion.

Plate 3.5 shows a nodule 16 days after inoculation. These nodules appeared as protuberances, approaching spherical in shape, up to about 1 mm in diameter. There is a well developed bacteroid region and development of conducting vessels from the root stele has started. Dense cell packing, larger cell size and degeneration of nucleii within the bacteroid region is evident. The meristem is clear, and there is evidence of formation of inclusion granules in the cytoplasm of cells surrounding the bacteroid region.

At the next stage of development (Plates 3.6a,b-3.7) (22 days after inoculation) haemoglobin formation was beginning. There is now no evidence of a meristem. The conducting system is better developed, and the open, aerated structure of nodules, similar to that described for soybeans (Bergersen, 1968) is becoming apparent. The development of starch-like granules in the cytoplasm of cells surrounding the bacteroid region (Plate 3.6b) is more apparent than in younger nodules. Under higher magnification, the dense granular appearance of the cytoplasm of invaded cells was apparent, (Plate 3.7). The staining technique used here did not differentiate the actual bacteroids. The cortical zone between two bacteroid regions may indicate the beginning of

lobe formation (Plate 3.6b, 3.7).

Plate 3.8 shows the well developed conducting system along a nodule 28 days after inoculation. At this stage nodules were approximately 2 mm long and 1 mm wide. These sections were made either above or below the bacteroid zone of the nodule and therefore show an extensive area of cortical cells containing starch-like granules. The conducting system has apparently formed around the upper or lower (or both) surfaces of the bacteroid zone, and not through the centre of the nodule. Greater magnification of these sections (Plate 3.9a) shows the abundance, shape and structure of cytoplasmic granules, the dense staining cytoplasm, and diffuse degenerate nucleii in the bacteroid-containing cells.

Fortythree days after inoculation, an active meristem is again apparent in sections of the tip of the nodule (Plate 3.9b). Cells from the meristem apparently form bacteroid-containing cells to the inside, and large vacuolated or empty husk or cortical cells to the outside (cf. Dart and Mercer, 1965c). The open aerated structure of the bacteroid region (cf. Bergersen, 1968) is clear from this section. This zone clearly occupies almost the entire volume of the nodule. The granule-containing cortical cells, the conducting system and the nodule husk are confined to a narrow layer around this zone. The nodules at this age had increased considerably in size compared with the previous harvest, to approximately 3 x 2 mm.

Plates 3.10 and 3.11a show sections of nodules taken 20 weeks after inoculation. These nodules were well developed and appeared to be active. Plate 3.10 shows the strand of conducting tissue between the root stele and nodule, and the basal portion of the bacteroid tissue. The structure and arrangement of cells are again clear in this section. Cells of the surrounding cortical tissue are much more closely packed than those of the bacteroid region. Changes in nuclear structure are clear from these plates. Plate 3.11a shows a photomicrograph of a section through the distal portion of the nodule shown in Plate 3.10. The meristematic region, and area of differentiating bacteroid tissue are clear. Several strands of conducting tissues are visible. At this

stage, the development of a lobed structure, typical of older nodules of these species, was well defined.

DISCUSSION

The similarity in structure and development of nodules of gorse and broom to those of other species was striking. The lack of any evidence to the contrary suggests that the mode of infection was through the root hairs as in Trifolium spp, Pisum sativum, Medicago spp, Vicia spp.and others.

Growth of nodules of herbaceous legumes ceases after a relatively short time (Bergersen, 1968). The longevity of nodules of gorse and broom and of other woody species such as Sesbania grandiflora (Harris et al, 1949) and Caragana arborescens (Allen et al, 1955), resulted from continued or renewed meristem activity. Presumably this was accompanied by degeneration of older tissues, leading to the characteristic appearance of these perennial nodules (Pate, 1958, 1961a). It is obvious from the plates that layers of cork-like cortex were maintained on the outside of roots and nodules, hence the typical woody appearance of these nodules.

Vascular systems of gorse and broom nodules developed at an early stage and grew to encircle the nodules, with branches running through the cortex as in Caragana arborescens (Allen et al, 1955) and Trifolium subterraneum (Bergersen 1970b). Nodule rootlets characteristic of some other woody legumes, e.g. Sesbania grandiflora (Harris et al, 1949) and C.arborescens (Allen et al, loc.cit), were not observed in gorse or broom. The existence of starch-like granules in the cytoplasm of cortical cells has been also observed in S.grandiflora (Harris et al, loc.cit), but not in soybeans (Bergersen, 1968). If these granules are an endogenous energy source for the nodule, it would explain the relatively long life of excised nodules of these two species, as distinct from the short lived soy bean nodules (see Ch.IV and V).

Apart from their structural adaption to longevity, and possibly their high proportion of bacteroid tissue compared with S.grandiflora

(Harris et al, 1949) or C.arborescens (Allen et al, loc.cit.), the structure of these nodules was similar to that of other legume nodules.

Comparison of the structure and development of nodules and roots (Plate 3.11b) showed quite clearly that nodules on these species are not modified roots, as is the case with non-legumes (Silvester, 1968), but are specialized structures within the root cortex.

In this experiment the time taken for the first visible nodule to appear (12-16 days) was rather longer than the 4-7 days reported for Medicago sativa (Munns, 1968a) and Trifolium subterraneum (Gibson, 1967b). This difference probably reflects 2 factors:-

(1) the lower growth rate of woody species gorse and broom,

(2) the effect of delayed inoculation (Dart and Pate, 1959).

While the structure of gorse and broom nodules was typical of legumes, and the growth of roots, although rapid, was not unusual, the growth and development of shoot systems was different from that of most legumes. In the juvenile growth phases, however, both species were very similar to herbaceous legumes. The shoot modifications shown by adult plants appear peculiar to these two species.

CHAPTER IV

ACETYLENE REDUCTION BY EXCISED GORSE AND BROOM NODULES

INTRODUCTION

Independent observations of the reduction of acetylene to ethylene by nitrogenase enzyme preparations of Clostridium pasteurianum (Dilworth, 1966) and Azotobacter vinelandii (Schollhorn and Burris, 1967) led to development of this system as a new and powerful, if indirect, tool in the study of nitrogen fixation. This system is much quicker, simpler, and considerably more sensitive than use of the stable isotope $^{15}N_2$, or detection of nitrogen fixation by Kjeldahl analysis. Since its first discovery in these two bacterial systems, reduction of acetylene to ethylene has been observed in legume nodules (Koch and Evans, 1966) and their cell-free extracts (Koch et al, 1967 a,b; Klucas and Evans, 1968), non-legume nodules (Sloger and Silver, 1967; Silver, 1967, 1969), endophyte from leaf nodules of Psychotria bacteriophila (Silver, 1969), blue-green algae (Stewart et al, 1967, 1968, 1971; Cox and Fay, 1969; Stewart, 1969; Vanderhoef et al, 1972; Ohmori and Hattori, 1972) as well as in many nitrogen fixing bacteria(e.g.Kelly, 1968; Mahl and Wilson, 1968; Biggins and Postgate, 1969; Weinard et al, 1971; Dobereiner et al, 1972).

The general association of this reaction with a wide range of nitrogen fixing systems, is further circumstantial evidence of the similarity of the biochemical mechanism of all known nitrogen fixing enzyme systems. The use of this assay technique was extensively reviewed and evaluated by Hardy et al (1968). This system has been used in a range of ecological studies (e.g. Stewart et al, 1967, 1968, 1971; Moustafa et al, 1969; Stewart, 1969; Vanderhoef et al, 1972; Waughman, 1972) and biochemical studies (Kelly, 1968; Klucas and Evans, 1968; Moustafa and Mortenson, 1968, 1969; Biggins and Postgate, 1969; Moustafa, 1969; Bergersen and Hipsley, 1970; L'vov et al, 1971; Pankhurst et al, 1972). Much of this work has been summarized in a

series of papers (Chatt and Fogg, 1969).

It is not possible at present, to use the acetylene reduction technique as a measure of the amount of nitrogen fixation occurring within an ecosystem, as the quantitative relationship between nitrogen fixation and acetylene reduction varies considerably in different conditions (Bergersen, 1970a; Magee and Burris, 1972). However, it is possible to compare rates of acetylene reduction under different experimental conditions. In this study the acetylene reduction technique was used in experiments studying properties of the nitrogen fixing systems of excised gorse and broom nodules.

MATERIALS AND METHODS

Commercial grade ethylene, acetylene, argon and oxygen were used throughout the experiments described in this chapter.

Acetylene and ethylene were analysed on a Perkin-Elmer F 11 gas chromatograph fitted with a hydrogen flame ionization detector and a stainless steel column (4 feet x 1/8 inch O.D.) of 'Porapak T' (Varian Aerograph Co., California) at 100°C. Nitrogen (25 - 30 ml/min) was used as carrier gas. Gas samples were taken in 1 ml tuberculin syringes fitted with 26g x $1\frac{1}{2}$ inch needles, and 0.5 ml was used for each injection. Water was allowed to enter the flasks to replace the volume of gas removed.

For experiments on excised nodules, 10 litres argon: oxygen(75:25/v:v) gas mixture was made up by water displacement. Acetylene was stored over water in a separate flask fitted with a serum seal. The aspirator containing the argon:oxygen gas mixture was connected to the manifold as were nodule incubation flasks. The manifold and flasks were then flushed, at least six times, by evacuating to 0.5 atmosphere and restoring the pressure to one atmosphere with the argon:oxygen mixture. Magee and Burris (1972) demonstrated that evacuating incubation systems in such a manner had no effect on acetylene reduction by excised soybean nodules. After removal of an appropriate volume of gas mixture from the incubation flask by syringe, acetylene to make

approximately 0.15 atmosphere was added from another syringe. Thus the final gas mixture was approximately argon: oxygen: acetylene, 64: 21: 15 (v:v:v). The volume of nodules in one experiment was almost constant at less than 8% of total volume. For convenience the volume of nodules was ignored in calculating results of all experiments.

Calibration

A standard curve was constructed to relate height of peaks measured on the recorder to the amount of acetylene or ethylene injected. For the preparation of standard gas mixtures a glass manifold was constructed. This manifold consisted of a length of glass tubing with a vacuum tap and "Quickfit" B19 socket at each end. A row of twelve B14 "Quickfit" sockets was fitted on one side of the manifold and one socket on the other. Ten 100 ml and two 200 ml "Quickfit" flasks with vacuum taps on sidearms were fitted into the row of sockets. These flasks were also equipped with serum seals in B19 "Quickfit" screw fittings. A mercury manometer was fitted into the single socket on the reverse side.

Argon and oxygen cylinders were connected to one end socket and to the third flask respectively. Acetylene and ethylene were introduced separately into the evacuated system and stored in the first and second flasks respectively. After each gas was introduced, the manifold with remaining flasks was flushed with argon and evacuated again. With four flasks open to the manifold experimental gases were let in turn into the system, and the pressure of each gas read on the manometer. The final pressure was made to one atmosphere with argon. By applying the Universal Gas Equation $PV = nRT$ to partial pressures thus obtained, and to temperature, it was possible to calculate the concentration of each gas.

After closing the taps two flasks were removed and allowed to equilibrate in a water bath. Six 0.5 ml aliquots were injected into the gas chromatograph using a gas-tight syringe. Two new flasks were connected to the manifold and evacuated. The gas in the remaining flasks was let into

the system, more acetylene added if necessary, and the pressure made to one atmosphere with argon. Samples were taken from these flasks and this process was repeated until the lower limit of detection of the gas chromatograph was reached.

The standard curve obtained is shown in Fig.4.1. There was a linear-logarithmic relationship between the amount of acetylene or ethylene injected and the peak height on the recorder. Point A on the graph for acetylene and point G on that for ethylene were obtained from analysis of a different gas mixture to the other points on each graph. Of the remaining points, B, C and D all had extra acetylene added during each dilution. The facts that: (a) all these points on the graph for acetylene form a straight line, and (b) point G falls on the same straight line as the other points for ethylene, substantiate the validity of the method used in making standard gas mixtures. For the analytical system used the maximum amount of either acetylene or ethylene which could be determined was 2-3 µmoles per 0.5 ml aliquot. At greater concentrations than this, the peaks spread out, and therefore measurement of peak area would have been necessary. In practice, ethylene concentrations of this order were approached only rarely.

INCUBATION EXPERIMENTS

Experiment I

Gas samples in this experiment were analysed on a Varian Aerograph Series 1200 gas chromatograph connected to a Hitachi recorder. As standard curves could not be prepared for this equipment, results are expressed in arbitary units (height of ethylene peak on recorder (in inches) x 2 x incubation volume). These units are proportional to the concentration of ethylene present.

Nodules from two year old gorse plants (5.42 g f.w.) or from one year old broom plants (3.29 g f.w.) were incubated in 100 ml "Quickfit" conical flasks fitted with serum seals. Incubations were started within 30 minutes of picking the first nodule. Gassing was carried out on a metal manifold to which

syringe needles were attached. Eleven gas samples were taken during the 6 hour incubation at 23°C. Results are presented in Fig.4.2.

No correction was made for the amounts of gas removed during sampling or for weights of nodules used in the incubations. For these reasons, and because the gas chromatograph column was run at a 70°C for the gorse and 100°C for the broom nodule samples, the results obtained for the two species were not comparable. In spite of this, it was apparent that acetylene reducing activity continued for at least four hours in broom nodules, and five hours in gorse nodules.

This time is considerably longer than the 1 - 2 hours observed for acetylene reduction and $^{15}N_2$ fixation in other legumes. (Aprison and Burris, 1952; Bond, 1959, 1963; Nutman, 1959b, 1963; Stewart, 1966; Koch and Evans, 1966; Dixon, 1967; Hardy et al, 1968; Mague and Burris , 1972).

Experiment II

Excised gorse nodules (1.30 - 1.79 g f.w.)and excised broom nodules (0.6 - 1.0 g f.w.) were incubated for one hour at five temperatures-16.0°C, 20.0°C, 24.0°C, 25.5°C and 30.5°C.(all $\pm$ 2.5°C), in 45 ml "Quickfit" tubes fitted with serum seals. A syringe needle connected to a short length of water-filled plastic tubing was inserted through each serum seal to allow gas pressure inside the tubes to equalise with atmospheric pressure. These tubes were kept in place throughout the whole experiment. After tubes had equilibrated in each temperature bath for 20 minutes, acetylene to make a concentration of 0.15 atmosphere was added. Gas samples were taken from each tube after one hour, and analysed on the Perkin-Elmer F-11 gas chromatograph. Results were expressed in terms of three measurements of the amount of nodule material:-

1. amount of protein, water extracted from the nodules (Layne, 1957).
2. nodule fresh weight before incubation.
3. dry weight of nodule residue remaining after protein extraction.

Unfortunately, both protein extracts from the 16°C incubations were

lost before measurement.

The results (Fig.4.3) indicate a temperature optimum at 20°C for both species. There were no large differences among the three ways of expressing activities. As the most convenient and reproducible measure was that based on dry weight of the nodules, all results from subsequent experiments were expressed in this way.

It is also clear from the graphs, that there was a large difference between the acetylene reducing ability of the two species. At the optimum temperature, activity of broom nodules was approximately seven times that of gorse nodules. This possibly results, at least partly, from differences in ages of plants from which nodules were excised. Nodules of both species contained similar concentrations of protein. If the nitrogenous enzyme system represents the same proportion of total water-soluble protein in nodules of both species, then the energy transfer or reductant transfer systems must be less efficient in gorse nodules than in broom nodules.

The temperature optimum observed here (viz.20°C) was considerably lower than the optimum for ^{15}N fixation in soybean nodules (Aprison et al, 1954) and acetylene reduction in Alnus glutinosa (Wheeler, 1971), but was similar to the optimal temperature for $^{15}N_2$ fixation by Alnus viridis (Benecke, 1970).

It has been noted in the past that nodules of most legumes cease $^{15}N_2$ fixation or acetylene reduction relatively soon after excision (Aprison and Burris, 1952; Bond, 1959, 1963; Nutman, 1959b, 1963; Stewart, 1966; Koch and Evans, 1966; Dixon, 1967; Hardy et al, 1968; Mague and Burris , 1972). This limited duration of activity following excision could be caused by substrate or oxygen depletion, a build-up in inhibitor, or exhaustion of endogenous energy reserves. If it is caused by substrate or oxygen depletion, then restricting the rate of acetylene reduction by utilising slightly sub-optimal temperatures, or acetylene or oxyten concentrations should result in longer periods of lower activity than under optimal conditions. The total amount of ethylene formed under these slightly sub-optimal conditions would be similar to that

formed under optimal conditions. If, however, cessation of activity results from increasing concentrations of a competitive inhibitor (e.g. hydrogen, see Sprent, 1969), then sub-optimal acetylene concentrations would shorten the period of activity and supra-optimal acetylene concentrations would lengthen it.

Sprent(1971) showed that drying of detached soybean nodules caused reduction in acetylene reduction. When fresh weight was reduced to 80% of its turgid value, acetylene reduction ceased. These changes were associated with gross and microscopic changes in the structure of nodules (Sprent, 1971, 1972a). These changes however, do not explain the normal cessation of activity as, reduction of water content to 80% of turgid value caused proportionate reduction in acetylene reduction (Sprent, 1971), not the relatively rapid cessation usually observed. Also, Sprent (1969) was able to re-start acetylene reduction in excised soybean nodules, by renewing the gas phase. The changes she observed at 80% turgid fresh weight, were, however, irreversible (Sprent, 1971, 1972a).

To ensure that longevity of the gorse and broom nodules observed in Experiment I did not result from sub-optimal temperature, oxygen or acetylene concentrations, the effect of these three variables on the time course of activity were tested in a series of three experiments.

Experiment III

This experiment was designed to examine more closely the various time course of acetylene reduction at various incubation temperatures for excised gorse and broom nodules.

Gorse nodules (3.1 - 4.0 g f.w. (0.5 - 0.75 g d.w.)) and broom nodules (2.4 - 3.2 g f.w. (0.4 - 0.7 g d.w.)) excised from plants in their second season were incubated in the 100 - 200 ml "Quickfit" flasks used in constructing the standard curve. Incubations were carried out at six temperatures, $15^{o} \pm 1.0^{o}C$, $17.75^{o} \pm 0.25^{o}C$, $20.0 \pm 1.0^{o}C$, $22.0^{o} \pm 1.0^{o}C$, $24^{o} \pm 0.1^{o}C$ and $26.1^{o} \pm 0.1^{o}C$. Flasks containing nodules were allowed to equilibrate for

20 minutes at each temperature, before acetylene (to a concentration 0.11-0.13 atmosphere) was added and excess pressure released. Incubation was started within $2\frac{1}{4}$ hours of picking the first nodule. Gas samples were taken over the next 22 hours, most samples being taken at 20°C - the optimum temperature shown in Experiment II.

The results, expressed as μmole ethylene produced per gram of nodule dry weight, over the incubation period, are presented in Figs.4.4, 4.5.

In this experiment, acetylene reducing activity of broom nodules continued for seven hours at the optimum temperature (22°C) and then stopped completely (Fig.4.4A). At 20°C and 24°C, lower activity continued for a longer time than at the optimum temperature. Thus, total amounts of ethylene produced at 20°C and 24°C were only slightly less than that produced at the optimum (22°C). At 18°C, ethylene production ceased after approximately 16 hours. At 16°C acetylene reduction continued for most of the 22 hour incubation period, however the rate was greatly reduced. Thus, the total amount of ethylene formed in 22 hours of activity at 16°C was only 75% of that produced in 7 hours at 22°C. These effects are consistent with substrate, energy or oxygen depletion being the factor limiting length of activity following excision.

At 26°C acetylene reducing activity of broom nodules was severely restricted (Fig.4.4A). This possibly reflected a degree of temperature inactivation of the enzyme systems involved in acetylene reduction. If this was so, nitrogen fixation by broom in the field may also be expected to be severely reduced by prolonged soil temperatures higher than 26°C.

Gorse nodules appear to reduce acetylene to ethylene at a considerably longer time after excision than broom nodules (Fig.4.4B). Activity of gorse nodules continued at all temperatures for the whole 22 hour incubation period. The total amount of ethylene produced by gorse nodules at the optimum temperature was approximately half of that produced by broom nodules at 20°, 22° or 24°C.

As acetylene reduction by gorse nodules was still occurring at all

temperatures, the amounts of ethylene produced in 22 hours reflect the relative reduction rates at each temperature.

For both these species maximum acetylene reduction occurred at 22°C (Fig. 4.5). This was rather lower than the 25°C optimum for $^{15}N_2$ fixation in soybeans (Aprison et al, 1954) and for acetylene reduction by Alnus glutinosa nodules (Wheeler, 1971). In contrast to the results of Experiment II (Fig.4.3), there was little temperature response in a one hour incubation period. A marked response of acetylene reduction by broom nodules to temperature was clearly apparent after 2 hours, and this response became accentuated with longer incubations (6 - 8 hours). There was only a small response to temperature in gorse.

The differences between the results of Experiments II and Experiment III probably stem from differences in activities of nodules used. Similar amounts of excised broom nodules in terms of incubation volume were used in both experiments. However in Experiment II the maximum activity was 120 μmoles ethylene produced per g d.w. of nodule per hour and in Experiment III it was approximately 12 μmoles per g d.w. of nodule per hour. No explanation for the 2°C difference in temperature optimum between the two experiments is apparent. It is possible that because of the larger incubation vessels used in Experiment III, the nodules and gas mixtures were not as well equilibrated in that experiment as in Experiment II.

Nodules of both species were more adversely affected by high temperatures than they were by temperatures below the optimum. A rise in temperature of 4°C (22° - 26°C) depressed the activity of gorse nodules by nearly 50% and that of broom nodules by 75%, whereas at 16°C, 6° below optimum, activity was only 30% and 57% lower than at optimum for gorse and broom respectively. This is in contrast to the effect of sub- and supra-optimal temperatures on acetylene reduction by excised nodules of Alnus glutinosa (Wheeler, 1971). In that species a 6-fold increase in acetylene reduction was observed between 14° and 25°C and with decreases of only 20% and 50 - 55% at 5° and 12.5°C respectively above the optimum.

