VARIATION IN THE SENSITIVITY OF NODULATION AND NITROGEN FIXATION TO NITRATE IN ANNUAL *MEDICAGO* SPECIES

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DECLARATION

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ABSTRACT

Annual species of *Medicago*, or medics, are important pasture legumes in the neutral to alkaline soils in southern Australia, but as with all legumes, their nodulation and nitrogen fixation processes are retarded by soil nitrate. The principal objective of this study was to ascertain whether an observed tolerance to nitrate among medic species (J. H. Silsbury, unpublished) could be substantiated, and if possible, to understand the underlying factors responsible.

A series of experiments in sand culture involved the addition of different concentrations of nitrate to well-grown and nodulated annual medics, 37-40 d from inoculation, with the assessment of nitrogenase activity (Acetylene Reduction (AR) assay) over the following 4-6 d. All species, including *Medicago truncatula*, *M. littoralis*, *M. rugosa* and *M. tornata* showed similar levels of inhibition, which was 50% and 75% for 2.5 and 5 mM nitrate respectively after 4 d. One experiment included *M. sativa,* and it was less tolerant than the other medics, whereas *Trifolium subterraneum* in the same experiment showed similar inhibition of nitrogenase activity to the annual medics. In the annual medics there was a strong negative correlation between nitrate concentration in the root and shoot tissues and nitrogenase activity of the plants.

Nodulation in *M. rugosa* cv. Sapo in hydroponics with three strains of *R. meliloti* was superior to that in sand while the opposite was the case for *M. truncatula* cv. Borung. That in *M. polymorpha* cv. Serena was better in sand with *R. meliloti* strains WSM540 and CC169 but with WSM826 hydroponics was considerably superior. *Medicago truncatula* was a principal species under examination, so an evaluation of host x strain relationships was made to ensure that the most suitable strain of *Rhizobium meliloti* would be used. This resulted in the replacement of the recommended commercial inoculant strain (CC169) with

the strain WSM540, for subsequent studies.

In a series of experiments, several cultivars of six medic species were evaluated for the effects of 1 mM nitrate on initial rates of nodulation. *M. rugosa* cultivars were the most tolerant, showing only 30-48% inhibition compared to 80-95% inhibition in *M. truncatula* cultivars 14 DAI. Individual cultivars of *M. tornata* (Tornafield), *M. murex* (Zodiac) and *M. scutellata* (Kelson) were equally tolerant to the *M. rugosa* cultivars while *M. littoralis* and *M. polymorpha* cultivars were relatively sensitive. The perennial medic, *M. sativa* showed almost complete suppression of nodulation with 1 mM nitrate. By comparison *Trifolium subterraneun*, although showing some delay to initial nodulation in the presence of nitrate, showed only a small impairment in nodulation, compared to the controls, at 14 days after inoculation.

The *M. rugosa* cultivars had a lower rate of nitrate uptake, 50-60% of that in *M. truncatula* cultivars. The other medics had intermediate levels of nitrate uptake and overall there was an approximate inverse relationship between nitrate uptake and inhibition of nodulation. The K_m value