Experiment IV

Gorse nodules (5.8 - 6.0 g f.w. (0.95 - 1.75 g d.w.)) from plants in their second season were incubated at 22°C in each of five gas mixtures of different known oxygen tensions in duplicate flasks. These mixtures were made up in the incubation flasks. After flushing six times with argon/acetylene (80: 20; v:v), gas pressure was reduced to 0.5 atmosphere and oxygen was introduced. The gas pressures were measured on the manometer and total pressure in the flasks made up to one atmosphere with argon. Samples were taken at five intervals up to seven hours and again after 20 hours. The results are presented in Figs. 4.6 and 4.7.

Figure 4.6 shows that an oxygen concentration of 0.176 atmosphere was sufficient for maximum initial activity. The rate of ethylene production at this low oxygen concentration reduced with time (between 1 and 3 hours) presumably as the oxygen concentration was reduced by respiration. This oxygen concentration (0.176 atmosphere) was therefore probably only slightly less than the optimum concentration for the initial rate of acetylene reduction by gorse nodules. However an initial concentration of 0.225 atmosphere or greater was necessary for longer incubations. Thus an initial oxygen concentration of 0.274 atmosphere permitted maximum linear activity for seven hours. Acetylene reduction then ceased at this oxygen concentration. A higher oxygen concentration (0.354 atmosphere) completely inhibited acetylene reduction for the entire 20 hour incubation period. Presumably some tissue respiration occurred within the nodules during this time. This, however, did not reduce the oxygen concentration sufficiently to permit acetylene reduction to commence. Some acetylene reduction occurred at nominally zero oxygen concentration. This probably reflected the oxygen present in nodule tissue or a small concentration of oxygen in the argon-acetylene mixture. This gas mixture was made up over water and no specific steps were taken to ensure that no oxygen was present. As no ethylene production occurred at 0.354 atmosphere oxygen and none after

seven hours incubation at 0.274 atmosphere oxygen it is unlikely that endogenous ethylene production by excised nodules contributed to the measured activity at the lower oxygen concentrations.

At 0.176 atmosphere oxygen, acetylene reduction apparently continued at a measurable rate between 7 and 20 hours incubation. The amount of ethylene present after 20 hours incubation at this oxygen concentration was 50% more than the amount present after seven hours. An initial oxygen concentration of 65% of optimum thus reduced the amount of ethylene produced in 20 hours to 72% of the amount produced at the optimum concentration. At 20% and nominally 0% of the optimum concentration, ethylene production over 20 hours was 16% of the maximum amount produced. It is clear that sub-optimal oxygen concentrations restrict the rate of acetylene reduction by excised gorse nodules. This activity, however, continues for a considerably longer time than the seven hours observed at optimum oxygen concentration. There was therefore no evidence that depletion in oxygen supply caused cessation in activity in excised nodules.

Shapes of the curves in Figs.4.7 are very similar to those obtained for $^{15}N_2$ fixation of excised nodules of Casuarina cunninghamii, Hippophae rhamnoides, Myrica gale, and Pisum sativum (Bond, 1961) and Myrica cerifera (Silver, 1969). Bond (1964) also showed that $^{15}N_2$ fixation decreased with increasing oxygen tensions above 0.2 atmosphere for excised Casuarina cunninghamii nodules. In Glycine max nodules, oxygen tensions up to 0.5 atmosphere increased $^{15}N_2$ fixation (and respiration) but oxygen tensions above that decreased $^{15}N_2$ fixation (Burris et al, 1955; Bergersen, 1962, 1966; Bergersen and Turner, 1968; Hardy and Knight, 1969). Wilson and Fred(1937) showed that nitrogen fixation by nodules of intact Trifolium pratense increased with increasing oxygen tensions up to 0.2 - 0.4 atmosphere and decreased above this.

Acetylene reduction by G.max nodules has been reported to require optimal oxygen concentrations similar to(Bergersen 1970a;Magee and Burris, 1972) or much lower than (Koch and Evans, 1966) for $^{15}N_2$ fixation.

Koch and Evans (1966) showed that 0.2 atmosphere oxygen was optimal for acetylene reduction by excised soybean nodules. Bergersen (1970a) showed that 0.5 atmosphere oxygen was optimal when 0.1 atmosphere acetylene was used, but was inhibitory when using 0.005 atmosphere acetylene. However Mague and Burris (1972), observed maximum acetylene reduction at 0.7-0.9 atmosphere oxygen in that species. No inhibition of acetylene reduction was observed in those studies at oxygen concentrations above optimum, at acetylene concentrations of 0.1 atmosphere or greater. The difference between the effect of high oxygen concentrations - greater than 0.5 atmosphere - on acetylene reduction and nitrogen fixation reflects the much greater solubility in water of acetylene than nitrogen, (Bergersen, loc.cit).

Optimal oxygen concentrations for nitrogen fixation alters with nodule age (Bergersen, 1962; Krikunets & Belima, 1971). This probably explains some of the variations in optimal concentrations for acetylene reduction by soybeans mentioned above.

The optimum oxygen concentration observed by Koch and Evans (1966) for acetylene reduction by soybean nodules (viz. 0.2 atmosphere) was similar to that observed in this experiment with excised gorse nodules.

Breis or bacteroid suspensions require much lower oxygen concentrations for nitrogen fixation than do intact nodules (Bergersen, 1968, 1969; Bergersen and Turner, 1967, 1968). If suitable sources of energy or reducing power were provided, oxygen was not required for acetylene reduction or nitrogen fixation by breis, bacteroid suspensions or cell free extracts (Koch et al, 1967, a,b).

Thus the effect of sub-optimal or optimal oxygen concentrations on acetylene reduction by gorse nodules appeared similar to the effect on nitrogen fixation by legume and non-legume nodules studied by Bond(1961). The optimum oxygen concentrations for gorse and the species used in Bond's (loc.cit) study were 0.12 atmosphere for Hippophae rhamnoides nodules, approximately 0.2 atmosphere for Pisum sativum and approximately 0.27 atmosphere for Casuarina cunninghamii. These last two optimal oxygen

concentrations are similar to those shown for acetylene reduction in gorse in this present study (Fig.4.7).

Severe depressions in nitrogen fixation were observed by Bond (1961) at 0.40 atmosphere oxygen for all species. In no case, however, was nitrogen fixation completely inhibited at this concentration as it was in gorse. Similar results for Alnus nodules had been observed by Bond in an earlier study (quoted in Bond 1961).

Parker and Scutt(1960) suggested that oxygen and nitrogen compete as terminal hydrogen acceptors in Azotobacter. Other workers using whole nodules or nodule breis confirmed this view (Bond, 1961; Bergersen, 1962, 1969, 1970a; Bergersen and Turner, 1968). In this experiment optimal acetylene reducing activity was observed at an initial concentration of 0.2 to 0.27 atmosphere oxygen. The optimum oxygen concentration was slightly higher for longer incubation periods. Activity fell off rapidly at higher concentrations to nil at 0.35 atmosphere oxygen. These results also support the view that oxygen and acetylene compete in acetylene reduction.

Experiment V

For this experiment the actual gas mixture used was Ar:O_2,60:40 (v:v). Mixtures of known acetylene concentration were made up in a manner similar to that used for the oxygen concentrations in Experiment IV (see page 32). Gorse nodules (6.1 - 7.1 g f.w. (1.0 - 1.4 g d.w.))were incubated at 22°C in duplicate flasks at each acetylene concentration. Results are presented in Fig.4.8.

There appeared to be an initial inhibition of acetylene reduction at the highest acetylene concentration (0.203 atmosphere). However, this temporary inhibition could have been caused by exposure of the nodules to high oxygen tensions during the preparation of the gas mixtures. This was the only experiment (except at high oxygen concentrations in Experiment IV) in which nodules were exposed to a gas mixture containing an oxygen concentration shown to be inhibitory (viz. 0.4 atmosphere).

Incubation mixtures were prepared serially from the lowest acetylene concentration to the highest. Nodules at the highest acetylene concentration (0.203 atmosphere) were therefore in contact with the high oxygen concentration for approximately ten minutes longer than those at the lowest acetylene concentration.

Bergersen (1962, 1969) showed that increasing oxygen tensions increased the oxygenation of the leghaemoglobin, giving competitive inhibition of acetylene reduction. Reduction in the oxygenation of leghaemoglobin by respiration, once the oxygen concentration in the flask was lowered, would allow acetylene reduction to start.

The slopes of the graph at 0.203 atmosphere acetylene, between $3\frac{1}{2}$ - 7 hours, after the initial inhibition had ceased, and at 0.131 atmosphere acetylene from 0 - 3 hours are very similar. Both these concentrations of acetylene were probably initially supra-optimal (Fig.4.8).

The analytical system used was not capable of detecting changes in the high concentrations of acetylene. Some decrease with time was noted, however, at lower concentrations.

At all acetylene concentrations, ethylene production continued for considerably longer than the seven hour incubation period. This was especially true in the highest acetylene concentration, (0.203 atmosphere) where only 50% of the final amount of ethylene was produced in the first seven hours.

Excised soybean nodules show maximal acetylene reducing activity at 0.1 atmosphere (Koch and Evans, 1966) to 0.2 atmosphere acetylene (Magee and Burris, 1972). Nodulated roots and soil samples were saturated at only 0.025 atmosphere acetylene (Hardy et al, 1968). Moustafa (1969) used a gas atmosphere of 0.65 atmosphere argon, 0.25 atmosphere O_2 and 0.1 atmosphere acetylene and gas compositions of this order seem typical for this work and for work with $^{15}N_2$ (Aprison and Burris, 1952; Magee and Burris, 1954; Virtanen et al, 1954, 1955; Bond, 1961; Sloger, 1969; Silver and Magee, 1970; Waughman, 1972). Such oxygen concentrations would be supra-optimal

for short incubations and sub-optimal for longer incubations in gorse nodules (Fig.4.7). This acetylene concentration is sub-optimal for gorse (Fig.4.8).

DISCUSSION

In Experiment V both sub- and supra-optimal acetylene concentrations enabled acetylene reducing activity to continue for a considerable length of time. With sub-optimal acetylene concentration, the rate of ethylene production was less than at higher concentrations. This effect was similar to those observed in Experiment III with sub-optimal temperature and in Experiment IV with sub-optimal oxygen concentration. There were, however, differences between the effects on the rate of acetylene reduction of supra-optimal temperature or oxygen concentration and the effect of supra-optimal acetylene concentration. Whereas acetylene reduction was severely inhibited by high temperature and oxygen concentration, it was unaffected by a high acetylene concentration. This effect was similar to that observed by Koch and Evans (1966) with excised soybean nodules.

Although acetylene reduction ceased after seven hours at 0.131 atmosphere acetylene, ethylene production evidently continued for some time further at 0.203 atmosphere acetylene. This suggests that normal cessation of acetylene reduction by gorse nodules after approximately seven hours incubation was caused by increasing concentrations of an inhibitor in the gas phase. Sprent (1969) suggested that this inhibitor may be hydrogen. If this were so, then this inhibitor may be expected to be more effective at acetylene concentrations below optimum. This effect was not observed in this experiment. Thus it was unlikely that cessation of acetylene reduction at optimal temperature, oxygen or acetylene concentration was caused by such an inhibitor. This longevity at high acetylene concentrations was probably caused by use of an oxygen concentration that was sub-optimal at this acetylene concentration. The temporary inhibition of acetylene

reduction by incubation in high oxygen concentrations showed that such inhibition was reversible.

The lack of inhibition of acetylene reduction by supra-optimal oxygen concentrations in excised soybean nodules (Koch and Evans, 1966) was an effect of competition from supra-optimal acetylene concentrations. Acetylene is approximately 65 times more soluble than nitrogen in water (Bergersen, 1970a). Thus apparently optimal acetylene concentration (0.1 atmosphere) are, in fact, much greater than those needed to saturate the nitrogenase enzyme system. Concentrations of 0.025 atmosphere acetylene, (Hardy et al, 1968) would suffice for activity, provided oxygen concentrations were not too high. Bergersen (1970a) observed inhibition of acetylene reduction at 0.5 atmosphere oxygen when the acetylene concentration was 0.005 atmosphere, but not when it was 0.1 atmosphere.

These results and results of Experiments IV and V indicate complex interactions among oxygen and acetylene concentrations, rate of acetylene reduction, and time course of acetylene reduction after nodule excision. Lack of time precluded further investigation of these interrelationships and the extension of these results to broom nodules.

However, shapes of the graphs of time course of acetylene reduction at the optimal temperature (Figs.4.4 A,B) suggest that oxygen or acetylene concentrations were optimal or supra-optimal for broom but possibly sub-optimal for gorse. This suggests a lower oxygen or acetylene requirement for broom than for gorse.

It was clear in each of these experiments (III, IV and V) that sub-optimal conditions enabled acetylene reduction to continue for a longer time, at a rate lower than at the optimum conditions. When temperature and oxygen and acetylene concentrations were optimal, the time course of acetylene reduction after excision appeared linear for 7 - 9 hours with cessation of activity after this (Figs. 4.4A, 4.6, 4.8). There was no evidence that this cessation in activity was caused by depletion of oxygen or acetylene supply.

There was also no evidence that it was caused by increasing concentrations of an inhibitor. All results presented were consistent with cessation in activity being caused by depletion of endogenous energy reserves.

This time course differs considerably from those reported for excised nodules of other legumes using both acetylene reduction and $^{15}N_2$ fixation. For those other species tested (soybean and Pisum sativum) activity ceased 1 - 3 hours after excision (Aprison and Burris, 1952; Nutman, 1959b, 1963; Bond, 1959, 1963; Stewart, 1966; Koch and Evans, 1966; Dixon, 1967; Sloger and Silver, 1967; Hardy et al, 1968; Mague and Burris, 1972).

Sprent (1969), however, observed a constant rate of acetylene reduction in excised soybean nodules for at least 8 hours after excision. She was able to re-start acetylene reduction by larger nodule samples at its previous rate, after it had ceased, by renewing the gasphase. She suggested that the usual cessation of acetylene reduction in legume nodules, resulted from increasing concentrations of hydrogen or by diminished oxygen supply. These limitations were overcome by keeping the quantity of nodule material small per unit volume of gas phase (10 - 100 mg nodules in 7 ml of gas - 70-700 ml/g of nodule). It was important not to allow the nodules to dry out (Sprent, loc.cit, 1971, 1972a). Normal time course of cessation after 1 - 3 hours constant acetylene reduction was observed when using a gas volume of 7.7ml/g of nodules (Sprent, 1969). Other workers have however, observed short-lived acetylene reduction or nitrogen fixation using gas volumes recommended by Sprent (loc.cit.) (e.g.Dixon, 1967; Hardy et al, 1968). In experiments reported in the present work, gas volumes of 20-60 ml/g nodules used. In several cases acetylene reductions continuing considerably longer than 7 - 9 hours were observed in excised gorse or broom nodules. Nodules of non-legumes have previously been observed to continue acetylene reduction or nitrogen fixation for considerably longer after excision than those of soybeans or peas (Bond, 1957, 1959; Benecke, 1968, 1970; Silvester, 1966; Silver, 1969). Continued activity for up to 24 hours, observed in Hippophae rhamnoides and Myrica nodules by Bond (1957, 1959) and by

Silver(1969), probably resulted from the use of sub-optimal concentrations of oxygen or nitrogen (or acetylene) or sub-optimal temperatures.

Several authors have commented on the longevity of non- legume nodules after excision. This has been attributed to a basic difference between legumes and non-legumes nodules (Bond, 1957, 1959, 1963; Nutman, 1959b, 1963; Stewart, 1966; Silver, 1969; Becking, 1970). However, this present work shows that gorse and broom nodules are similar to non-legume nodules in this respect. The difference is therefore not between legumes and non-legumes but between the herbaceous species generally used in experiments on excised legume nodules (e.g.soybeans and peas), and the woody legumes (e.g.gorse and broom) and non-legumes (viz.Coriaria arborea, Alnus spp, Hippophae, Myrica spp, Casaurina). This difference may lie in larger endogenous energy reserves in nodules of the woody species, or if Sprent's (1969) suggestion is correct, in the lack of a hydrogenase associated with the nitrogenase enzyme.

Using an average factor of fresh weight to dry weight of 5.5 - 5.8 in these nodules, comparison can be made with results of other workers (Table 4.1). The activity of broom nodules was rather lower than that of many other legumes, but similar to that of most non-legumes. Broom nodules had a 3.5 fold greater reducing activity than gorse nodules. Thus activity of gorse nodules was considerably less than that of other legumes and many non-legumes (e.g. Myrica cerifera, Sloger and Silver, 1967; Silver, 1969) but was similar to that of Casuarina esquisetifolia (Silver, 1969).

Acetylene reduction by non-symbiotic nitrogen fixing systems is generally considerably less than fully effective symbiotic systems. Weinard et al (1971) showed that acetylene reduction in symbiotic systems was approximately 100 times as great as in non-symbiotic systems.

The differences between woody species (non-legumes and gorse and broom) and non-woody legumes (e.g. G.max, Trifolium repens) reflect three factors:-

(a) the amount of active tissue relative to total nodule weight,

(b) the effectiveness of the symbiotic associations,

(c) the conditions (season, time of day, gas mixture etc.) of the experiment.

Hardy et al (1968) and Wheeler (1971) showed that there was a strong diurnal fluctuation in the activity of excised soybean and alnus nodules respectively. The importance of light intensity at the time of harvest was emphasized by Bergersen (1970a).

Of the above factors, effectiveness of the symbioses would probably be paramount. Many herbaceous legumes used by other workers, have been used extensively in agriculture, and rhizobia forming their symbioses extensively developed for maximum activity. On the other hand, rhizobia forming nodules on gorse and broom plants used in this study were those found in in the wild state. The nature of the endophyte in non-legumes nodules is not yet fully confirmed. It is not, therefore, surprising that gorse and broom and non-leguminous nodules should be considerably less active than those of many herbaceous legumes.

CHAPTER V

METABOLISM AND TRANSLOCATION OF FIXED NITROGEN WITHIN GORSE AND BROOM

A. FREE AMINO ACIDS OF XYLEM SAP AND OF SOLUBLE NITROGEN POOL OF ROOT NODULES OF GORSE AND BROOM

Introduction

Relatively few studies have been made comparing the amino acid content of xylem sap of different plant species. Bollard (1957b) in a study of the nitrogenous compounds in xylem sap of 110 species, showed that the amides, asparagine and glutamine, were the major amino acid components in all but 5 species in which citrulline predominated, and 5 in which allantoic acid was more important than the amides. The relative importance of the two amides present in 8 woody species of the Rosaceae varied with the species and with the time of year at which samples were taken.

Wieringa and Bahkuis (1957) equated the appearance of asparagine in xylem sap of pea plants, with formation of effective nodules and noted an increase in the nitrogen and amino acid content of sap at that time. This, together with studies of Pate and his school (Pate, 1962b, 1966; Pate et al, 1964, 1965; Brennan et al, 1964; Pate and Grieg, 1964; Pate and Wallace, 1964) show that asparagine is the major form in which nitrogen is translocated in those legumes that they studied.

Free amino acids of legume nodules have been the subject of two comparative studies. Hunt (1951) and Sen and Burma (1953) studied the free amino acids of roots and nodules of a total of eight species. The general patterns they observed were very similar in all the herbaceous and semi-herbaceous species. The differences were in the wide range of minor compounds which these authors were unable to identify. Asparagine was shown by Wheeler and Bond (1970) to be the major free amino acid of nodules of the non-legumes - *Myrica* spp, *Hippophae*, *Elaegnus*, *Connothus* and *Casuarina*

with substantial amounts of glutamine also present. This latter amide was shown to be the major free amino acid in Coriaria (see also Silvester, 1968), while citrulline predominated in Alnus spp. This last observation correlated with the earlier observed importance of citrulline in xylem sap of this genus (Bollard, 1957b), in the free amino acids in roots, nodules and leaves (Miettenin and Virtanen, 1952), and in the metabolism of fixed nitrogen in Alnus nodules (Leaf et al, 1958).

Bleeding sap was used in the study of physiological rhythms in legumes by Grieg et al (1962), who observed considerable diurnal variations in the quantity and in the ^{14}C content of sap of Pisum arvense after exposure of the leaves to $^{14}CO_2$. Silvester (1968) used bleeding sap from partly or wholly detopped Coriaria plants, and also sap obtained by the vacuum extraction technique developed by Bollard (1957a,b).

Materials and Methods

As gorse and broom do not bleed after being detopped, the most convenient method of extracting sap was to use air pressure (40 lb/sq.inch) on the root system contained in a specially constructed metal box (see Plate 5.1).

A series of experiments were conducted to determine the free amino acids of xylem sap and of the soluble nitrogen pool of root nodules of gorse and broom. In all experiments the amino acids present were determined by two dimensional thin layer chromatography on 10 cm x 10 cm layer of mixed cellulose/silica gel (Turner and Redgewell, 1966). Water saturated phenol (1st dimension) and butanol:acetic acid:water (5:1:4 v:v:v, upper phase) (2nd dimension), were used as solvents. Amino acids were detected by spraying the plates with ninhydrin (5 g/l in ethanol) and heating to approximately 100°C for 15 minutes.

i) Free amino acids of sap

Experiment 1. Gorse seedlings collected in the field were transplanted into perlite or into sterilized soil: perlite (1:1) in four inch

plastic pots. $5\frac{1}{2}$ weeks later, the pressure-extracted sap from 4 plants from each rooting medium was collected and the free amino acid content determined.

As there were no apparent variations in amino acid patterns of these individual plants, the remaining sap was combined and constituent amino acids identified by co-chromatography with authentic amino acids samples, and from the colour of the ninhydrin spot. Fig.5.1 is a diagram of a typical chromatogram.

Experiment II. The amino acid pattern of sap extracted under pressure from 6 gorse and 6 broom plants which had been grown for $5\frac{1}{2}$ months in sand: perlite: soil (4:3:1) was determined. To check for the presence of glutamine and asparagine, 50 µl portions of saps were hydrolysed with equal volumes of concentrated hydrochloric acid, at 100°C overnight in sealed tubes. After drying in vacuo over NaOH pellets to remove the acid, the residue was dissolved in 50µl of 100 ml/l isopropanol and amino acids in the hydrolysate determined. Plates 5.2 A,B and 5.3 A,B show typical chromatograms of these saps before and after hydrolysis.

At the same time, sap from a broom plant with black degenerated nodules was analysed for free amino acids (Plate 5.4).

Results. It was clear from these analyses that the predominant amino acid in sap of plants with effective nodules was the amide asparagine. Alanine and γ amino-butyric acid were present in lesser but still significant amounts while aspartic and glutamic acids and serine were present in small amounts. Valine and leucine (and in young plants, citrulline) were present in trace amounts.

The amino acid concentration of sap of plants with ineffective nodules was much lower than in those with effective nodules. The amino acid pattern was characterized by a complete lack of asparagine and a relatively high proportion of γ amino-butyric acid.

ii) Free amino acids of nodules

Experiment I. Nodules from young gorse plants (as used in 1) were

extracted in methanol: chloroform: water (12:5:3 v:v:v)m.c.w.) (acc.Bielski and Turner, 1966).

A diagram of a typical thin layer chromatogram of the free amino acids is given in Fig. 5.2.

Results. This pattern showed a large amount of asparagine, and lesser amounts of glutamic acid, alanine, γ amino-butyric acid, and leucine, and a wide range of amino acids present in small amounts or traces only.

Experiment II. The free amino acid patterns of the m.c.w. extract of nodules from gorse and broom plants, later in their second year of growth, were analysed before and after hydrolysis (as in Experiment i, Exp.2) (Plates 5.5, 5.6).

Results. These patterns showed again the preponderance of asparagine. In these extracts, however, glutamic acid was present in significant amounts. the other amino acids, glycine, aspartic acid, alanine and γ amino-butyric acid (and leucine in gorse), being present in trace amounts only. Thus the range of free amino acids present in significant amounts in m.c.w. extracts of these nodules was much more limited than in younger nodules (ii, Exp.I) or in sap (i). The presence of a faint spot running slightly ahead of asparagine and near alanine on these chromatograms, and the increase in concentration of glutamic acid after hydrolysis, suggest that glutamine was present in quite significant amounts in sap and nodule extract of these plants. (This amide is hydrolysed in the acid solvents used in this study). In addition to this concomitant increase in aspartic and glutamic acids on hydrolysis of the amides, there was an increase in glycine in one case (gorse nodules, Plate 5.5.) and some slower moving ninhydrin positive components appeared. This suggests the occurrence of some form of low molecular weight, bound amino acid compounds in the nodules. Butler and Bathurst (1958) found that such amino acids form a large proportion of the amino acids in the 800 ml/l ethanol soluble fraction of legume nodules, and Bollard (1957b) indicated that peptides were often present in xylem sap. A summary of semi-quantitative estimations of the amino acid composition of extracted saps and nodule extracts from this series of experiments is given in Table 5.1.

Discussion

When compared at the same age, the free amino acid composition of nodule extract or of xylem sap was the same for both species.

The patterns of major free amino acids in nodules or in sap of young gorse plants was generally the same as has been observed in nodules of other legumes (Hunt, 1952; Zelitch et al, 1952; Sen and Burma, 1953; Bathurst, 1954; Wieringa and Bakhuis, 1956; Butler and Bathurst, 1958), in legume bleeding sap (Pate, 1962b, 1966; Grieg et al, 1962; Brennan et al, 1964; Pate and Grieg, 1964; Pate et al, 1964, 1965; Pate and Wallace, 1964) or in nodules of several non-leguminous genera (Wheeler and Bond, 1970).

Thus, free amino acid patterns of nodules and extracted sap of most nitrogen fixing plant genera which have been studied are similar. The predominant amino acids in the herbaceous legume Ornithopus sativa were glutamine and glutamic acid (Kennedy, 1965a, 1966a, b), not asparagine as in these and many other species. Also, two genera of non-leguminous nitrogen fixing plants differ from this generalization. In Alnus nodules, citrulline was shown to be the major free amino acid (Miettenin and Virtenen, 1952; Leaf et al, 1958; Wheeler and Bond, 1970). Glutamine and glutamic acid predominate in Coriaria nodules (Bollard 1957b; Silvester, 1968; Wheeler and Bond, 1970). In older gorse and broom plants, the range of amino acids found was much smaller than in young plants. Thus, patterns found in those plants were not directly comparable with those observed by other workers who, in the main, studied herbaceous legumes. In spite of this, however, the great preponderance of asparagine in the amino acid pattern is in common with, although more marked, that in most other species. Miettenin (1955) showed that the importance of the amides increased with age. However, many other factors such as mineral nutrition (Prianishnikov, 1951) and light (Miettenin, 1955) are also important in determining the relative proportions of glutamine and asparagine.

The appearance of asparagine in sap has been shown to be associated with the formation of active nodules in peas (Wieringa and Bakhuis, 1956). The lack of this amino acid in sap of broom plants with ineffective nodules (Plate 5.4)

and its great preponderance in the sap of effectively nodulated plants, and in the soluble pool of nodules at both ages studied, indicated that it was the major compound in which nitrogen was stored and translocated following fixation in gorse and broom.

B. ^{15}N LABELLING STUDIES

i) Metabolism of fixed $^{15}N_2$ in excised nodules

Introduction. It is now generally accepted (Bergersen, 1965, 1969) that ammonia is the first stable product of nitrogen fixation by legumes. After short exposures (approx. 1 min.)of excised nodules to $^{15}N_2$, 90 - 100% of the ^{15}N label was found in the ammonia fraction of the soluble portion of nodule nitrogen (Bergersen, 1965). The label was then rapidly incorporated into amide and amino groups of amino acids (Bergersen, loc.cit.; Kennedy, 1965a, 1966, a,b). The same also seems to hold for nitrogen fixing non-legumes such as Coriaria arborea (Silvester, 1968).

Kennedy (loc.cit.) was able to identify the primary amino compounds in Ornithopus sativa nodules as glutamine and glutamic acid.

Equilibrium in soybean nodules was reached after about 20 mins. when 20% of the total level was present in the ammonia fraction, and a similar amount in the amides (Bergersen, loc.cit.). Silvester (1968) concluded that glutamine was the most important free nitrogenous compound in nodules of Coriaria arborea and Kennedy (loc.cit.) showed glutamic acid and glutamine also to be the most important amino compounds in nodules of Ornithopus sativa. Asparagine accounted for a further 10% of the label after 4 minutes incubation of O.sativa nodules in an ^{15}N enriched atmosphere. Kennedy (loc.cit.) recovered from the various fractions only 66-87% of the total ^{15}N label found in the nodule extract.

Two experiments were conducted to compare the course of metabolism of newly fixed nitrogen in excised gorse and broom nodules with the findings mentioned above.

Experiment I. Nodules from gorse plants approximately 3 months old were excised and incubated at 24°C for seven time intervals from 10 minutes to 4 hours, in an atmosphere of argon; oxygen: nitrogen (96 atom %xs $^{15}N_2$) (70:20:10). Vials (20 ml) fitted with serum seals were used as incubation vessels. The incubation was stopped at the required time by addition of 700 ml/l ethanol. Separate samples were used for each incubation time.

The tissue was ground and extracted twice more in 700 ml/l ethanol. Combined extracts from each sample were divided into two unequal portions (a. twice as large as b.) for analysis:-

a. after acidification and rotary evaporation at 35–40°C,

   i. the ammonia content was determined by distillation under reduced pressure at pH. 9 (Bergersen, 1965),

   ii. the amide content was determined by hydrolysis in boiling 10M KOH for 30 minutes (Bergersen, 1965), and

   iii. the residue was subject to Kjeldahl analysis

b. the amino nitrogen content was determined by the method of Kennedy (1966b) after hydrolysis of the amide present in 1M NaOH for 3 hours at 100°C, and neutralization with 0.5M H_2SO_4.

The ammonia recovered from each fractionation step was determined by titration with 0.005M H_2SO_4. After further acidification with 0.05M H_2SO_4, samples were dried and the ^{15}N enrichment determined on a mass spectrometer (Hulston and Shilton, 1958) housed at Institute of Nuclear Sciences, D.S.I.R., Wellington. For this, the ammonia was oxidised to nitrogen by alkaline hypobromite in micro-Rittenberg flasks (Silvester, 1968).

Experiment II. Nodules from gorse and broom plants in mid-second season were excised and incubated in an $^{15}N_2$ enriched atmosphere as above, in 30 ml "Quickfit" test tubes fitted with serum seals. The incubation was stopped after 1, 5, 8, 15, 30, 60, 90, 214, 360 and 540 minutes by plunging one tube at each time into ethanol/dry ice (-78°C) and adding cold (-20°C) m.c.w. extractant. Separate nodule samples were used for each incubation time. The crushed nodule material was extracted 3 times by the method of Bieleski and Turner (1966).

The third of these extractions contributed 4.5-5.5% of the total nitrogen extracted. Two lots of control nodules for each species, not exposed to $^{15}N_2$, were extracted and analysed similarly.

Duplicate aliquots were taken for total nitrogen determination by Kjeldahl analysis. The remainder was acidified with 2 drops of 100 ml/l H_2SO_4, and reduced to a small volume by rotary evaporation at 37°C. Ammonia was separated from the extract by the method of Henderlong and Schmidt (1966) and determined by nesslerization of an aliquot (Middleton, 1960). The amides were hydrolysed in 0.5M H_2SO_4 at 100°C for 3 hr and the hydrolysates re-applied to the columns. Ammonia derived from hydrolysis of the amides was separated and analysed as previously. The eluants remaining after nesslerization were acidified with two drops 100 ml/l H_2SO_4, and reduced to a small volume. The ^{15}N enrichment of N_2 derived from ammonia by reaction with alkaline hypobromite was determined by Dr.W.B.Silvester, Botany Dept.,University of Auckland, on a A.E.S. M.S. 10 mass spectrometer fitted with a single collector.

In early samples analysed it was found that fine brown colloidal precipitate (thought to be polyphenolic in nature) caused blocking and consequent very slow running of the columns. This was overcome by centrifuging the extracts and adding the pellet of precipitate only at the final stage.

Acid hydrolysis of the eluate caused coagulation of any of this precipitate eluted from the column and hence made this step much simpler. Using this procedure 98.9-102.1% of ammonia and 98.8-105% of amide was recovered from standard solutions containing more than 100 µg of each. Great difficulty was experienced during mass spectrometric analysis, as potassium chloride in the eluate made drying and evacuation difficult, and slowed the reaction of hypobromite.

Results and Discussion

Experiment I. Results are given in Table 5.2 and Figs.5.3 - 5.5. It was apparent that the first and most highly labelled fraction was ammonia, while labelling of the other three fractions, amide, α-amino and total, was all

very similar. However, as ammonia and amide fractions were very small in comparison to the total fraction, they constituted only a relatively minor portion of the total label detected. The total amounts of ^{15}N label incorporated into the ethanol fraction, and the amount in the fraction termed "non-α-amino nitrogen" were obtained by summation of the Kjeldahl, ammonia and amide fractions and as the difference between Kjeldahl nitrogen and α-amino nitrogen respectively, rather than by direct measurement. The "non-α-amino" fraction corresponds to the deficit in Kennedy's (1965a, 1966a,b) results. This large and important fraction, was, however, not identified in these experiments, but possibly consisted of purines and pyrimidines. These are biosynthesised in animals and micro-organisms from aspartate, glycine, glutamine and/or ammonia. As few, if any, enzymes involved in these syntheses have been identified in plant tissues, identity of this fraction must remain conjecture.

Because the amide nitrogen constituted a very small proportion (less than 0.9%) of the total label at all times, it has been included with the ammonia fraction in Fig.5.5. It was necessary to add considerable amounts of non-labelled "carrier" nitrogen during the analysis of this fraction. Thus enrichments measured were very low. These results must therefore be interpreted with caution.

Enrichments measured in all other fractions were much greater than in amide and hence their real importance is beyond doubt.

The amount of nitrogen represented by the "non-α-amino" fraction was 2-3 times that of the α-amino pool, but it was turning over at a considerably slower rate, hence the rate of incorporation of ^{15}N into the "non-α-amino" fraction was only slightly greater than that in the α-amino fraction.

Incorporation of ammonia into glutamine and glutamate is generally accepted as the first step in its utilization in plant and animal metabolism. However, these two amino acids were relatively minor components of the soluble nitrogen pool of gorse nodules (Table 5.1). Glutamine and glutamate pools within these nodules must therefore be turning over rapidly. The high total ^{15}N content of the α-amino pool indicates the importance of transaminase

reactions in metabolism of fixed nitrogen within these nodules. Enzymes catalysing two such reactions (alanine amino transferase and aspartate amino transferase) have been demonstrated in roots of Phaseolus vulgaris (Abbadi and Shannon, 1969). These and 3 other enzymes involved in the incorporation of ammonia into amino acids were observed at specific activities in nodules of Pisum sativum 2-4 times higher than in roots and 5-10 times higher than in shoots (Grimes and Turner, 1971).

Experiment II. In this experiment only ammonia and amide fractions were determined. The m.c.w.extract of a sample of control nodules used in this experiment was chromatographed to identify free amino acids present. Results of this were presented earlier (Section Aii, Exp.II).

Enrichments obtained from incubated samples were compared with control samples, (3 for gorse and 2 for broom) which had not been exposed to $^{15}N_2$. Control levels were 0.351 - 0.361 atom % for gorse and 0.350 - 0.389 atom% for broom. Only ^{15}N levels higher than these were considered as enriched. The average of these levels was taken as zero level. Results are presented in Table 5.3 and Figs.5.6 - 5.8. To enable clearer interpretation, results from shorter incubations are presented in expanded form in Fig.5.9.

It can be seen from Fig.5.9 that the amide fraction rapidly became more highly enriched than the ammonia from which it was supposedly derived. This situation remained true for the whole incubation period with gorse (Fig.5.6A). However, with broom the ^{15}N enrichment in these two fractions increased in an approximately parallel manner although the amide fraction was consistently more enriched than the ammonia fraction at all incubation times (Fig.5.6B). To explain such an observation in excised nodules of Ornithopus sativa, Kennedy (1965a, 1966a) postulated the existence of two pools of ammonia within nodules. The smaller active ammonia pool became saturated with ^{15}N label rapidly, and during extraction was diluted by the mass of unlabelled ammonia from the other pool.

Enrichment of the ammonia fraction of gorse nodules was very low, especially in the first 30 mins (Table 5.3A, Fig.5.9A). Enrichment in this

fraction was still considerably lower at 360 minutes than in the total or amide fractions (Table 5.3A, Fig.5.6A). Thus, the active ammonia pool of gorse nodules must be too small to measure by these techniques. Some interchange between this active pool and the non-active pool obviously occurred in longer incubations. However, the amount of this interchange must presumably have been small, because of the low final enrichment in this fraction.

The active ammonia pool of broom appeared to be much larger than that of gorse. It was apparently fully saturated with ^{15}N label after 1-1.5 minutes incubation and remained so for at least 30 minutes (Table 5.3B, Fig.5.9B). By 60 minutes incubation however, considerable incorporation of ^{15}N label from the active pool into the non-active pool had occurred (Table 5.3B, Fig.5.6B).

Assuming that the active ammonia pool of broom nodules was saturated at 15-30 minutes at 0.024 atom %xs, then the active pool size was only approx - imately 0.025% of the total ammonia present, or 6 μmoles of ammonia/g f.w. of nodule. This is considerably less in proportion to nodule weight, although similar in size to that in serradella and soybean nodules (Kennedy, 1965a, 1966a, and Bergersen, 1965 respectively).

Accurate measurement of enrichments of the ammonia fraction in 214 and 540 minute exposures of gorse nodules was impossible because of the small amount of sample obtained.

Figs.5.7 and 5.8 emphasise the relative importance of the two fractions isolated here in the overall metabolism of fixed ^{15}N within excised gorse and broom nodules. It is clear from these graphs, and from Table 5.3B, that the ammonia fraction in broom, contained all the label at 1 minute incubation (the concentration of ^{15}N in the amide fraction at this time, must be treated with scepticism because of the very low enrichment in this fraction compared with that in the ammonia fraction), and 2.2 to 6% of the total label up to 90 minutes. However, in broom, the ammonia pool contained less than 1.5% of the total nitrogen in the samples, while the amide fraction contributed 15-19% of the total nitrogen, and contained 10-100% of the label. In gorse, the ammonia pool constituted less than 1% and amide 10-18% of the total nitrogen. The ammonia

fraction contained 6 to 13% of the total label, and the amide fraction approximately 20 to 50% and 100% of the ^{15}N label (Table 5.3A, Fig.5.7,5.8). Thus, the ammonia fraction, although containing a high proportion of the label (for broom) at short exposures, constituted a very small proportion of the total soluble nitrogen of the nodules. The small size of the ammonia pool in gorse made measurement of ^{15}N enrichment impossible in most cases. This made it impossible to draw any conclusions on the place of this pool in the metabolic path of fixed ^{15}N . However, in broom it is clear from Fig.5.8B that the first fraction to receive label was ammonia. After one minute incubation this fraction contained 100% of the ^{15}N label. This proportion of label rapidly fell with an accompanying rise in the ^{15}N content of the amide fraction. Thus the initial path of fixed $^{15}N_2$ in these nodules was from ammonia, the first stable product of fixation (Bergersen, 1965), to amide - presumably asparagine (see Part A). From the similarly high proportion of total label in the amide fraction of gorse after 5 - 30 minutes exposure, a similar sequence may be presumed to occur in those nodules as in broom.

Comparison of results of this experiment (Table 5.3, Fig.5.6 - 5.9) with those of Experiment I (Table 5.2, Figs.5.3-5.5) shows several differences.

In Experiment I amide and ammonia pools were similar in size (Table 5.2) but in Experiment II the amide fraction was 20 times larger than the ammonia fraction. The total soluble nitrogen pool was slightly smaller in Experiment II than in Experiment I. The amide pool in Experiment II was 2 to 5.5 times, and the ammonia pool approximately 1/7 the size of respective pools in Experiment I.

The incorporation of label into the amide fraction occurred much more rapidly in Experiment II than in Experiment I.

In Part A of this chapter, considerable differences were noted with age in the relative importance of asparagine and other compounds. With increasing age, the amount of asparagine increased and other amino acids decreased (Table 5.1). This increased importance of amide in older nodules therefore contributed to a large extent to the apparent differences in results of these two experiments. It is also apparent from these experiments that the ammonia

pool decreases in size with age and its rate of turnover into the amide pool increases with age.

Table 5.2 and Fig.5.5 show that a high proportion of label existed in ammonia and amide at 10–20 min. This rapidly decreased with a concomitant rise in the proportion of ^{15}N label in the α-amino fraction. This then decreased, and the "non-α-amino"fraction became more important.

Combining the conclusions of these two experiments shows that the metabolic sequence of fixed $^{15}N_2$ in these nodules was:-

$$^{15}N_2 \longrightarrow {}^{15}NH_3 \longrightarrow {}^{15}N\text{-amide} \longrightarrow {}^{15}N\text{-}\alpha\text{-amino acids} \longrightarrow {}^{15}N - \text{"non-}\alpha\text{-amino" compounds}$$

As nodules age, the ^{15}N-amide pool apparently becomes a larger proportion of the total soluble nitrogen content. It is presumably involved both as a storage compound and in translocation.

The sequence given above is similar to that postulated for Coriaria (Silvester, 1968) and Ornithopus sativa (Kennedy, 1965a, 1966a,b). However, because of the small amounts of α-amino acid present, separation and identification of the individual ^{15}N labelled compounds involved, as carried out by Kennedy (loc.cit.) was impossible.

The pre-eminent position of glutamate between $^{15}NH_3$ and ^{15}N amide must be assumed, as glutamate is the only known compound into which ammonia is incorporated in amino acid synthesis. Because of the apparent lack of glutamine in nodules of gorse and broom, and large amounts of asparagine present, it is unlikely that glutamine plays an important role in the metabolism of fixed nitrogen. Asparagine takes over the role which glutamine plays in Coriaria (Silvester, 1968) and Ornithopus sativa (Kennedy, loc.cit.).

It was also apparent from the two experiments discussed in this section, that fixation had continued for considerably longer than the 1-2 hours normally observed in excised legume nodules (Aprison and Burris, 1952; Kennedy, loc.cit.). This showed clearly that the longevity after excision observed during acetylene reduction experiments (Ch.IV) was not an artifact of that method, but was a real effect. This supports conclusions reached in Chapter IV.

The 2 to 3 fold higher activity of broom nodules than gorse nodules, observed in acetylene reduction experiments was also observed here.

ii) Translocation of fixed nitrogen

Although Pate and his school have made extensive use of ^{14}C labelled photosynthate to study nitrogen transport of Pisum arvense, Lupinus angustifolia and Vicia atropurpurea, little use has been made of ^{15}N labelling in such studies.

Bond (1956) used ^{15}N to study translocation of fixed nitrogen in xylem tissues of Alnus glutinosa and obtained substantial ^{15}N enrichment (up to 0.12 atom %xs) of xylem sap after exposure to an atmosphere containing nitrogen enriched to 36 atom %xs $^{15}N_2$.

Sylvester (1968) identified the major form of ^{15}N translocation in xylem of Coriaria arborea as glutamine.

Oghoghorie and Pate (1972) studied the routes of translocation and the distribution of fixed $^{15}N_2$ and absorbed $^{15}NO_3$ in Pisum arvense.

To enable ^{15}N labelling pattern of sap of gorse and broom to be studied a closed circuit apparatus was designed to enable nodulated roots on whole plants to be incubated in $^{15}N_2$ containing gas mixtures and to enable the plants to be changed without allowing atmospheric contamination of the $^{15}N_2$ gas. The apparatus is depicted in Figs. 5.10 a,b. Five litres of a gas mixture of Ar: O_2:N_2 (4.74 atom %xs $^{15}N_2$) was made up in a gas reservoir by displacement of water. After each exposure, the decrease in gas volume was made up to 5 l with oxygen. Six plants of each species were sealed in a split rubber bung in petroleum jelly and incubated for 3 and 6 hours. Six control plants of each species, not been exposed to $^{15}N_2$, were included in the experiment to provide a base ^{15}N level. After incubation, shoot systems were removed below the first branch, the outer layer stripped off, and the stem sealed into a short length of plastic tubing, which was then forced into a rubber bung and sap forced out under 40 lb/square inch gas pressure (Plate 5.1). Before collection of sap was started, the cut stem ends and the plastic tubing were thoroughly rinsed with distilled water and the washings discarded. After being under pressure for 45 min , the gas pressure was released and the tubing again washed with distilled

water. These washings were combined with the collected sap. The sap collected from the 6 plants in each group was combined and an aliquot from each combined lot taken for analysis of total nitrogen by Kjeldahl digestion. 50 µl of each was taken to test for the presence of ammonia (acc. Middleton, 1960). No ammonia was detected in any sample. Amino acids present in the sap of control plants were determined by thin layer chromatography of 20 µl portions of combined sap. (Section Ai, Experiment II). The remaining sap volume was reduced to dryness by rotary evaporation, and the residue dissolved in 0.6 ml 100 ml/l isopropanol. Amino acids present in this solution were then separated by high voltage electrophoresis on Whatman No 3MM paper using a pH 1.95 formic acid/acetic acid buffer. The electrophoresis was run for 25 minutes at 5.0-5.5 kv (75-80 ma). Fifty to one hundred µl of isopropanol solution, representing 100-150 µg total nitrogen was used for each separation run.

This sytem separated components of the sap into three major bands, the positions of which were determined by comparison with standard amino acid run on each side of the electrophoresis paper. Major constituents of these bands were:-

1. asparagine
2. alanine

and 3. γ- amino butyric acid

respectively. After elution with distilled water, and reduction in volume by rotary evaporation, amide nitrogen of Band 1 was analysed as ammonia released by acid hydrolysis. This ammonia was separated by the method of Henderlong and Schmidt (1966) as before. The recovered amino acid and that from the remaining two bands was then subjected to the ninhydrin destruction procedure of Kennedy (1965b) and the resulting ammonia recovered by steam distillation from alkaline solution and determined by titration against 0.005M H_2SO_4. The ammonia samples were acidified, reduced in volume, and the ^{15}N level of nitrogen produced by reaction with hypobromite determined by mass spectrometry by Dr.W.B.Silvester, Botany Dept., University of Auckland as before. The ^{15}N level of a sample of the gas mixture was determined at the same time. Results are given in Table 5.4. There was considerable loss of α- amino nitrogen from the asparagine band during

separation of ammonia resulting from hydrolysis..

Had recovery of this fraction been complete, considerably more than 100% recovery would have been obtained. An explanation for this was not apparent.

Analyses of the nitrogen content of the bands gives quantitative confirmation of observations made earlier (Section Ai) on the importance of asparagine in the transport of nitrogen in these plants.

As little glutamine was detected in the xylem sap of these plants (Section Ai, Experiment II), it must be concluded again that the amide fraction was almost entirely asparagine.

Nitrogen in the other compounds became labelled to only a very small extent and in all cases carried less than 5% of the total label even at 6 hour incubation.

Expressing ^{15}N enrichment in the compounds as a percentage of $^{15}N_2$ enrichment of the gas phase gives a measure of the proportion of recently fixed nitrogen in the xylem sap. Table 5.4 shows that 4% of the total nitrogen being translocated in gorse after 6 hours incubation was fixed from the $^{15}N_2$ enriched atmosphere. In broom 7.5% of translocated nitrogen was fixed in this atmosphere. At 3 hours no recently fixed nitrogen appeared in the broom xylem sap. Nearly 9% of amide nitrogen present in the broom sap was labelled nitrogen. Thus, transfer of fixed nitrogen mainly as asparagine through the nodule to the xylem must be a slow process. In studies of the structure of nodules of these species (Ch.III) the xylem within the nodules was seen to develop early and grow to encircle the bacteroid tissue. This xylem branched from the root stele. As no xylem elements were visible within the bacteroid tissue, transfer of fixed nitrogen through this tissue to the nodule xylem presumably occurs from cell to cell. This is a considerably slower process than translocation in sieve tubes.

The lower activity of gorse nodules than broom observed earlier with excised nodules (Ch.IV, Ch.V,Part Bi) was reflected here in the ^{15}N enrichment in translocated nitrogen.

Pate and his co-workers have made extensive use of petiole or stem bleeding sap to study translocation of ^{14}C labelled sap in nodulated plants. They showed considerable variation in the volume, total nitrogen content, ion concentration

and ^{14}C activity from Lupinus angustifolium and Pisum arvense (Grieg, et al,1962; Pate, 1962 b).

According to Pate and Grieg (1964), glutamine and asparagine constitute 60-80% of the nitrogen in bleeding sap of P.arvense and L.angustifolium and up to 90% of ^{14}C activity in this sap when plants were decapitated after exposure to $^{14}CO_2$. These figures are very similar to those obtained here with $^{15}N_2$ labelling.

The α-amino nitrogen in alanine (Band 2) and γ-amino butyric acid (Band 3) are labelled to a much lower extent than amide (Band 1). The amount of ^{15}N in these other two bands was very small.

Thus it is clear that gorse and broom share with other legumes the pre-eminence of the amide group in both metabolism within the nodule and translocation of fixed nitrogen in the transpiration stream. The pre-eminent position of asparagine was rather more accentuated than in species such as L.angustifolium and P.arvense (Pate, 1962b; Grieg et al, 1962; Pate and Greig, 1964) and especially Ornithopus sativa (Kennedy, 1965a, 1966 a,b). This situation is further accentuated as the plants age. In the woody non-legumes Myrica, Hippophae, Eleagnus, Ceanothus and Casuarina, asparagine appears to be the most important free amide in nodules (Wheeler and Bond, 1970) and thus, gorse and broom are more similar to these woody species than to herbaceous legumes. The two nitrogen fixing genera which differ from this generalization are Alnus (Miettenin and Virtanen, 1952; Leaf et al, 1958; Wheeler and Bond, 1970) and Coriaria (Silvester, 1968; Wheeler and Bond, loc.cit.).

Thus, gorse and broom must be considered from the longevity of nodules, the amino acid metabolism within nodules and the amino acid pattern in the translocation stream, as more typical of woody nitrogen fixing species than of the well-studied legumes, which are, in the most part, herbaceous.

CHAPTER VI

EFFECT OF TEMPERATURE AND LIGHT ON GROWTH, NODULATION AND NITROGEN ASSIMILATION BY GORSE AND BROOM

INTRODUCTION

The effects of varying temperature and light regimes on plant growth are complex. With nodulated plants, these effects are even more complex as the system includes another component - the microbial symbiont, another growth process - nodulation (Joffe et al, 1961; Small et al, 1968) and another enzymatic assimilation process - nitrogen fixation (Joffe et al, loc.cit.; Dart and Mercer, 1965a).

Studies on the effect of light on plant growth are also complicated by the temperature rise often associated with increased light. Meyer and Anderson (1959) pointed out that such local heating effects often associated with high light intensity could result in suppression of nodulation and nitrogen fixation, an effect used by Wilson (1940) in proposing his C/N ratio theory of nodulation.

As expected, considerable variations have been observed in the response of different legumes to temperature. Jones and Tisdale (1921) observed different nodulation responses in soybean, pea, clover and lucerne in the range 18-40°C. Humphries (1967) listed temperature optima for several sub-tropical or tropical legume species. They ranged from 27°/22°C for Glycine wightii c.v. Cooper (Cooper glycine) to 36°/31° for stylo. Ketalloper (1963) gave 20°/14°C as the optimum temperature regime for Vicia faba and 23°/17°C for Lupinus nanus and Pisum sativum.

Variations in susceptibility to temperature have also been observed within a genus. Small and Joffe (1968) studied 5 African and 3 European clovers all with temperature optima of approximately 26°C. They showed that the African species were better able to withstand high temperature extremes. Strains of clover rhizobia isolated from Northern Scandinavia were better adapted for growth, nodulation and nitrogen fixation at 10°C than were those isolated from Southern

Scandinavia (Ek -Jander and Fahreus, 1971). There were no significant differences between strains when grown at 20°C. Lie (1971a,b) studied a pea cultivar isolated in Iran resistant to the majority of Rhizobium strains at 20°C, and requiring a short period of a higher temperature for successful nodulation.

Kunelius and Clark (1970) showed that although the optimum temperature for growth and nitrogen fixation of Lotus corniculatus inoculated with several strains of Rhizobium was 18° - 24°C, several cultivars grew equally well at 30°C when supplied with ammonium nitrate.

Thus, there are differences between species in temperature response of nitrogen fixation and between plants relying on fixed nitrogen and those receiving combined nitrogen. Differences also exist between bacterial strains on the same host (Pate, 1961b, 1962a; Kunelius and Clark, 1970; Gibson, 1967c; Ek-Jander and Fahreus, 1971), and between host varieties of the same species or different variety x strain combinations (Gibson, 1961, 1963, 1965, 1967b; Kunelius and Clark, loc.cit.; Williams, 1972).

Such differences between species and varieties in both temperature optima and sensitivity to high temperature, caused the differences between the effects noted by Ludlow and Wilson (1970) and those observed by Pate (1961b, 1962a) and Meyer and Anderson (1959) and also probably the differences in temperature optima reported by two different groups for Phaseolus vulgaris viz. 25°C optimum with inhibition of infection at 30°C (Barrios et al 1963), and 29°C optimal for nodulation (Small et al, 1968). Ludlow and Wilson (1970) showed that growth of 10 tropical legumes was greater at 30°C than at 20°C, whereas Pate (1961b, 1962a) observed inhibition of nitrogen fixation above 27°C in Vicia atropurpurea and Medicago tribuloides, and Meyer and Anderson (1959), inhibition at 30°C in Trifolium subterraneum. One of four cultivars of T. subterraneum examined by Williams (1972) showed no inhibition of growth between 15°C and 30°C. The other three varieties exhibited marked inhibition of growth at 30°C. Similar differences were observed by Mes (1959b) between other temperate and tropical legumes.

Meyer and Anderson (1959) and Pate (1961b, 1962a) attributed their results

to the specific inhibition of nitrogen fixation at elevated temperatures. However, it is probably better to express the effect of temperature on nitrogen assimilation and growth by arranging 4 processes in order of thermo-sensitivity thus:- nitrogen fixation > nodulation > nitrogen uptake > root growth (Joffe et al, 1961; Barrios et al, 1963; Small and Joffe, 1968; Small et al, 1968; Gibson, 1971). Temperature was shown to affect nitrogen fixation by altering the rate of fixation and not by permanently affecting the nitrogen fixing system (Gibson, 1969b). Thus a nightly reduction of 10°C alleviated temperature inhibition to a large extent (Pate, 1962a).

Gibson (1971) showed that in T. subterraneum, sub-optimal root temperatures retard root hair infection more than they affect later stages of nodulation viz. nodule initiation and nodule development (including bacteroid tissue development and degeneration) or nitrogen assimilation. Supra-optimal temperatures stimulated root hair infection and speeded nodule formation. Nodule initiation was little affected by temperature in the range 20° - 30°C and below this temperature infection and nodule initiation were affected to the same extent (Gibson, 1967b, 1971). Nitrogen fixation was not affected by low temperatures (8°C) as much as the early stages of nodulation viz. root hair infection and initial bacteroid development (Gibson, 1967c, 1971). The highest temperature at which nodules appeared and that at which the maximum rate of root extension occurred was 33°C (Gibson, 1967b). The time of appearance of the first visible nodule was least, and the rate of increase in nodule numbers and hence the number of nodules after 24 days, greatest at 30°C. Below this temperature, there was an increase in the time to first visible nodule, and a decrease of nodule appearance so that at 7°C, only 50-80% of plants, depending on the strain of Rhizobium, were nodulated after 24 days, (Gibson, 1967b). A strain of Pisum sativum normally resistant to a large number of strains of Rhizobium leguminosarum at 20°C has been shown to nodulate effectively at 26°C (Lie, 1971a). A period of 1 day at 26°C on the second or third day after inoculation was shown to be sufficient to ensure nodulation (Lie, 1971b). The first three days after inoculation were most sensitive to inhibitory temperatures

for nodulation in Phaseolus vulgaris (Barrios et al, 1963). A similar situation was observed in the effect of pH on nodulation of Medicago sativa (Munns, 1968a). It therefore seems likely that the process most sensitive to pH and temperature is the same in both cases - root hair infection.

Gibson (1966a) showed that for T. subterraneum, lower root temperatures (5° or 10°C) restricted translocation of nitrogen to the shoots in both nodulated plants and unnodulated plants, supplied with combined nitrogen. Up to 18°C, he observed a progressive increase in absorption or assimilation and translocation of nitrogen with variations in distribution with different strains of Rhizobium. Above 20°C, host variety effects on translocation appeared to predominate.

Legumes are generally considered to have high light requirements. However. this requirement too, shows considerable variation (Blackman and Black, 1959). For example, whereas a reduction of light level by 50% from 800 ft candles reduced nitrogen fixation in Medicago tribuloides and Vicia atropurpurea by 25% (Pate, 1961b, 1962a), reducing the light level to 75% of full daylight, depressed yield of Stylosanthes humilis by 44-47% (Sillar, 1967). Further reduction in light level to 48% of daylight resulted in 7% mortality, and to 38% full daylight, 33% mortality.

Dart and Mercer (1965a) made a lengthy study of complex interactions among temperature, light and applied nitrogen on nodulation and growth of Vigna sinensis.

Much of the work on the effect of light levels has been carried out by shading pots, with very little attempt to control temperatures within shaded compartments. Quite apart from the cooling effects of shading, local heating of leaves can result from high light intensities and these temperature effects can combine to hide, or at least complicate, any direct light response.

MATERIALS AND METHODS

After transplanting into sand:perlite (1:1) in 4 inch plastic pots and inoculating, plants were grown for approximately 4 weeks in a glass house. During this time uninoculated plants were supplied with combined nitrogen, at

100 mg/l, as nitrate. When they were transferred to growth cabinets, nodulation and nitrogen fixation were well established in inoculated plants. Plants were chosen at random from inoculated and uninoculated populations and arranged randomly in rows in each half of each growth cabinet.

Lighting in each cabinet consisted of 48 x TLMF80w/33Rs, 80 watt fluorescent tubes, augmented by 18 x 60 watt incandescent strips. This gave a measured light intensity at the end of the experiment of 1320 $\pm$ 80 foot candles. Half of each cabinet was shaded with plastic fly-screen to give a light intensity of 803 $\pm$ 43 foot candles. Temperatures in the cabinets were monitored continuously. They were:-

16.1 $\pm$ 1.7°C.

22.5 $\pm$ 1.2°C.

25.6 $\pm$ 1.7°C.

29.2 $\pm$ 1.7°C.

Temperatures were held constant for 16hr. day and 8 hr.night. One cabinet was run at each temperature. Humidity was set at 80%.

There were 2 species (gorse and broom)and two sources of nitrogen (fixed nitrogen - nodulated - and combined nitrogen - unnodulated) for each of the two light intensities and four temperatures. Nitrogen was supplied to unnodulated plants at 100 mg/l as nitrate for 5 weeks and at 200 mg/l thereafter. Each treatment consisted of 9 replicates. Nine plants of each species were taken from each nitrogen treatment at the start of the experiment to comprise the initial harvest, and shoot heights of all plants were recorded at the start of the experiment. Each plant received 40 ml of nutrient 3 times weekly and was watered with distilled water daily for the first 6 weeks, and twice daily until harvest after 14 weeks. Shoot heights were measured and tops and unnodulated roots weighed dry..

Nodulated roots were deep frozen for later nodule analysis and dry weighing.

RESULTS AND DISCUSSION

Two weeks after placing in cabinets, good growth was apparent at 22.5°C and 25.6°C for both light levels, with best initial growth at 25.6°C.

Growth at 16.1°C appeared slower than at 22.5°C, whilst at 29.2°C young growth was chlorotic, especially in gorse. This latter appearance was maintained throughout the experiment. Several plants at 29.2°C died before the completion of the experiment. Only plants alive at harvest were included in the harvest.

Results are summarized in Tables 6.1A,B with initial harvest data in Table 6.2. Data was analysed as a completely randomised factorial experiment of 4x2x2 design with 9 replicates, by analysis of variance using Duncan's Multiple Range Test. Square root transformations of plant dry weight were similarly analysed (Fig.6.1, Table 6.3). It can be seen from these data that there was a large variability among replicates and this tended to mask responses.

The optimal temperature for dry weight, shoot heights, nodulation and shoot nitrogen was 22.5°C for nodulated plants of both species (Tables 6.1A,B, 6.4, Fig.6.1). This is lower than the optimum for initial growth, 25.6°C. The optimum temperature for nitrogen fixation-from nitrogen content (Tables 6.1A,B, 6.4,Fig.6,2) - 22.5°C was very similar to that observed for acetylene reduction by excised nodules (Ch.IV). Dry weights of gorse plants at this optimum temperature were significantly ($P = 0.05$) greater than at any other temperature. Growth of nodulated gorse was significantly less in low light than in high light at both 22.5°C and 25.6°C. The growth optimum at 22.5°C was less clearly defined in unnodulated gorse and light level had no significant effect on its growth. (Table 6.1A, 6.3, Fig.6.1A). Differences in growth and nitrogen assimilation between nodulated and unnodulated gorse plants are clearly shown in Fig.6.1A, 6.2A.

With both nodulated and unnodulated broom, temperature and light responses were not as apparent as in nodulated gorse, although dry weight of nodulated plants was still greatest at 22.5°C. (Tables 6.1B, 6.3, Fig.6.1B).

The highest temperature (29.2°C)caused a large, significant reduction in

shoot height, dry weight and nitrogen content for both species, and in nodule dry weight for gorse. Depressions in dry weight and nitrogen content were greater in nodulated than in unnodulated plants (Tables 6.1A,B, 6.4, Fig.6.2). The difference in dry weight responses with the two nitrogen sources, resulted in a significant temperature x nitrogen source interaction for both species (Table 6.3). Meyer and Anderson (1959) also observed a significant temperature x nitrogen source interaction with Trifolium subterraneum. This they attributed to a specific inhibition of nitrogen fixation at supra-optimal temperatures. In this experiment, the amount of nitrogen fixed, when expressed per mg nodule weight, per nodule, or as a multiple of the average nodule weight was markedly reduced at 29.2°C compared with other temperatures (Table 6.5).

Approximately 300 mg of nitrate nitrogen was added to unnodulated plants in the course of this experiment. Of this, up to half was absorbed. In a separate glasshouse experiment 70% of nitrate nitrogen supplied at 100 mg/l was absorbed (Ch.VII). This uptake corresponds closely with the observed uptake of ammonium nitrate by Vigna sinensis (Dart and Mercer, 1965a). It is therefore unlikely that growth of any unnodulated plants was restricted by a lack of nitrate.

Nodulation responses to light and temperature were different for the two species (Table 6.1A, B, 6.4, Fig.6.3). There was no significant nodule weight response to temperature for broom. However, for gorse, nodule weight was significantly higher at 22.5°C than at 29.2°C, both under high light. There was a significant effect of light on nodule number for both species (Tables 6.1A,B, Fig.6.3). Nodules were scattered over the whole root systems and not restricted to the older parts of roots, and there was a large increase in nodule number during the experiment (compare Tables 6.2 with 6.1A,B). The effect of temperature and light on growth of individual nodules was generally small. For both species, however, there was a significant increase in the number of nodules per gram of root tissue at 29.2°C compared with all other temperatures. This is similar to effects noted by Pate (1962a) and Gibson (1969a, 1971). Both these authors described it as a compensating effect for the decrease in nodule effectiveness at

the extreme of temperature range.

Higher nitrogen concentration in both shoots and roots of unnodulated plants of both species at 29.2°C than at 22.5°C, indicate that growth was impaired more than nitrate uptake at this temperature (Table 6.1A,B). The reverse was true in nodulated plants. Pate (1961b, 1962a) attributed a similar observation to limited photosynthesis at lower temperatures but adequate nitrogen fixation. It is more likely that nitrogen fixation was limited by supra-optimal temperatures to a much greater degree than other growth processes.

The nitrogenase enzyme system is well known for its lability at low temperatures (Moustafa, 1969), and for its sensitivity to denaturation by oxygen (Hardy et al, 1971a), polyphenolics etc. (Bergersen, 1971a). This sensitivity probably reflects a greater general instability than most other enzyme systems. Hence, nitrogen fixing processes catalysed by the nitrogenase enzyme system would probably be more sensitive to supra-optimal temperature than most other processes involved in plant growth.

The increase in concentration and total nitrogen content in nodules as temperature decreased (Tables 6.1A,B) was opposite to the effect observed by Gibson (1969a). This suggests that limitation of bacteroid development and accumulation of starch noted at 21°C by Dart and Mercer (1965a) for Vigna sinensis (optimum temperature about 27°C), did not occur in this experiment. The proportion of nitrogen fixed or assimilated which was transported to the shoots tends to decrease above and below the optimum temperature (Table 6.5). This trend was similar to that reported by Gibson (1966a) and presumably reflects the optimal activity of plant metabolism which may be expected at the optimal growth temperature.

Gibson (1969a) proposed a criterion of effective strains of Rhizobia as being ones which not only maintain high rates of fixation, but which also release high proportions of fixed nitrogen for use in general plant growth. It is clear from nitrogen content (Tables 6.1A,B) and nitrogen distribution (Table 6.5) figures that, in this experiment, strains of Rhizobia on broom better fulfill this criterion than do those for gorse. Higher nitrogen fixing or acetylene reducing

ability for excised broom nodules than for excised gorse nodules was noted in Ch.IV and V, and higher nitrogen content of broom plants than of gorse plants in Ch.VII and IX.

An increase in shoot/root ratio with lowered light intensity, attributed by Dart and Mercer (1965a) to a compensating effect at lower light levels, occurred in only 2 temperatures in this experiment (nodulated broom at 22.5°C and 29.2°C). However, a higher shoot/root ratio for nodulated plants than for unnodulated plants (Table 6.1A,B) was also noted by those authors (Dart and Mercer, loc.cit.). This would be expected if it is assumed that nitrogen was potentially the major limiting plant nutrient in this experiment, as root development necessary to absorb nitrogen required for plant growth would be greater for unnodulated plants than when nitrogen was supplied by nodules. This contention is supported by the shoot/root ratio response to temperature in gorse, where root development relative to shoot development was least at the optimum temperature for nitrogen fixation. In this species reduction in nodule development with excess temperature, paralleled reductions in root and total plant growth (Table 6.1A). In broom where this parallel reduction in nodule growth and root or plant growth did not occur, changes in shoot/root ratio with temperature were more complex (Table 6.1B).

The temperature optima for growth and nitrogen fixation by both gorse and broom are similar (22° - 23°C). This is considerably lower than ranges quoted for tropical legumes (Dart and Mercer, 1965a; Humphries, 1967), but is similar to that of many temperate species (Ketalloper, 1963; Gibson, 1963; Kunelius and Clark, 1970). Similarly, reduction in growth at high temperatures, was like that observed with temperate legume species (e.g. Medicago tribuloides and Vicia atropurpurea Pate (1961b, 1962a), Trifolium subterraneum, Gibson (1963), Lotus corniculatus, Kunelius and Clark (1970), and contrasts with the lack of such sensitivity to moderately elevated temperatures in tropical species (Mes, 1959a,b; Ludlow and Wilson, 1970).

The general pattern of temperature response fitted fairly closely with those of pea and clover (Jones and Tisdale, 1921). Gorse and broom appear to be

more sensitive to high temperatures than many species of Trifolium (Meyer and Anderson, 1959; Gibson, 1961, 1963, 1965; Small and Joffe, 1968), or Phaseolus vulgaris (Barrios et al, 1963; Small et al, 1968).

Reductions in growth at lowered light intensity were clearly not as severe as in Stylosanthus humilis (Siller, 1967) and again matched more closely that observed by Pate (1961b, 1962a) with two temperate legumes. As gorse and broom showed adequate growth, nitrogen fixation and nitrate uptake at the relatively low light levels used in this experiment, it is clear that their light requirements are not high.

Similarities in nitrogen concentrations of unnodulated and nodulated plants of both species at both light levels in most temperatures (Tables 6.1A, B) show that growth and nitrogen fixation or nitrate uptake were similarly affected by change in light intensity.

It is apparent from this experiment, that gorse and broom must be classified as legumes with relatively low optimal temperatures for growth, nitrogen fixation and assimilation, and with relatively high sensitivity to temperatures above this optimum. They are more sensitive to temperature increase when relying on fixed nitrogen for growth than when supplied with combined nitrogen as nitrate. Nitrogen fixation by these species may therefore be expected to be more sensitive to added nitrate at high ambient temperatures, than at low. Light requirements of gorse and broom also appear to be relatively low when compared with tropical or sub-tropical species.

CHAPTER VII

THE EFFECT OF PHOSPHATE AND COMBINED NITROGEN ON GROWTH AND NITROGEN FIXATION BY GORSE AND BROOM

INTRODUCTION

Reports in the literature indicate that there are two main responses by nodulated plants to combined nitrogen. These are best differentiated as:-

1. a large and significant increase in nodulation, plant growth and nitrogen fixation,
2. a decrease in nitrogen fixation compensating approximately for increased combined nitrogen intake.

Responses of the first type have been obtained when plants (both legumes and nodulated non-legumes) are grown in solution - usually non-aerated (Quispel, 1954; MacConnell and Bond, 1957a; Stewart and Bond, 1961; Stewart, 1963a; Daly, 1966; Silvester, 1965; Small, 1969). This effect is probably best regarded as a severe depression of nitrogen fixation in control treatments caused by experimental conditions. Small amounts of combined nitrogen increased size and vigour of plants in other treatments.

The second, more general type of response, occurs with long term applications of combined nitrogen in soil or sand, provided adequate steps are taken to ensure a continued supply of combined nitrogen and to control pH. (Thornton and Nicol, 1936; Jensen, 1948; Mulder, 1949; Huber, 1956; Stewart, 1963b; Cartwright, 1967; Benecke, 1968; Law and Armitage, 1970; Lee and Smith, 1972).

Small amounts of combined nitrogen applied before nodule initiation can assist plants over the initial period of nitrogen starvation as the first nodules are developing. If the supply of combined nitrogen is not continued, responses to combined nitrogen may be observed (Richardson et al, 1957; Pate and Dart, 1961; Dart and Mercer, 1965a; Ezedinma, 1964; Small, 1969). Such applications of nitrogenous fertilizers can therefore be a useful agronomic practice, especially where nodulation is normally slow. Oghoghorie and Pate (1971)

report slightly increased nodule numbers, nodule weight per root and acetylene reduction and greatly increased average nodule weight in Pisum arvense supplied with low levels of nitrate. This could well reflect application of combined nitrogen before effective nodules were formed, as the effect on acetylene reduction, most marked in early harvests (18 days after sowing) when nodules were becoming fully effective, was disappearing by final harvest at age 30 days.

There have been reports of differences between sources of combined nitrogen in their effect on nodulation and plant growth. Thus, Richardson et al (1957) and Diatloff (1967) observed a greater inhibition of nodulation by nitrate, than ammonia. Law and Armitage (1970) showed that while heavy dressings of urea had only a transient, if any, effect on root growth and nodulation by Trifolium repens, similar applications of ammonia severely reduced both. These effects could, however, result from increasing soil acidity associated with uptake of ammonia (Mulder, 1949; Barker et al, 1966). Ohkawara (1928), Thornton and Nicol (1936), Munns, (1968a,b,c), and Benecke (1968) all used nitrate as a source of nitrogen and hence avoided pH problems associated with the use of ammonia. Quispel (1954), Richardson et al (1957), Stewart (1963b) and Diatloff (1966) compared nitrate and ammonia. MacConnell and Bond (1957a), and Stewart and Bond (1961) used ammonia as a nitrogen source, but controlled the pH of the solution. Jensen (1948), Pate and Dart (1961), Dart and Mercer (1965a), Abu-Shakra and Bassiri (1972), and Lee and Smith (1972) used ammonium nitrate. Apart from three reports mentioned earlier, there appeared to be little or no difference in plant growth response among the three sources of nitrogen provided pH of the culture medium was adequately maintained (Thornton and Nicol, 1936; Small, 1969).

Subba-Roa and Vasantha (1965) detected changes in amino acid composition of nodulated Trifolium alexandrum plants and their exudates, when supplied with nitrate. Weissman (1972 a,b) observed differences in enzyme and nucleotide patterns of unnodulated Glycine max between plants grown on ammonia and those utilizing nitrate as a source of nitrogen. Cycling assimilated nitrogen through shoots back to roots of nodulated Pisum arvense was much greater in

plants receiving $^{15}NO_3$ than in those relying on $^{15}N_2$ fixation (Oghoghorie and Pate, 1972). This possibly reflected more generalised absorption of nitrate, compared with specialized structures for nitrogen fixation.

Using excised roots with cut ends embedded in agar, Cartwright (1967) showed a difference between effects of urea and nitrate on nodulation of Phaseolus vulgaris. The number of nodules was markedly increased by increasing concentrations of sucrose in the agar medium. The author correlated effects observed with the levels of free inorganic nitrogen in the roots. Thus, the effect of combined nitrogen, can, in this case be directly attributed to availability of nitrogen from either the external medium or internally within the roots.

Small and Leonard(1969) showed in peas and subterranean clover, that increasing the supply of nitrate in the culture solution, rapidly decreased translocation of ^{14}C labelled photosynthate to nodules and increased its translocation to growing root tips and out of nodules.

Nodule structure is also affected by combined nitrogen. Supplying ammonium nitrate to Medicago sativa and Trifolium subterraneum resulted in rapid nodule degeneration similar to that of cell organelles (Dart and Mercer, 1965b).

Mahl and Wilson (1968)and Strandberg and Wilson (1968)studied the effect of combined nitrogen (as nitrate or ammonia) on growth and acetylene reduction by nitrogen fixing bacteria Klebsiella pneumoniae and Azotobacter vinelandii. They concluded that addition of combined nitrogen to the culture medium caused repression of nitrogenase enzyme synthesis. Ohmori and Hattori (1972) showed that while nitrate had no effect on nitrogenase formation or activity in the blue-green alga Anabaena cylindrica, ammonia completely repressed its formation, but had no effect on its activity. In contrast to this, L'vov et al (1971) observed complete inhibition by ammonia of nitrogenase in cell free extracts of Azotobacter vinelandii. Moustafa et al (1969) and Hardy et al (1971) showed that there was a marked reduction in nitrogenase activity in the field after application of nitrogenous fertilizer to white clover and soy beans respectively. They did not consider the mechanism of this action.

Generally, nitrogen fixing plants (legumes, non-legumes, and algae) require relatively high levels of phosphate for maximum growth (van Schreven, 1958; Andrew, 1962; Stewart, 1963b; Benecke, 1968). The high phosphate requirement was shown by most tropical legumes (except Stylosanthes humilis and Lotononis bainesii) and by the temperate legume Medicago sativa when tested on two low phosphate status soils (Andrew and Robins, 1969a, b). When grown in pots in soils of low in available phosphate, S.humilis showed marked responses to moderate amounts of applied superphosphate (125 kg/ha), but no further response to greater amounts (Robinson and Jones, 1972). This response was especially marked when adequate sulphur was supplied. Andrew (1966) showed that this low phosphate requirement of S.humilis was associated with its ability to absorb ^{32}P from solutions both high or low in phosphate at a faster rate than would three other legumes or barley.

In almost all of eleven species tested by Andrew and Robins (1969b), nitrogen concentrations in plant tops increased with increasing phosphate supply. There were also close correlations between nitrogen and phosphorus concentrations in the tops provided phosphorus was not absorbed in excessive amounts. Increases in plant nitrogen concentration of S.humilis with applied phosphate were also observed (Robinson and Jones, 1972).

Rossiter and Kirton (1956) reported a range of sensitivities to phosphate in lupins, subterranean clover and barrel medic. Snaydon and Bradshaw (1962) showed a range of response to phosphate even within a species (Trifolium repens) depending on the phosphate status of soils from which parent populations were obtained. Specht and Groves (1966) claimed that there were no great differences in phosphate requirements of various Australian native and introduced legumes or non-legumes. Their results must, however, be treated with skepticism, as no effort was made to maintain the phosphate concentration in the medium in which plants were grown during the 46 week experiment.

Two experiments were conducted to attempt to classify gorse and broom with respect to their response to combined nitrogen and phosphorus.

EXPERIMENT I

Plants used in this experiment were broom seedlings extremely deficient in nitrogen and phosphorus. They were inoculated by pouring 5 ml of a suspension of crushed nodules, over the surface of the perlite rooting medium. By the time nutrient treatments were started, tops of inoculated plants were showing signs of effective nodulation on roots, and were considerably more healthy than those not inoculated.

Plants (10 per treatment) were grown individually in perlite in 2 inch plastic pots, arranged in blocks in a glasshouse. Five nitrogen (as $NaNO_3$) levels, 0, 5, 10, 25 and 50 mg N/l, and 5 phosphate levels, 0, 1, 5, 10 and 30 mg P/l, at each nitrogen level were used. These treatments were applied to both nodulated and unnodulated plants. Plants were watered daily with distilled water. Nutrient solution (100 ml per 10 plant treatment) was applied at approximately weekly intervals. Ten nodulated and unnodulated plants were taken as the initial harvest.

Treatments were started in April and the experiment harvested 20 weeks later. At harvest, plants were washed from the perlite, nodules counted and plants bulked for dry weight and nitrogen analysis.

Results and Discussion

Growth responses to added nitrogen were apparent in unnodulated plants after 4 weeks. However, although growth of nodulated plants was apparent when treatments were started, differences between treatments took much longer to appear than in unnodulated plants. Results are summarized in Table 7.1 and Figs.7.1 – 7.4. As plants were bulked for dry weighing, no statistical analyses could be carried out on the data.

It is clear from Table 7.1, Fig.7.1, that in nodulated plants, provided sufficient phosphate ($\geqslant$ 5 mg/l) was applied, there was a large increase in plant dry weight at 5 mg nitrogen/l compared with the no nitrogen treatments. This increase became larger as phosphate levels increased, but there was little

additional response in plant growth to higher levels of nitrogen.

When no combined nitrogen was applied there was a small increase in growth with between 15 and 30 mg phosphorus/l. However, apart from an apparent growth maximum at 25 mg N/l and 15mg P/l, response to phosphate was almost linear at all nitrogen levels. There was a 5.5 to 7.5 fold increase in dry weight between 0 and 30 mg P/l at 5 and 10 mg N/l. Plant growth at 30 mg P/l declined as the nitrogen level increased above 5 mg/l. Thus, it would appear that the two highest levels of applied nitrogen severely inhibited nitrogen fixation.

With the exception of a small decrease at 1 mg P/l at moderate nitrogen levels ($\geqslant$ 10 mg/l), there was a general increase in nodule number with increasing phosphate (Table 7.1, Fig.7.2). The maximum number of nodules was found on plants supplied with no combined nitrogen, phosphate at 30 mg P/l. The number of nodules on these plants was 4.3 times that in corresponding zero phosphate treatment.

Apart from high nodule numbers at 25 mg N/l, 15 and 30 mg P/l, number of nodules decreased with increasing nitrogen from a peak at 10 mg N/l (zero phosphate), 5 mg N/l (1, 5 and 15 mg P/l) or zero nitrogen (30 mg P/l). Thus low levels of applied nitrogen overcame to some extent, inhibition of nodulation caused by lack of phosphate. Higher levels of applied nitrogen however, inhibited nodulation.

As expected, dry weight of unnodulated plants increased with nitrogen at all phosphate levels and increased with applied phosphate at 25 and 50 mg N/l. The lack of response to phosphate at the 3 lower levels was probably caused by a severe lack of nitrogen at these levels. (Table 7.1, Fig.7.3).

Nitrogen concentration in nodulated plants was affected only slightly by adding combined nitrogen at each phosphate level. It was, however, considerably increased with increasing phosphate (Table 7.1, Fig.7.4). As this trend accentuated growth responses, total nitrogen content was increased 12.5 times at 5 mg N/l, between 0 and 30 mg P/l. In unnodulated plants, nitrogen concentration and total nitrogen content followed similar trends to dry

weight, although smaller in magnitude. Both increased with nitrogen concentration at each phosphate level and with phosphate at 25 and 50 mg N/l.

The main features of these results were:-

1) a large growth increase,

2) a large increase in total nitrogen content, and

3) a large nodulation response of nodulated plants

between 0 and 5 mg N/l, except at 30 mg P/l. This indicated that nitrogen fixation was unable to supply plant's full nitrogen requirements under these conditions of severe phosphate stress. Similar, although larger, responses than these have been noted previously in solution culture (MacConnell and Bond, 1957a; Stewart and Bond, 1961; Daly, 1966; Silvester, 1968; Small, 1969). Great care must be exercised in comparing responses of nodulated and unnodulated plants in this experiment. As there were large differences in size and nitrogen content of plants at the start of the experiment (Table 7.1), any direct comparison of these two blocks of plants is invalid.

EXPERIMENT II

Pre-Treatment

This trial was set up in September. Two hundred and eighty unnodulated plants of each species were placed overnight in a suspension of effective strains of Rhizobium. They were then transplanted into sand: perlite (1:1) in silver-painted bottomless quart size bottles held neck downwards in small plastic pottles. These pottles acted as supports and caught excess solution. These plants represented nodulated treatments. They were watered thoroughly daily from then until experimental treatments were started 35 days later. Four days after transplanting, plants were given nutrient solution lacking N and P, and after a further 4 days, 10 ml of nutrient solution with 25 mg P/l but lacking nitrogen, to encourage nodulation. After 10 more days they were re-inoculated with 1 ml of an overnight culture of Rhizobium placed on the surface of the sand:perlite and given 10 ml of 0 mg N/l, 15 mg P/l nutrient

solution.

Twenty-four days after transplanting, young nodules began to appear as small, waxy white protuberances on the root systems of some plants and at 31 days tops of most plants began to show signs of effective nodule nitrogen fixation.

After these pretreatments had been applied for 25 days, 200 further plants of each species (unnodulated treatments) were transplanted without inoculation into autoclaved bottles filled with sand:perlite. All plants were watered with distilled water from this stage. Twenty eight additional plants were taken to provide initial harvest data.

Experimental Treatments

Combined nitrogen at 0, 5, 25, 50 and 100 mg/l, as nitrate or ammonia, was provided for each plant. Gorse plants receiving 25 mg N/l as nitrate were given phosphate at two additional levels (0 and 5 mg P/l), all other plants received phosphate at 25 mg N/l.

Tagged nitrogen solutions were used as nitrogen sources throughout the experiment. Nitrate solutions were prepared by diluting 54 atom % excess $Na^{15}NO_3$ with stock $NaNO_3$, to provide 0.684 atom % excess enriched nitrate solutions. Ammonia solutions were similarly diluted to 0.854 atom % excess from 95 atom % excess $(^{15}NH_4)_2\ SO_4$. ^{15}N enriched compounds were obtained from Isocommerz, Berlin. Enrichments of nutrient solutions were verified by mass spectrometric analysis of ammonia recovered from the solutions by Markham Distillation. Devarda's Alloy was used to reduce nitrate to ammonia.

Plants were arranged in randomized blocks, each of which contained one plant of each treatment. Every third block contained only nodulated plants. There were thus 14 replicates of nodulated treatments and 10 of unnodulated treatments. For statistical analysis results of these were treated as two separate experiments for each species.

Experimental treatments were started on 25th October (35 days after transplanting the first plants). Plants were given 20 ml of nutrient solution

per week for the first 6 weeks, 20 ml twice per week for 5 weeks, and 40 ml twice per week until they were harvested after 20 weeks of experimental treatments.

After harvest, plants were divided into roots and shoots. Roots of 160 nodulated plants from 8 blocks chosen at random were taken as a sub-sample for nodule analysis. Nodules were removed, counted and dry weighed. All root systems in the other 6 blocks were dry weighed without removing nodules.

Dry plant parts were bulked in treatments, ground in a Tema Ball Mill, and subjected to Kjeldahl analysis. The ammonia distillate was subjected to mass spectrometry after reduction to nitrogen with alkaline hypobromite (see Ch.V).

Mass spectrometric analyses were carried out by Dr.W.B.Silvester of University of Auckland.

Results and Discussion

Initial harvest data are presented in Table 7.2. For convenience, effects of phosphate and of combined nitrogen on plants will be discussed separately.

a. Phosphate. Relevant data are presented in Table 7.3 A and B. These data are extracted from fuller tables (Table 7.4 - 7.9) and as analysis of variance was carried out on all dry weight and nodule data for all N and P treatments, the same significance figures are used here as in Table 7.4-7.9.

In unnodulated plants there was no significant effect of phosphate on dry weight or shoot height (Table 7.3 A). Maximum nitrate absorption occurred at 5 mg P/l. Reducing phosphorus supply to zero reduced shoot growth more than root growth. Thus the shoot:root ratio was significantly less at 0 mg P/l than at 5 or 25 mg P/l. In nodulated plants, in contrast shoot:root ratio increased significantly when phosphate supply increased from 5 mg P/l to 25 mg P/l. This difference in response between nodulated and unnodulated plants, probably reflected an adequate supply of nitrogen to maintain growth in the former, and nitrogen starvation in the latter.

While some growth and nitrogen fixation did occur in nodulated plants when no phosphate was supplied (Table 7.3B), a 2-fold increase in dry weight

and a 2.75-fold increase in nitrogen fixation resulted from supplying 5 mgP/l. A further doubling of growth and nitrogen fixation occurred when the level of phosphate supplied was increased to 25 mg P/l. Nodule initiation was not significantly reduced at 5 mg P/l compared with 25 mg P/l, but at 0 mg P/l it was reduced to less than $\frac{1}{4}$ of that at 5 mg p/l. Nodule growth was retarded more by the decrease from 25 mg P/l to 5 mg P/l than was nodule initiation. Thus average weight per nodule at 5 mg P/l was half that at 25 mg P/l. Decreased nodule initiation at 0 mg P/l caused a slight increase in average nodule weight compared with that at 5 mg P/l. Total weight of nodules per plant at 0.mg P/l was not significantly less than at 5 mg P/l.

Growth responses to phosphate observed in this experiment were similar in magnitude, although generally smaller than those observed in Experiment I (Table 7.1, Figs.7.1 - 7.4).

Nodule efficiency (amount of nitrogen fixed, expressed in terms of nodule number, nodule dry weight or nodule nitrogen content), increased with decreasing phosphate supply. This correlates with observed low phosphate requirements for nitrogen fixation by isolated bacteroids (Bergersen, 1969). However, total nitrogen fixation and plant growth were reduced, similarly by moderate phosphate stress (25 to 5 mg P/l). With more severe stress(0 mg P/l),the amount of nitrogen fixed was more severely limited than was growth. The main factor restricting nitrogen fixation at 5 mg P/l compared with 25 mg P/l was therefore reduced nodule development. At 0 mg P/l reduced nodule initiation reduced this further.

The amount of nitrate nitrogen absorbed increased with decreasing phosphate supply. This presumably reflected the reduction in nitrogen fixation at lower phosphate levels compared with plants supplied with adequate phosphate.

In Experiment I, growth of plants at 25 mg N/l supplied with 5 mg P/l was equal to, or greater than that at 30 mg P/l. While some growth at low phosphate levels may be due to phosphate present in the rooting medium - this was not

tested - available phosphate content of perlite is very low. Thus, these plants appear to have an ability to grow adequately in soils of low phosphate status, although phosphate responses may be obtained (Egunjobi, 1967). In the experimental conditions used here, however, the proportion of plant nitrogen derived from fixed atmospheric nitrogen decreased as phosphate status lowered.

In their ability to grow at low levels of available phosphate, these species are similar to others such as Stylosanthes humilis and Lotononins bainesii (Humphries, 1967; Andrew and Robins, 1969a). However, the reasons for this ability in gorse and broom were not investigated.

b. Unnodulated plants. As expected unnodulated plants show approximately linear increases in growth and nitrogen absorption with increasing nitrogen supply (Tables 7.4, 7.5). Death of all gorse cuttings given no nitrogen and many of those at 5 mg N/l as well as negligible growth (cf. Table 7.2) of broom at zero nitrogen, indicate paucity of nitrogen in the rooting medium.

Because all or most plants at zero or 5 mg N/l (as NH_4) died, these data could not be included in analysis of variance. Generally low dry weight and nitrogen concentration in comparison with nodulated plants (Tables 7.6, 7.9), show that nitrogen was severely limiting at all levels. The maximum amount of nitrogen absorbed (broom at 100 mg NO_3-N/l) was 72.4% of that supplied.

Growth of both species and nitrogen content of broom at 100 mg N/l were lower with ammonia than with nitrate. This suggests that uptake of ammonia at this level was limited, probably by increasing acidity in the sand/perlite medium. However, differences in growth between gorse on ammonia and nitrate were not accompanied by similar differences in nitrogen content of plants. As growth differences in gorse were of smaller magnitude than those in broom, it is possible that gorse was not as susceptible to the inhibiting effect of increasing acidity as broom.

c. Nodulated plants. Final harvest data for plant nitrogen content and

^{15}N enrichment for nodulated plants are given in Tables 7.6 and 7.7 respectively.

Nodulation responses during this experiment were determined on a sub-sample of 8 of the 14 replicates. For this reason, data for nitrogen content and fixation, and dry weight of roots and nodules were calculated as weighted means of two sets of data. These sets were roots and nodules which had been taken as a sub-sample and analysed separately (8 replicates), and the remaining 6 replicates of root systems with nodules attached.

Added nitrogen had only minor effect on nitrogen content(expressed on a dry weight basis) of these plants. There was a peak in nitrogen content (both total N and N concentration) for both roots and shoots of nodulated plants of both species at 50 mg NH_4- N/l, for gorse at 50 mg NO_3 - N/l and for broom at 25 mg NO_3- N /l (Table 7.6).

From the nitrogen enrichment data presented in Table 7.7, it is possible to obtain some information on translocation within plants of combined nitrogen absorbed by root systems. Nodules of both species - site of fixation of atmospheric nitrogen - contained considerably less ^{15}N enrichment than the root systems to which they were attached. Oghoghorie and Pate (1972) made a similar observation in nodulated Pisum arvense supplied with $^{15}NO_3$.

Even at 100 mg N/l most nitrogen metabolised within nodule was fixed from the atmosphere, in preference to combined nitrogen. Ammonia appeared to be more efficient at replacing fixed nitrogen than did nitrate.

Assimilated ammonia constituted 8 and 7.6% of nodular nitrogen at 100 mg N/l for gorse and broom respectively. Assimilated nitrate constituted 2.6 and 3.4% respectively. Sprent (1972b), using ^3HOH, showed that nodules can remove water from the transpirational stream in Vicia faba and Glycine max. Thus the effect noted in this experiment probably did not result from the unavailability of absorbed nitrate or ammonia to nodules, but presumably reflected the ready availability of fixed nitrogen for metabolism within nodules.

At lower levels of nitrogen application, especially 0 and 5 mg N/l, shoot

systems contained a considerably lower enrichment than roots. As the level of combined nitrogen increased, so did the proportion of label transferred to shoots. There was a disproportionately large increase in the enrichment of shoots between 50 and 100 mg N/l in all cases. This doubling of the supply of combined nitrogen caused a 3 fold increase in shoot enrichment. Similar findings were observed by Oghoghorie and Pate (1971, 1972) with nodulated Pisum arvense supplied with ^{15}N enriched nitrate. This suggested that at low levels of applied nitrogen, absorbed nitrate or ammonia was metabolised and utilized in the root systems and shoot nitrogen was supplied largely from atmospheric nitrogen fixed by nodules. Weissman (1972a) studied effects of nitrate or ammonia nutrition on soybean exudate and root enzyme patterns. He concluded that large quantities of ammonia absorbed as such, or formed in roots by reduction of nitrate, were assimilated into amide. Much of it, rather than entering the transpirational stream was metabolised, stored or utilized in protein synthesis.

In this present experiment, atmospheric nitrogen fixed in nodules was the form preferentially translocated to shoots. Only at the highest level of combined nitrogen (100 mg N/l) were considerable amounts of assimilated combined nitrogen translocated. At this level, 35 - 45% of shoot nitrogen was absorbed as combined nitrogen. At 50 mg N/l the corresponding range was 4-16%. This greater translocation of fixed nitrogen than assimilated nitrogen presumably reflected a lower rate of protein synthesis and turnover in nodules than in the remainder of the root system.

This difference was associated in both species with a higher nitrogen concentration and total content in plants supplied with ammonia than in those supplied with nitrate (Table 7.6).

It is possible to derive an equation to calculate the amount of nitrogen fixed during the experiment from the nitrogen content at harvest and the ^{15}N enrichment of this nitrogen and of nutrient solutions used.

If t = total nitrogen (mg) present in each plant part at final harvest,

a = total nitrogen (mg) present in each plant part at initial harvest,

b = amount of nitrogen (mg) fixed during the experiment,

c = amount of nitrogen (mg) absorbed during the experiment,

and y and x are ^{15}N enrichments (atom % excess) of solution and plant nitrogen respectively then

$$t = a + b + c \qquad (1)$$

nitrogen absorbed was enriched at x atom % xs,

then as mg xs ^{15}N in plant = mg xs ^{15}N absorbed

and mg excess ^{15}N = mg N (total) $\times \frac{\text{atom \% xs}}{100}$

$$\text{then } \frac{tx}{100} = \frac{cy}{100}$$

$$c = \frac{tx}{y}$$

$\therefore$ substituting in (1) and rearranging

$$b = t\left(1 - \frac{x}{y}\right) - a$$

Nitrogen fixation data calculated with this equation are presented in Table 7.8.

In one case only was there any marked increase in the amount of nitrogen fixed when combined nitrogen was supplied. This was in gorse supplied with 25 mg NO_3 - N/l where 13.5% more nitrogen was fixed than at 0 mgN/l. The amount of nitrogen fixed at each level of combined nitrogen, expressed as a percentage of total increase in plant nitrogen, decreased as the amount of combined nitrogen increased. The absolute amount of nitrogen fixed by gorse was not severely reduced up to 50 mg/l. Hence, for gorse, the increasing nitrogen content (Table 7.6) over the range of 0 - 50 mgN/l generally reflected an increasing amount of nitrogen absorbed from the nutrient solution. Nitrogen fixation was reduced at 100 mgN/l to 55 - 60% of that at 0 mgN/l. Thus, under these conditions, nodules on gorse were unable to provide its total nitrogen requirements. In this respect, these results showed a similar, although much smaller, trend to that reported by Stewart and Bond (1961) for Alnus glutinosa and Myrica gale in solution culture.

In broom, however, the response was somewhat different from that of gorse in this experiment. In Experiment I described earlier, a relatively large increase in dry weight and nitrogen content was noted in broom to small amounts of added nitrogen. In this second experiment, however, nitrogen fixation and, generally, nitrogen content, were reduced by added nitrogen compared with levels in the absence of combined nitrogen. Small amounts of added nitrogen inhibited nitrogen fixation. In this respect, ammonia had a greater effect than nitrate. Oghoghorie and Pate (1971) observed a similar reduction in nitrogen fixation by Pisum arvense supplied with nitrate. This effect was, however, contrary to effects reported by Stewart and Bond (1961) and Silvester (1968).

The nitrogen fixing symbioses of broom was, in this experiment, able to provide the total nitrogen requirements of the plants. The difference between gorse and broom probably reflected a greater nitrogen fixing efficiency by broom nodules than by gorse. This was also shown in results presented in Chapters IV, V and VI.

The amounts of nitrogen fixed by these species during this experiment, 132 and 165 mg per plant in 20 weeks for gorse and broom respectively, were very similar to those reported by Stewart and Bond (1961) for Alnus glutinosa and Myrica gale (63 and 84 mg respectively in 10 weeks). Becking (1970) in his review quoted 500 mg as the amount of nitrogen fixed by Alder in one 48 week season and the amount fixed by Ceanothus velatius in 18 weeks as 268 mg per plant. This figure for C.velatius is considerably higher than those obtained in this experiment, although the former is similar in magnitude. Although experimental conditions used by Becking (loc.cit.) were not quoted, they must be considerably more conducive to nitrogen fixation by alder than were those used by Stewart and Bond (1961). Figures quoted by Becking (1970) therefore probably represent a truer estimate of nitrogen fixing ability by these woody non-legumes than those of Stewart and Bond (1961). It would seem, therefore, that broom under these conditions was able to fix a similar amount of nitrogen to that fixed by Myrica (Stewart and Bond, 1961). Nitrogen fixation by Alnus was approximately 25% greater than this and by gorse 35% less. This assumes that

comparisons over different lengths of experiment are possible.

Final harvest plant dry weight and shoot height data are given in Tables 7.9A, B.

There were only slight responses in shoot, root and total dry weight and shoot height to combined nitrogen. Dry weight at 50 and 100 mg N/l was significantly ($P < 0.05$) greater than when no nitrogen was supplied with gorse only (Table 7.9A). Variability of growth of these plants possibly hid other responses.

Only with broom supplied with 100 mg N/l were dry weight and shoot height of plants supplied with ammonia, significantly different from those supplied with nitrate (Table 7.9B). At this level, nitrate supplied plants weighed 25% more than those supplied with ammonia. The nitrogen concentration within these ammonia-fed plants was higher than those supplied with nitrate. Thus, although there was a 25% difference in total dry weight, there was only a 4.6 and 6.6% difference in nitrogen fixation and absorption respectively (Tables 7.6, 7.8)between plants supplied with different nitrogen sources.

Distribution of nitrogen within plants was very similar for both sources.

The difference in growth on the two nitrogen sources probably reflected inhibition of growth of plants supplied with ammonia, rather than stimulation of those supplied with nitrate. This possibly reflects increasing acidity associated with ammonia uptake (Mulder, 1949; Barker et al, 1966).

The effect, must, however, have been indirect rather than a direct inhibition of ammonia uptake. As neither pH of the growth medium, nor chemical composition of plants was determined, it was not possible to determine possible effects of ammonia uptake on absorption of other nutrients. As there was no similar difference observed with gorse, it was possible that that species was less susceptible to increased acidity than was broom. In both species, however, increasing supply of combined nitrogen (both sources) caused increased root growth compared with shoot growth. Thus, in all cases at 100 mg N/l shoot root ratio was less than at zero nitrogen (Table 7.9 A,B).

Growth responses observed in this experiment fitted broadly into the pattern of response observed by other authors, notably Mulder (1949), Allos and Bartholomew (1955), Samuels and Landrau (1953) and Landrau et al (1953). They contrasted with results obtained in the experiment described earlier in this chapter and more markedly with those obtained by other workers with non-legumes (Stewart and Bond, 1961; Stewart, 1963a; Daly, 1966; Silvester, 1968).

As there was no significant difference between the two nitrogen sources in their effect on nodulation ($P = 0.2$), nodule data for both sources were combined for regression analysis. Regression lines of nodule weight and nodule number on level of applied nitrogen were highly significant for both species (Table 7.10, Fig.7.5, 7.6).

With gorse, both nodule weight and nodule number were significantly lower at 100 mg N/l than at 0, 5 and 25 mg N/l. Nodulation in broom, however, appeared to be slightly more sensitive to applied nitrogen than gorse. Apart from the anomalous depression at 5 mg N/l, nodule weight at 100 mg N/l was significantly less than at all other levels. Nodule number at 50 and 100 mg N/l was significantly lower than at 0 and 25 mg N/l. Overall trends, as shown by the slopes of regression lines (Fig.7.5, 7.6) were remarkably similar in both species. There was a 40–50% reduction in both measures of nodulation from the lowest to the highest level of applied nitrogen (Table 7.10).

This response was similar to that in Pueraria phaseoloides (Samuels and Landrau, 1953). In that species, 270 kg N/ha as ammonia depressed nodule number by 83%. Diatloff (1967) noted complete inhibition of nodulation at 168 mg NO_3/l in soybeans, although at 56 mg/l nodulation was increased, and 120 kg N/ha (as NH_4NO_3) reduced soybean nodule numbers by 60% and nodule weight by 43% (Abu-Shakra and Bassiri, 1972).

Again the response was in direct contrast to those shown by MacConnell and Bond (1957), Stewart and Bond (1961), Silvester (1968), and Small (1969). In some of those reports nodulation was almost doubled at small levels of nitrate or ammonia (when pH was controlled).

Nodulation data (Tables 7.9 A, B) and nitrogen content and fixation figures

(Table 7.7,7.8) were used to calculate nodule efficiency of plants. These efficiency data, expressed in three ways viz. as mg nitrogen fixed per nodule, per mg nodule weight, or per mg nodule nitrogen are given in Table 7.11.

These figures were obviously fairly variable, and there were several anomalous low values which did not fit into the general trend. For example, when expressed in terms of the number of gorse nodules at 5 mg NH_3- N/l or broom nodule nitrogen at 50 mg NO_5-N/l, efficiency appears considerably lower than other values. There appeared, however, to be a peak in nodule efficiency for all measures and for both species at 25 or 50 mg N/l. Efficiency at 100 mgN/l was similar to, or lower than that at 0 mgN/l. Oghoghorie and Pate (1971) observed similar effects of applied nitrate on acetylene reduction by Pisum arvense nodules.

In most cases, nodule efficiency of plants supplied with nitrate was greater than those supplied with ammonia. This was most marked when efficiency was expressed in terms of nodule nitrogen content. This difference reflects the higher nodule nitrogen content on the ammonia fed plants compared with nitrate fed plants(Table 7.6). Jensen (1948) observed large (70-80%) reductions in nodule efficiency (N fixed per mg d.w. of nodules) in Medicago tribuloides and Trifolium subterraneum following heavy applications (300-400 mg N/pot of 6-8 plants) of combined nitrogen. Levels of nitrogen applied during this experiment were considerably less than this and reductions in efficiency of this magnitude were not observed.

CHAPTER VIII

OXYGEN REQUIREMENTS AND THERMODYNAMICS OF SYMBIOTIC NITROGEN FIXATION. THEORETICAL DISCUSSION

Of the authors who observed large increases in nodulation, plant growth and nitrogen fixation with combined nitrogen (responses of the first type, Ch.VII), Quispel (1954) studied Alnus glutinosa, MacConnell and Bond (1957a) and Stewart and Bond (1961), A.glutinosa and Myrica gale, Stewart (1963a) M.gale and Casuarina spp., Daly (1966) A.rugosa and Silvester (1968), Coriaria arborea. MacConnell and Bond (1957a) also included Ulex europaeus in their study, and Small (1969) studied several varieties of Trifolium africanum. Thus it is clear that this type of response is not peculiar to either leguminous or non-leguminous nitrogen fixing plant symbioses.

One interesting result in the work of MacConnell and Bond (1957a) was the response of Myrica gale. The lack of effect of combined nitrogen on growth, nodulation and nitrogen fixation in that species was in direct contrast to large responses with Alnus glutinosa and Ulex europaeus. These authors concluded ".... the Alnus nodules in nitrogen-free solution failed by a larger margin than those of Myrica to satisfy the plants' requirements for nitrogen and to permit maximum nodulation". Thus, nitrogen-free solution failed to permit maximum nodulation in Alnus under the experimental conditions used.

It should be noted here that Myrica nodules possess so-called "nodule roots" which grow upwards. These were considered by Bond (1952) to be an adaptation for growing in wet anaerobic bog conditions. These rootlets were necessary for maximum activity by excised Myrica nodules (Bond, 1961). Thus an aeration effect may have caused the differences between Alnus and Myrica (MacConnell and Bond, 1957a ; Stewart and Bond, 1961).

Virtanen and von Hausen (1936), Bond (1951) and Ferguson and Bond (1954) have shown that nodulation and nitrogen fixation of peas, soybeans, and red clover, respectively were very dependent on the oxygen concentration of

the rooting medium. Unnodulated plants supplied with combined nitrogen were much less dependent on aeration than were those relying on nitrogen fixation. Thus while nitrogen fixation may be severely restricted by lack of oxygen (cf. effect on excised nodules - Ch.IV), the uptake of combined nitrogen may not be.

Nodule initiation was not nearly as sensitive to lack of oxygen as nodule development and nitrogen fixation (Ferguson and Bond, 1954). Thus, under conditions of restricted oxygen supply, small amounts of combined nitrogen would enable plants to make considerable growth responses and hence increase the size and number of nodules present. Considerably more nitrogen fixation would occur under these conditions than when no combined nitrogen was supplied. Thus nodulation and nitrogen fixation may not be directly affected, but these nodulation and nitrogen fixation responses reflect growth stimulation accompanying addition of combined nitrogen.

This contention is supported by the observations of MacConnell and Bond (1957a) for Alnus and Myrica and Stewart and Bond (1961) for Alnus, that while the actual weight of nodules per plant was lowest at zero nitrogen, relative to enhanced growth of plants, nodule development was successively depressed with increasing amounts of combined nitrogen added.

Slight stimulation of nodulation on nitrate containing media as shown by Gibson and Nutman (1960) for clover and lucerne may also be partly ascribed to growth limitations caused by inadequate aeration. Gibson (1967a) subsequently showed that plant growth in test tube culture can limit growth by limiting aeration.

Ferguson and Bond (1954) could obtain little response to oxygen with T. pratense grown in sand. This they attributed to limited growth of plants in their experiments. However, differences in growth were apparent in their first experiment (with enclosed roots in solution) at an early stage of growth - shortly after the first signs of nitrogen fixation appeared. Thus, it would appear that the higher oxygen availability in sand than in solution, was a major contributing

factor in their results.

Greenwood (1969) stated in his theoretical study on the effects of water-logging soils on oxygen availability to roots, that even short periods of water-logging can seriously affect later yields of wheat. Continuous or repeated inundations of root systems in solutions, would likewise, seriously affect plant growth, especially in sensitive plants such as those relying on nodulation and nitrogen fixation.

Excised soybean nodules incubated in distilled water reduced acetylene at a much slower rate than those incubated dry (Schwinghamer et al, 1970; Mague, 1971; Mague and Burris, 1972). Nodules of soybeans and Vicia faba from water-logged soil exhibited much less acetylene reducing activity than those from soil watered to field capacity (Sprent, 1972b). These effects were attributed to oxygen deficiency.

Loveday (1963) and Cradwell (1969) studied the effect of limitations in oxygen diffusion rates (as measured by a platimum electrode), on growth of subterranean and white clover respectively. Their figures show reduction in root, shoot, or nodule growth at oxygen diffusion rates less than $8-10\times10^{-8}$ g/cm^2/min but normal growth at rates greater than $15 - 20 \times 10^{-8}$ g/cm^2/min .(theoretical rate of diffusion of oxygen in air is about 3.2×10^{-4} g/cm^2/min). Thus root growth is unlikely to be restricted in most soils (Greenwood, 1969). Using solution culture, however, the situation is considerably different from that in soil. Air saturated water has an oxygen concentration of 6.4×10^{-6} g/cm^3 (cf, in air 0.2 atmos = 2.9×10^{-4} g/cm^3). The diffusion coefficient of oxygen in water is 10^{-4} of that in air. Therefore growth limiting oxygen diffusion rates would be reached much more rapidly in water than in air. In fact, Greenwood(1969) stated that oxygen concentrations in water-saturated aggregates could fall from that in air-saturated water to zero in 0.1cm if root respiration rates were high. Rates of oxygen transfer through most plants according to Greenwood(loc.cit.) are sufficient to satisfy the metabolic oxygen requirements of only short portions of root of normal mesophytic species.

The optimal oxygen concentration for nitrogen fixation by excised nodules is 3 - 7 x 10^{-4}g/cm^3(0.2 - 0.5 atmos)(see Ch.IV). Assuming that low leghaemoglobin levels in nodules grown in solution are a response to low oxygen levels (see Ch.IX), then oxygen requirements under these conditions would be more like that of bacteroids. Bergersen and Turner (1968) gave this as 0.12 atmos, (1.72 x 10^{-4}g/cm^3). As this is several times higher than the concentration of oxygen in air-saturated water (6.4 x 10^{-6}g/cm^3), nitrogen fixation in solution culture would therefore be severely limited by limited oxygen supply.

Root and nodule growth probably have similar oxygen requirements. Nitrogen fixation is more sensitive than these. Oxygen concentration in solutions in which plant roots are growing would rapidly fall to low levels. This would restrict firstly nitrogen fixation and later, nodule growth. Small amounts of combined nitrogen added to nodulated plants under these conditions, would clearly aid plant growth by partial removal of nitrogen stress. Responses of the type observed in Experiment II, Ch.VII (Tables 7.8A,B. Figs.7.5,7.6) represent a truer general response of nodulated plants to combined N than large growth, nodulation and nitrogen fixation responses.

The mechanisms of depression of nodulation and nitrogen fixation by combined nitrogen are in doubt. Bergersen (1969) showed that the action is not one of end-product inhibition. However, ammonia completely inhibited nitrogenase activity in cell free extracts of Azotobacter vinelandii (L'vov et al,1971). Addition of ammonia to the culture media of nitrogen fixing bacteria and blue-green algae caused repression of further nitrogenase enzyme synthesis (Mahl and Wilson,1968; Strandberg and Wilson,1968; Ohmori and Hattori,1972). However, this mechanism would be too slow in plants to explain results obtained by Moustafa et al (1969) and Hardy et al (1971a) who observed a reduction in acetylene reduction by white clover and soybean nodulated roots after application of nitrogenous fertilizer. Nor does enzyme repression explain nodule degeneration observed by Dart and Mercer (1956b) in Medicago tribuloides and Trifolium subterraneum when supplied with 30 mgN/kg(on soil basis).

Thornton and Nicol (1936) and Munns (1968b) showed that one effect of

added ammonia and nitrate was to reduce root hair curling and formation of infection threads and to increase the number of infection threads aborting. Lessened I.A.A. production in the presence of ammonia, increased breakdown by nitrate (Tanner and Anderson, 1963), and reduced poly-galacturonase formation in the presence of nitrate (Fahreus and Lungren, 1959; Subba-Roa and Vasantha 1965) would affect this phase of nodulation.

Dart and Mercer (1965a) and Munns (1968a) showed that only the first crop of nodules after addition of nitrate was affected. The next crop, 2-3 weeks later was not markedly affected. These findings suggest that one mode of action of added nitrate on nodulation is internal, for example, through C/N balance (Wilson, 1940; MacConnell and Bond, 1957a; Gibson and Nutman, 1960), with some hormonal control (Stewart, 1966).

Raggio et al (1957) and Cartwright (1967) suggested that the effect of combined nitrogen on nodulation resulted from accumulation of free nitrogen compounds and depletion of carbohydrates within roots rather than a local effect of any particular nitrogen compound.

These mechanisms do not explain nodule degeneration (Dart and Mercer, 1965b), reduced nodule efficiency (Table 7.11)(Jensen, 1948) or nodule activity (Moustafa et al, 1969; Oghoghorie and Pate, 1971) when combined nitrogen is supplied.

Use of nitrate instead of fixed nitrogen as a source of nitrogen causes changes in nitrogen metabolism. The ratio $\frac{\%N}{D.W.}$ (Haydock and Norris, 1967), amino acid composition (Subba-Roa and Vasantha, 1965), and enzyme and nucleotide pattern (Weissman, 1972a,b) within seedlings depend on the source of nitrogen.

Wheeler (1971) observed a rapid translocation of ^{14}C labelled photosynthate to nodules. This photosynthate is necessary for continued nitrogen fixation (Virtanen et al, 1955; Bergersen, 1971a). The flow of photosynthate to nodules reduced by application of combined nitrogen (Small and Leonard, 1969). Reduction of nodule activity (Moustafa et al, 1969; Hardy et al, 1971a) and, presumably, nodule degeneration follow (Dart and Mercer, 1965b).

It is important at this stage to consider possible differences in energy changes associated with assimilation of the three main sources of nitrogen(viz. NO_3, NH_4 and N_2). Gibson (1966b) showed that the carbohydrate requirement of nitrogen fixation (including nodule development and maintenance) similar to, or only slightly greater than those for uptake of combined nitrogen (as NH_4NO_3).

From Baylis' (1956) figures, it is possible to calculate the changes in Gibbs Free Energy functions during reduction of nitrate or nitrogen to ammonia. Uptake of ammonia would result in very little or no free energy change preceeding amino acid formation.

As some reductant in nitrogen fixation is supplied at the level of ferredoxin, which is very similar to H_2 (Bergersen, 1969), the best equation for nitrogen fixation is:-

$$\tfrac{1}{2}N_{2(aq)} + 3H^+_{(aq)} + H_{2(aq)} + \ddot{e} = NH_{4(aq)}^+ \quad G^o = -19 \text{ kcal} \qquad (1)$$

Nitrate reduction can be represented by:-

$$NO_{3(aq)}^- + 10H^+_{(aq)} + 8\ddot{e} = NH_4^+{}_{(aq)} + 3H_2O \quad G^o = -162.6 \text{ kcal} \quad (2)$$

The equation $G = G^o + RT \ln \frac{\text{activities of products}}{\text{activities of reactants.}}$

can be applied to correct for assumed biological concentrations (0.01 mol/l ammonia and nitrate) and nitrogen concentration in air-saturated water(0.67mmol/l). The functions of Gibbs Free Energy of equations (1) and (2) with pH are depicted in Fig.8.1. This suggests that nitrate is the energetically preferred nitrogen source at all pH levels below pH13.

This approach ignores however, the difference in numbers of electrons involved in reduction of these two nitrogen sources (7 electrons more for nitrate than for nitrogen). This would result in approximately 70-84 kcal/ mol ammonia less energy available from the electron transport chain when nitrate is reduced to ammonia than when nitrogen is fixed. Approximately 4-4.6 ATP molecules per electron pair are required for nitrogen fixation by Clostridium pasteurianum (Burris, 1969). This is equivalent to 16 kcal/mol ammonia according to equation (1). The ratio of ATP molecules to per electron pair

is also dependent on pH (Burris, loc.cit.) and on the ratio of the two proteins of nitrogenase (Bergersen, 1971b). These adjustments make nitrate and nitrogen energetically equivalent nitrogen sources, provided all energy obtained from reduction of nitrate is conserved. If it is not, then nitrogen fixation would be energetically preferred over nitrate reduction.

Bergersen (1971b) using slightly different equations and assuming different concentrations of reactants and products to that assumed here, showed that while nitrogen fixation could be expected to require 60-72 kcal/mol ammonia in living systems, nitrate reduction would need 20-60 kcal/ mol ammonia.

Ammonia may be slightly energetically preferred to the other two sources, as there are essentially no chemical reactions necessary in its absorption prior to amino acid formation. In this respect, it is interesting to note the greater incorporation of higher levels of assimilated ammonia than of nitrate (Table 7.6,7.8).

Oghoghorie and Pate (1971) showed, that at higher levels of supplied nitrate (>70 mg NO_3 - N/l) in vitro nitrate reductase activity of shoots of Pisum arvense was considerably greater than in roots. They suggested that this shoot nitrate reductase would sequester large amounts of photosynthate, thus depriving the root of substrates and reductant for nitrogen fixation.

Small and Leonard (1969) did not autoradiograph shoots of plants they supplied with $^{14}CO_2$ and nitrate, but they did observe alterations in translocation of ^{14}C labelled photosynthate in T.subterraneum when nitrate was supplied. This correlates with the much higher nitrate reductase activity in roots of Pisum arvense than in nodules when plants were supplied with nitrate (Oghoghorie and Pate, 1971).

Reduction in fixation of atmospheric nitrogen with added combined nitrogen, the effect predicted from the above discussion, occurred in both species in Experiment II,Ch.VII. The linear reduction in nodulation (Figs.7.5,7.6) and the limited dry weight responses (Table 7.9A,B) with combined nitrogen also support these conclusions. Substantial amounts of available combined

nitrogen may be expected to reduce nitrogen fixation in approximate proportion to the amount absorbed (Thornton and Nicol, 1936; Jensen, 1948; Mulder, 1949; Huber, 1956; Stewart, 1963b; Cartwright, 1967; Benecke, 1968; Moustafa et al, 1969; Hardy et al, 1971a; Oghoghorie and Pate, 1971).

The similarity between responses to combined nitrogen of plants grown in solution and those observed in Experiment I,Ch.VII, make it tempting to ascribe the latter response to limited aeration. The perlite rooting medium was noted to have packed down and considerable moss growth occurred on the surface. This could have reduced aeration and could explain some of the differences between results of these two experiments. Field responses would be more similar to those observed in Experiment II than to those of Experiment I.

CHAPTER IX

BORON REQUIREMENTS FOR GROWTH AND NITROGEN FIXATION IN GORSE AND BROOM

INTRODUCTION

Boron was first demonstrated as an essential plant nutrient in 1910 (see review by Skok, 1958).

Although there is no evidence that boron is required for nitrogen fixation per se, it has been shown to be necessary during tissue development (Skok, 1958). Boron deficiency restricts nodule development in Vicia faba by halting development of vascular strands within nodules. This results in restricted development of bacteroid tissue (Brenchley and Thornton, 1925). Calcium and phosphate are known to influence boron deficiency (Rene and Shive, 1941; Jones and Scarseth, 1941; Tanaka, 1967a).

Mulder (1949) growing Pisum sativum in solution culture, observed plant death from nitrogen deficiency at very low boron levels, while at slightly higher levels nitrogen fixation was not inhibited as seriously and boron deficiency symptoms became apparent in the tops. When combined nitrogen was supplied boron requirements appeared to be higher, - possibly reflecting higher growth rates. When plants were grown in soil, responses were rather different, with decreases in nitrogen fixation, even when no boron deficiency symptoms were observed in the tops.

When Trifolium subterraneum plants grown in solution containing varying levels of boron were transferred to solutions completely lacking this nutrient, levels of boron supplied in the original solution had marked effects on growth and dry matter accumulation following transfer(Bouma and Dowling, 1966; Bouma, 1969). Differences in leaf area increases between plants raised at different boron levels were apparent as soon as three days after such transfer (Bouma and Dowling, loc.cit.). In the early stages, boron deficiency had little effect on total dry weight increases but leaf growth increased in preference to root growth. Later, leaf growth was also restricted. Differences in patterns of

leaf dry weight increases were apparent between boron deficient plants transferred to solutions lacking boron and those transferred to complete solutions. Dry weight increases of existing leaves were much greater, and those of new leaves much less, in plants transferred to solutions lacking boron than in those transferred to boron sufficient solutions. Growth responses after removal of boron stress were characterised initially by preferential distribution of assimilates to roots (Bouma, 1969).

Samuels and Landrau (1953) and Landrau et al (1953), however, could obtain no response to boron in growth or protein content of the tropical legume Pueraria phaseoloides (Javinica) Benth (Tropical Kudza) on an acid (pH4) tropical soil. Boron has been shown to be necessary for optimal lucerne seed production in North Carolina, U.S.A. (Piland et al, 1941), Australia (Cradock, 1956) and Ohio, U.S.A. (McLean and Volk, 1958).

By comparing micronutrient levels in soils of a Wisconsin forest nursery with the health of nursery stock of 3 Pinus species growing there, Tanaka et al (1967) deduced that the critical soil concentration of boron was 0.3 mg/kg for those species. McLean and Volk (1958) stated that lucerne required a boron level greater than 0.5 mg/kg in soil for optimum growth. Normally grown lucerne plants contained 20-40 mg/kg boron, (Cradock, 1956; McLean and Volk, 1958; Sauchelli, 1969), but those which did not set seed normally contained 13 - 18 mg/kg (Cradock, loc.cit.).

Tissue levels for boron in pine forests in Tasmania were similar to these levels, although the lower sufficiency level for Pinus was lower than in lucerne. Healthy trees were shown to contain 14-25 mg/kg, while a concentration of 3-8 mg/kg was associated with corresponding degrees of die-back (Proctor, 1967).

Excised legume roots were shown to have boron absorption capacities intermediate between those of monocotyledonous plants, which had low boron absorptive capacities, and those of other dicotyledonous species, in which capacities were higher (Tanaka, 1967b). This correlates with the relative sensitivities of legumes, other dicotyledonous and monocotyledonous species to boron deficiency. Legumes have been shown to be more sensitive to boron deficiency

than other dicotyledonous species (McKay et al, 1962), but less sensitive than oats (Calder and Langille, 1963).

Rapid responses to deficient or sufficient levels of boron observed by Bouma and Dowling (1966) and by Bouma (1969) supported the conclusion (McIlraith, 1965) that absorbed boron was immobilised within most plants species. However in Bouma and Dowling's (1966) experiments, only when the original solutions were sub-optimal in boron, were increases in leaf area following transfer of plants to solutions lacking boron, significantly different from those on plants transferred to complete nutrient solutions. Bouma (1969) also showed similar differences for other growth measurements between plants raised in adequate solutions and transferred to solutions with or without boron and those raised at deficient levels before transfer. These results suggest a degree of mobility of boron within T.subterraneum plants, or a storage of excess amounts of boron.

As boron deficiency has been noted in nitrogen fertilized Pinus radiata grown on the Moutere Gravels (Appleton and Slow, 1966), an experiment was designed to test its effect on growth and nitrogen fixation by gorse and broom.

MATERIALS AND METHODS

Plant material used in this trial was identical to that used in a previous trial (see Ch.VI). Plants were carefully washed out of sand/perlite and roots washed in distilled water, distilled from a metal still, stored in polythene aspirators. Plastic containers in which roots were suspended in nutrient solution were painted with aluminium paint outside after soaking in 3% hydrochloric acid and were thoroughly rinsed in boron-free distilled water. Plants were suspended over holes in these containers on split rubber rings with their roots in half strength nutrient solution. Solutions were aerated with 9 aquarium gassing stones per container connected via a manifold to an air-compressor.

"Analar" grade chemicals were used without further purification in all nutrient solutions. Nine(broom) or ten(gorse) plants were grown in each

pot with one treatment per pot. Boron, as boric acid was supplied at the rate of 0.5 mg/l to half of the treatments and was omitted from the remainder. Nodulated (no combined nitrogen added) or unnodulated (supplied with 200 mg/l nitrate nitrogen) plants of each species were grown in both boron treatments. Ten days after setting up, half strength solutions were exchanged for full strength and this was repeated six more times before harvesting at 22 weeks. At harvest nodules from all plants in each treatment were removed rapidly and nitrogenase activities determined by the acetylene reduction assay (acc.Ch.IV). One hour incubations were carried out in 50 ml erlenmeyer flasks.

Boron contents of plants were determined by the carmine method of Hatcher and Wilcox (1950) after dry ashing for three hours at 550^{o}C with saturated barium hydroxide. Boron contents of roots and nodules were analysed together as there was insufficient nodule material for separate analyses.

RESULTS AND DISCUSSION

Data was analysed as a completely randomized factorial experiment of 2 x 2 design, using Duncan's Multiple Range Test.

Tables 9.1 and 9.2 show that in both gorse and broom there was a reduction in nitrogen content of plants at harvest (Table 9.2), and when nitrogen content of nodulated plants was expressed in terms of either nodule weight or number of nodules (Table 9.1). It was also reflected in acetylene reduction data, especially for gorse (Table 9.1). These differences between treatments reflected both an increase in nodule number and weight (Table 9.1) and a decrease in nitrogen content of plants grown without boron (Table 9.2).

There were several striking differences between gorse and broom in their nodulation response to reduced boron supply. While initiation of nodules in both was affected to a similar degree by reduced boron supply, the effect on nodule weight and activity of nodules was quite different in the two species (Table 9.1).

Both number and dry weight of nodules on gorse plants were increased by

75% and hence average weight per nodule was not affected by boron deficiency. In broom however, while the increase in number of nodules on boron deficient plants, was similar in magnitude to that in gorse, total dry weight of nodules on these plants was only 17% greater than on plants supplied with boron. As a result, the average weight of nodules on boron deficient plants was 40% less than that of nodules on plants supplied with boron. The decrease in amount of nitrogen fixed by boron deficient plants of both species during the experiment(nitrogen contents, Table 9.2), compared with corresponding boron sufficient plants, indicated that increases in number and total weight of nodules on those plants was not adequate to compensate for decreased activity of nodules grown under boron deficiency.

The restriction in activity of gorse nodules (both for nitrogen fixation and acetylene reduction) suggests that nodule development in that species could have been restricted by boron development (c.f. Brenchley and Thornton, 1925).

The lack of any great effect of boron deficiency on acetylene reduction by broom nodules suggests that this restriction in development of vascular and bacteroid tissue did not occur in that species. Brenchley and Thornton(1925) also however, showed that a considerable reduction in growth of nodules occurred with boron deficiency. A similar effect to this was shown for broom, but not for gorse in this experiment.

In broom, the acetylene reducing activity of nodules was much less affected by boron deficiency than was total nitrogen fixation when these were expressed in the same terms (e.g. nodule weight - Table 9.1). Thus, the difference between nitrogen fixing activity of boron deficient broom nodules, and that of those supplied with boron, was much greater than the reduction in their acetylene reduction activities, at harvest. This effect, and the effect on average weight of broom nodules, may be explained primarily in terms of a delaying or slowing effect of boron deficiency on nodule initiation. Thus, at harvest, nodules on boron deficient plants would be at an earlier stage of development than their counterparts supplied with boron. Hence they would have been active for a shorter period of time than those supplied with boron

although activity at harvest, as measured by the acetylene reduction assay, was similar in the two boron treatments. Any restriction in development of boron deficient nodules could be obscured by this effect.

As boron deficiency has been shown to affect the structure and development of plant cell walls in actively growing regions (Skok, 1958), formation of infection threads during the infection process of nodulation could well be affected by boron deficiency.

The effect of boron deficiency on acetylene reduction by gorse nodules at harvest, was similar to its effect on the amount of nitrogen fixed by gorse during the experiment, but average nodule weight for gorse was unaffected by boron deficiency. Thus any possible effect of boron deficiency on initiation and size of gorse nodules was minor compared with the effect on their activity.

It is difficult to ascribe these differences between responses of nodules of the two species to any single factor. There are probably at least two factors involved:-

i. differences in stage of development between the two species at the start of the experiment, and

ii. differences in degree of boron deficiency of the two species during the experiment.

As the weight and number of nodules (Table 9.1) and the size of plants at the start of the experiment was different in the two species, differences may be expected in the stage of initiation of development of nodules at that time. If the time at which initiation of nodules was sensitive to boron deficiency was short, then small differences in degree of development at the start of the experiment would have been magnified during the experiment. Differences in degree of boron deficiency could arise from either interspecific differences in boron requirements similar to those shown by Milliken (1956) or from differences in amount of boron available to boron deficient treatments from contamination in containers.

As expected, restriction of the supply of boron to plants resulted in greatly reduced uptake of boron in both species (Table 9.3). The ratio of

amount of boron in plants at final harvest to that in initial harvest was, in general, similar for both species in each boron x nitrogen treatment. Thus, any differences between species in the amount of boron absorbed during the experiment probably resulted from differences in initial sizes of plants used in the experiment. Boron concentrations in treatments lacking boron (Table 9.3) were associated with a significant reduction in shoot height in all treatments except for nodulated gorse, compared with plants supplied with boron, in spite of relatively high coefficients of variation (37.1% and 36.5% for gorse and broom respectively). Shoot die back is a symptom characteristic of boron deficiency (Skok, 1958). Thus a degree of boron deficiency affecting plant tops was achieved by omitting boron from the nutrient solution. Boron concentrations associated with this boron deficiency (9.4 - 9.6 mg/kg dry weight for gorse shoots and 6.4 - 7.3 mg/kg for broom shoots) were similar to those associated with moderate die back in Pinus forests in Tasmania (3-8 mg/kg) (Proctor, 1967), but lower than those associated with restriction in seed setting in lucerne (13-18 mg/kg)(Cradock, 1956). McKay et al (1962) showed that mimimum sufficient boron concentrations in beans - a species sensitive to boron deficiency - was 12 mg/kg. Normal P.radiata contained 14-25 mg B/kg(Proctor, 1967) and normal lucerne,clover, peas, and soybeans, 20-40 mgB/kg (McLean and Volk, 1958; Sauchelli, 1969). These normal levels are similar to those observed in this experiment (Table 9.3). It would thus appear that gorse and broom have similar boron requirements to other legumes and probably, to other woody species.

The marked differences in nodulation, nodule efficiency and boron uptake Tables 9.1,9.3) between plants supplied with boron and those without were reflected in the nitrogen content of plants (Table 9.2). Lack of boron had no effect on plant dry weights (Table 9.2). While total nitrogen contents of of both unnodulated and nodulated plants were low compared with other experiments, there were appreciable differences between the nitrogen status of plants grown with and those without boron, especially in nodulated treatments.

Nodules on all plants at harvest were yellow. In spite of this, they were still quite active. This,combined with the thick fleshy nature of root systems, suggested that aeration in solution was inadequate. However, similar root morphology in pea plants was reported by Lie and Brotonegro (1969) to result from use of Fe (III) EDTA as the source of iron in culture solution. This could also have affected root morphology in this experiment. However, van de Venter and Small (1972) observed signs of root toxicity only at 0.5 mM FeIII EDTA. This is greater than the concentration used in this experiment (viz. 3.75 mM). Van de Venter and Small (loc.cit), however, did not renew the culture solutions in the 3 or 6 week experiments. Toxicity symptoms may appear at much lower concentrations if solutions are renewed regularly.

In Chapter VIII it was shown that the use of solution culture in experiments on symbiotic nitrogen fixation can seriously impair nitrogen fixing ability of nodules. Growth of nodulated plants in this experiment was therefore probably restricted by the experimental conditions. Growth restrictions have been shown to reduce boron requirements of sunflower plants (Husa and McIlraith, 1965). In this experiment, there was a significant dry weight response to boron in unnodulated gorse plants (Table 9.2) where this growth restriction of solution culture did not occur, but there was no significant growth response in unnodulated broom plants. The very high variability(C.V.54-60% for dry weight) would hide any possible response. The nitrogen source effect observed in both species for shoot height and dry weight was at least partly attributable to differences in sizes of plants at the start of the experiment.

Nitrogen concentration in shoots of unnodulated boron deficient plants of both species was higher than in those supplied with boron. In nodulated plants of both species, nitrogen concentration in nodules of plants lacking boron was lower than in those supplied with boron. Thus, uptake and transport of nitrogen within plants were probably not primarily affected by boron deficiency.

Boron deficiency had no significant effect on nitrogen assimilation and activities of several enzymes of wheat seedlings studied by Harper and Paulsen

(1969), nor has it been found to be necessary for free-living nitrogen-fixing organisms or for cell-free nitrogenase enzyme systems (Hardy and Knight, 1969). It therefore appears probable that boron requirement of plants studied here was not in the nitrogenase enzyme system per se, but was indirect. There are two routes by which boron deficiency may affect nitrogen fixation. Firstly, as shown by Brenchley and Thornton (1925), boron deficiency restricted growth and development of nodules, thus presumably reducing their nitrogen fixing efficiency. The nitrogen fixing activity of nodules of both species and initiation or growth of broom nodules was shown in this experiment to be reduced or restricted by boron deficiency. Secondly, as well as affecting nodules directly, boron deficiency caused shoot-tip die-back in broom in this experiment. This would reduce the size of sinks for fixed nitrogen within plants and also probably reduce the supply of photosynthate to nodules.

Thus, although boron has not been shown to be required by the nitrogenase enzyme directly, its lack would seriously impair the plants' ability to grow and to fix nitrogen. Nitrogen transport within unnodulated plants was not greatly affected by boron deficiency. Thus, the effect of lack of boron on formation of new sinks or supply of photosynthate to nodules of nodulated plants, probably had less effect on nitrogen fixation than did its effect on the structure and development of nodules. Mulder (1949) observed such direct effect on nitrogen fixation in Pisum sativum in solution culture where seriously boron deficient plants died of nitrogen deficiency. However, at less deficient levels, boron deficiency was apparent in the tops before nitrogen deficiency. The different responses obtained by Mulder (1949) when plants were grown in solution culture or in soil can be explained in terms of a general reduction in nodule activity in solution below that normally occurring in soils(as discussed in Ch.VIII). Thus boron deficiency might be expected to reduce nitrogen fixation in plants grown in soil to a greater degree than in those grown in solution. Only at very boron deficient levels will a reduction in nitrogen fixation by plants grown in solution culture occur. Thus, one of the first effects of boron deficiency expected in legumes in the field would be a reduction in nitrogen fixing efficiency.

CHAPTER X

TRANSFER OF NITROGEN FROM GORSE AND BROOM

INTRODUCTION

Virtanen et al, (1932) reported, stimulation of growth of a non-nodulating plant species when grown in close association with nodulated legumes. Since then, several authors have attempted to demonstrate direct transfer of nitrogen from nitrogen fixing plants. Wyss and Wilson (1941) found that such transfer was more likely to occur under certain environmental conditions (e.g.long day, low temperature), but that this effect was not consistent. Since then several workers have studied exudation of compounds from roots with only limited success (Katznelson et al, 1954; Pearson and Parkinson, 1961; Rovira, 1961; Clayton and Lamberton, 1964; Ayers and Thornton, 1968). Agboola and Fayemi (1972) observed increases in yield of corn when it was grown with greengram (Phaseolus aureus).

Loss of nitrogen compounds from plants appears to depend on three main factors:-

1. the age of the plant (Pearson and Parkinson, 1961; Vancura, 1964; Cadgil, 1971a) - young seedlings can exude ninhydrin positive material during germination and early growth,
2. the mineral nutrition of plants - phosphate deficiency (Bowen, 1969) or foliar application of nitrate (Balassubramanian and Rangiswami, 1969) can increase exudation of amino acids, and
3. root damage (Katznelson et al, 1954; Ayers and Thornton, 1968).

Hale et al (1971) reviewed factors affecting root exudation.

Amounts of amino acids and/or sugars exuded is generally very small. Henzell (1962) calculated that only 0.6 - 1.7% of nitrogen fixed by six different legume species could be transferred to a grass. Whilst these amounts are clearly important to rhizosphere micro-organisms (Rovira, 1965; Vancura, 1964;

Ruschel and Dobereiner, 1965; Smith and Peterson, 1966) their direct importance to other plants is doubtful (Butler and Bathurst, 1956).

Butler and Bathurst, (loc.cit.) calculated that regular sloughing off of white clover nodules could contribute 80.6 kg N/ha/a to the soil. Root turnover could contribute an unknown smaller amount. This assessment, however, assumed a 6 week cycle of nodule turnover and assumed that all nodule nitrogen was released.

Simpson, (1965) showed that senescence of subterranean clover released considerable amounts of nitrogen, presumably through root and nodule decay. Killing or defoliation of white clover and lucerne produced only a temporary release of nitrogen, although lucerne did release small amounts of nitrogen during a cyclical defoliation system.

In gorse and broom however, there is no such short cycle of nodule turnover, and only limited root turnover occurs - at least in the first season. It is therefore unlikely that these mechanisms contribute large amounts of nitrogen to associated plants.

Considerable amounts of most plant nutrients may be returned to soil by leaching from leaves by rain (Mes, 1954; Cooke, 1967; Tukey, 1970a, b). Such leachates have been shown to contain appreciable amounts of a wide range of amino acids (Morgan and Tukey, 1964), especially if nitrogen fixing organisms are present in the phyllosphere (Ruinen, 1965). This nutrient cycling has been shown to return approx. 12 kg/ha/a of nitrogen (5% of total N returned) in a tropical forest in Ghana (Nye, 1961) and 3 kg/ha/a (10% of total N turnover) in a beech forest in New Zealand (Miller, 1963). However, Egunjobi (1967) observed only 1 kg/ha/a (0.7% of total N returned to soil) returned by leaching from gorse. Leaf washings from lupin containing little ninhydrin positive material had no effect on the nitrogen content of Pinus radiata (Cadgil, 1971a).

Cadgil (1968, 1971a) showed that nitrogen exudation from a dense germination of lupin seed significantly increased nitrogen content of Pinus radiata seedlings in sand. This mechanism can only operate early in growth of Pinus, when such dense germinations are occurring and cannot be a continuing source of nitrogen.

The major path of nitrogen transfer from woody legumes and non-legumes is probably in litter fall. Cadgil (1968) felt that this would be the major mechanism of transfer of nitrogen from Lupinus arboreus to Pinus radiata on sands. She later showed in pot trials that decaying lupin tops slightly increased nitrogen content of 3 and 6 month old P.radiata compared with those growing alone (Cadgil, 1971b). Amounts of litter and nitrogen accumulating can be quite large. For example, a forest of brigalow (Acacia harpophylla F.Muell) in arid areas of Australia accumulated 75 500 kg/ha of litter over several years. This represented 370 kg N/ha (Moore et al, 1967).

Stone and Fisher (1969) assumed that greater mineralization of soil nitrogen around young conifers caused differences in soil and in herbaceous vegetation beneath and around the trees. A similar effect was noted in certain seasons in pot trials in Kenya (Birch and Dougal, 1967). This resulted in increases in growth of 3 grasses grown in association with the legume (Desmodium uncinatum)and in soil nitrogen.

Two experiments were conducted to investigate underground and surface transfer of nitrogen from gorse and broom.

EXPERIMENT I. DIRECT UNDERGROUND TRANSFER OF NITROGEN TO PINES

Materials and Methods

This trial was set up in a heated glasshouse in May and harvested 19 months later.

Mycorrhizal Pinus radiata seedlings were planted in 5.5 kg of sterilized soil/sand (1/3) mixture in 4.5 l plastic pots. Well rooted nodulated gorse and broom seedlings were transplanted (2 per pot) into the requisite pots. Three levels of phosphate (as superphosphate) were applied to these pots. No legumes were transplanted into the remaining pots. Urea was applied as a source of nitrogen to some of these, phosphate was applied at one level (P_1), to others. The remainder were unfertilized controls. There were 9 replicates per treatment.

The fertilizer levels applied were equivalent to:-

P_0 - No superphosphate

P_1 - 280 kg/ha superphosphate (0.63 g/pot)

P_2 - 560 kg/ha superphosphate (1.26 g/pot)

N_0 - No urea

N_1 - 112 kg/ha urea (0.28 g/pot) applied to Pinus seedlings alone.

These levels were applied at the start of the experiment only. Treatments were arranged in a fully randomized (2 x 3 + 3) design. Pots were watered at least once daily and any leaves dropped on to the soil surface washed off when watering to minimize transfer of nutrients from litter.

After harvesting, pine tops were dry weighed and nitrogen content of ground needles determined by Kjeldahl analysis.

Results

Data for P.radiata were analysed using Duncan's Multiple range test (Table 10.1).

Clearly competition by gorse and broom, presumably for nutrients and water, severely restricted growth of pine seedlings. The very low nitrogen levels would suggest that lack of nitrogen was severely limiting growth. In a similar pot experiment over a 17 month period on Waimea soils, Knight (1968) suggested a similar effect occurred. Nitrogen levels in tops of Knight's(1968) P.radiata were similarly low (6-10 g /kg).

Application of both nitrogen and phosphorus raised the level of nitrogen in needles compared with unfertilized controls. Generally association of Pinus plants with either gorse or broom raised needle nitrogen levels compared with unfertilized controls. This however, probably reflects decreased growth by these plants.

From these results it would be expected in the field that competition by gorse and broom on low nutrient soils may mask any possible response from nitrogen transferred from legumes to trees. Suppression of tree growth by gorse,

has been noted in forests (Appleton and Slow, 1966).

EXPERIMENT II. LITTER COLLECTION - BROOM

Methods

Litter fall under broom was measured on a site on the bed of the Waiau River, close to the Lewis Pass Highway, $1\frac{1}{2}$ - 2 miles upstream from the Waiau Ferry Bridge. Three broom stands representing 3 ages (average ages 5,9 and 13 years - determined by ring counts on representative samples) were chosen, in this area. Six $\frac{1}{2}$ metre square wood-sided gauze-bottomed trays were placed in these stands, 3 in the oldest, 2 in the youngest and 1 in the 9 year old stand. A further three trays were distributed at the first sampling three months later. Floods between the second and third samplings swept away most of the island on which the 9 year old stand was situated. Collection was therefore terminated at this sampling. For completeness, data from this stand is included in the results.

Litter was separated into 3 samples:-

(a) twigs

(b) leaves

(c) pods, flowers, seeds etc.

These were oven dried (overnight at 90°C), dry weighed and bulked sample fractions ground in a knife mill for determination of nitrogen content.

Results and Discussion

Results are summarized in Table 10.2. These collections represent just over a year's litter fall. The quantities are very similar to those quoted by Daly (1966) for Alnus rugosa (Du Roi) Spreng in Canada - 5 600 kg/ha/a of litter (157 kg N/ha/a). Several values have been given for litter fall under Alnus rubra Bong. These range from 1 315 kg litter/ha/a (21 kg N/ha/a) (Tarrant and Trappe, 1971) to 9 700 kg litter/ha/a (175 kg N/ha/a)(Zavitkovski and Newton, 1971). The amount of litter falling from gorse growing on a strongly weathered yellow-brown earth in New Zealand was considerably higher than most of the amounts

quoted above - 11 800 kg/ha/a (Egunjobi, 1967, 1969). However, as nitrogen concentration in broom litter (Table 10.2) was higher than that from gorse (Egunjobi, 1967), the amount of nitrogen in litter in both species were similar (up to 200 kg N/ha/a and 225 kg/ha/a for broom and gorse respectively - Table 10.2, Egunjobi, loc.cit.). Concentrations of nitrogen in broom litter were very similar to those in litter from Lupinus arboreus (Cadgil, 1968), Alnus glutinosa, A.incana, A.inokumae and A.rubra (Tarrant and Trappe, 1971; Zavitkovski and Newton, 1971), and A.rugosa (Daly, 1966; Tarrant and Trappe, loc.cit.). A greater amount of litter of higher nitrogen content may be expected from more fertile sites (cf. Egunjobi, 1967, 1969).

At site 3, the only site at which records were complete, variation among trays ($\pm$ 4% litter, $\pm$ 7% nitrogen) was much less than that quoted by Egunjobi (1967) for gorse.

Variation for site 1 was considerable (3 to 6.8 x 10^3 kg litter/ha/a). However, as tray 2 was situated near the windward (Northwest) side of a relatively small island, considerable loss could be expected from this tray.

The canopy at site 1 (5 year stand) was still quite open, at site 2 (9 years) closing and at site 3 (13 years) was completely closed. A vigorous undergrowth of grasses was beginning to appear at this age.

It has been calculated that a C:N ratio of 20:1, corresponding to nitrogen concentration 17-19 g/kg, was necessary before any mineralization would occur (Harmsen and van Schreven, 1955; Iritani and Arnold, 1960). However their evidence was based on additions of nitrogen containing compounds or plant material to a soil-plant mulch or to soil. Ilmari (1967) showed that such additions could quite drastically alter soil microflora. As this must result in binding of additional nitrogen in developing microflora, nutrients would be released only upon death and decay of micro-organisms, as the system returns to its former state.

Danneburg et al (1968) showed that during 180 days of incubation, only a small proportion of ^{15}N added to soil as ammonium sulphate entered the insoluble "humus" fraction. The remainder was readily available. This

situation could be considered as much closer to normal, near-equilibrium field conditions, when most of the nitrogen of litter would be readily available for plant growth.

Thus gorse and broom would provide considerable amounts of nitrogen (175-200 kg/ha/a at age 10 - 13 years) in litter for associated plants. Because the concentration of nitrogen was higher in broom than in gorse litter (Egunjobi 1967, 1969), nitrogen present would probably be released more readily from broom than from gorse.

Transfer of nitrogen from gorse and broom to associated P.radiata probably occurs mainly in litter. Direct underground transfer is probably minimal, at least in the first 1 - 2 years. However, because of the deep rooting habit of these two species (Burns, 1935), competition for nutrients in surface layers of older stands would be less than in young stands. Nitrogen added to the surface as litter in such stands would be of considerable benefit to the ecosystem.

CHAPTER XI

GENERAL DISCUSSION - CONCLUSIONS

Although this project was initiated because of a problem in re-afforestation in the Nelson Conservancy, most of the experimental work was carried out at Lincoln College. It is tempting to apply results of this type of work directly to field situations. However, because of great differences between glasshouse and field conditions, such application of results is very difficult. With care, however, some comments on possible field responses may be valid. Experiments reported here were conducted to enable comparison of woody perennial legumes, gorse and broom, with nodulated non-legumes, more intensely studied herbaceous species,and other biological nitrogen fixing systems.

Shoots of adult plants of both gorse and broom, bore little morphological resemblance to those of herbaceous legumes. However, both species went through a distinct juvenile phase. During this phase broom plants appeared very similar to clovers, lucerne etc. The juvenile phase of gorse was less typically legume in appearance, but the juvenile-adult transition in this species was more pronounced than in broom.

Both species have deep extensive rooting habits (also noted by Burns, 1935)and this may be a significant factor in improving soils of poor physical structure such as the Moutere Gravels.

Structural development of nodules was the same for both species. Early stages were very similar to development of herbaceous legume nodules, but instead of degenerating after about 6 weeks(Butler and Bathurst,1956), meristematic activity resumed and more bacteroid tissue developed. Vascular tissue of gorse and broom nodules was better developed than that of herbaceous species. The structure and development of the nodules was very similar to that described in two other woody legumes Sesbania grandiflora (Harris et al,1949) and Caragana arborescens (Allen et al,1955), but nodule rootlets apparent in those

species have not been observed in gorse or broom.

Excised nodules were tested for acetylene reduction and ^{15}N fixation. Both species showed constant rates of reduction or fixation for considerable times following excision. This contrasted with the short lives of herbaceous legume nodules when excised, but was very similar to those of woody non-leguminous nitrogen fixing species. Cessation of acetylene reduction probably resulted from depletion of endogenous energy reserves. Large numbers of starch-like granules were observed in the cytoplasm of cells in the nodule. These granules were probably β-hydroxybutyrate - a known energy source in legume nodules. The numbers of these granules would indicate large energy reserves.

Thus, nodules of gorse and broom were shown to be well adapted to long life. They exhibited structural adaptions (viz. renewed nodule meristem activity and a well developed vascular system) to a slow turnover rate and a long life under natural conditions. They also retained their activity for a long time following excision. In this longevity, both on the plant and following excision, nodules of these two species were similar to many non-leguminous nitrogen fixing species. Longevity after excision has been described as a basic factor distinguishing non-legumes from legumes. However, results presented in the present work make it clear that nodule longevity is a characteristic of these two woody legumes, gorse and broom also. Legumes most extensively studied (*Trifolium spp*, *Lotus spp*, *Glycine max*, *Pisum sativum*) are entirely or mainly herbaceous while nodulated non-legumes are generally woody or have woody type nodules. Describing differences in nodule longevity as differences between legumes and non-legumes is erroneous. The differences are in fact, between woody and herbaceous species.

The rate of acetylene reduction in broom nodules was similar to that observed in several non-legume nodules. Gorse nodules however, showed considerably lower activity. This difference in activity between these species was also observed with $^{15}N_2$ fixation, translocation of fixed $^{15}N_2$ and in several plant growth trials.

As with other biological nitrogen fixing systems, the first detectable product of nitrogen fixation by excised gorse and broom nodules was ammonia. This was rapidly incorporated into amide and then α-amino groups and an unidentified fraction of similar size. This sequence of ^{15}N metabolism was the same as in most other nitrogen-fixing symbioses. Translocation of this fixed ^{15}N across the nodule to xylem appeared slow. As with most nodulated non-legumes and many legumes, the major free amino acid in young gorse and broom nodules and sap was asparagine. As plants aged the range of amino acids decreased and the preponderance of asparagine increased. Species which differ from the typical amino acid pattern are Ornithopus sativa (Kennedy, 1966a, b) and Coriaria arborea (Silvester, 1968) in which glutamine predominates and Alnus glutinosa (Miettenin and Virtanen, 1952; Bollard, 1957b; Leaf et al, 1958). These three species seem to be exceptions.

The optimum temperature for growth of gorse and broom plants, and for nitrogen fixation by plants and excised nodules, was relatively low ($22^{o}C$). Coupled with this low optimum was a high sensitivity to elevated temperatures. Maximum average weekly growth over 14 weeks in growth cabinets illuminated at 1300 foot candles for a 16 hour day was about 75% of that in a 20 week trial carried out in full daylight in a glasshouse. Further reduction of growth cabinet light level by nearly 40% to 800 foot candles had little effect on growth, nodulation or nitrogen fixation. Although strict comparison of the two experiments is not possible because of the different conditions under which they were conducted, these results indicate that gorse and broom have low light requirements. This response was similar to that observed in Medicago tribuloides and Vicia atropurpurea (Pate, 1961b, 1962a), but contrasted with the great sensitivity to reduced light exhibited by the tropical legume Stylosanthes humilis (Sillar, 1967). This property of gorse and broom would make them eminently suitable for growth as an undercover in forests.

The effects of temperature on plants in this experiment were probably exaggerated because the whole plant was exposed continuously to the regime. Gibson (1967b) stated that at low root temperatures, low shoot temperatures

slowed initial nodulation, but not subsequent nodule initiation in Trifolium subterraneum. At slightly higher root temperatures (14° and 20°C), marked increases in nitrogen fixation were observed with shoot temperatures in the range 5° - 20°C. Increases in dry weight and nitrogen content with shoot temperature were greater in those plants receiving mineral nitrogen than in those relying on nitrogen fixation (Gibson, 1971). Gorse and broom plants in this experiment receiving combined nitrogen appeared to be less susceptible to supra-optimal temperatures than those relying on fixed nitrogen. In field situations root and shoot temperatures would be different especially in deep-rooted plants such as adult gorse (Burns, 1935) and broom. Thus, at sub-optimal soil temperatures (20°C) increased shoot temperatures should increase speed of nodule formation, and nitrogen fixation, and to a greater extent, absorption of combined nitrogen. However, as root temperatures rise above optimum, nitrogen fixation would be drastically reduced. This, however, would be relieved by nightly decreases in temperature (Mes, 1959a).

In a glasshouse and where aeration of the rooting medium was sufficient, there was little, if any, dry weight response, to combined nitrogen in nodulated plants of either species. Combined nitrogen increased the total nitrogen content of gorse, and it was only at the highest level (100 mg N/l) that there was any large reduction in nitrogen fixation. With broom, increasing amounts of combined nitrogen decreased nitrogen fixation, but had little effect on total nitrogen content. Nodulation in both species was reduced in an almost linear fashion by increasing amounts of supplied nitrogen.

There were no significant differences between effects of the two nitrogen sources (nitrate or ammonia) on nodulation. There were, however, differences at 100 mg N/l between dry weight and nitrogen content of broom plants receiving nitrate and those receiving ammonia. No such differences were observed with gorse. It was suggested that differences between nitrogen sources may have resulted from increased acidity of the sand/perlite medium associated with ammonia absorption. If this was so, then gorse would grow

better than broom in soils of low pH such as the Moutere Gravels (Jackson, 1967; Noonan, 1969).

Although both gorse and broom showed responses to phosphate, they were also able to grow and fix nitrogen when receiving no additional supply. In this, they were similar to tropical legume species such as Stylosanthes humilis (Humphries, 1967).

In both species reduced boron supply reduced nitrogen fixation and boron uptake. Formation of nodules was not apparently affected. To compensate for decreased nitrogen fixation, nodule numbers were increased in both species. In broom, but not in gorse, the average size of nodules was greatly reduced by boron deficiency. Nitrogen transport within plants appeared to be unaffected.

Boron deficiency caused a reduction in shoot height in both species, but had no effect on dry weight. Boron concentrations in plants supplied with boron and those lacking it were similar to those observed by other workers in corresponding treatments in Pinus radiata and in other legumes.

As solution culture was earlier shown to restrict growth seriously by reducing nitrogen fixation, boron requirements of plants grown under these conditions would be lower than those growing in soils. Mulder (1949) showed that nitrogen fixation in Pisum sativum was restricted more by boron deficiency when plants were grown in soil than when grown in solution. Thus boron deficiency in the field would affect nitrogen fixation by gorse and broom to an even greater extent than observed in this work.

Norris (1956, 1968) stated that there were considerable differences between tropical and temperate legumes in responses to fertilizer applications. Although tropical species did show growth responses, these were much smaller and less dramatic than for temperate species. In this, and other work (Egunjobi, 1967), it has been shown that gorse and broom were able to grow at relatively low nutrient levels, and although they did respond to applications of nutrients, these responses were small compared with those of temperate species. Thus these two species, although temperate, show responses which Norris (1956)

typified as those of tropical species. Slow responses such as these would also be expected of woody plants in general. Thus nutrient responses similar to those exhibited by gorse and broom would be expected from woody legumes.

When gorse and broom were grown with Pinus radiata in pots, any possible direct transfer of nitrogen to the trees was masked by competition effects. However, transfer of nitrogen in litter was shown to contribute potentially large amounts of nitrogen to soil. Broom was shown to contribute up to 200 kg N/ha/a to an ecosystem in this way. This amount was similar to that contributed by gorse in another study (Egunjobi, 1967, 1969). Many authors have attempted to show that little of this nitrogen would be available to plants and most would be utilized by micro-organisms. However, in field situations, in conditions of relative equilibrium in stands of non-deciduous species such as gorse and broom where litter fall occurs in all seasons, nitrogen in litter would be released following microbial death.

It was noted in several experiments in this work that concentrations of nitrogen in broom plants were generally higher than in gorse plants receiving the same treatment. Nitrogen concentration in broom litter was likewise considerably higher than that reported elsewhere for gorse (Egunjobi, 1967, 1969). Amounts of nitrogen fixed were greater and rates of $^{15}N_2$ fixation and acetylene reduction by excised broom nodules faster than for similar gorse plants. Thus for use in forests in overcoming nitrogen deficiency in Pinus radiata, broom would be more effective than gorse. If, however, the suggestion that broom was less tolerant of acid soil conditions than gorse was true, then this would restrict its use on the Moutere Gravels. More work would be necessary to clarify this point.

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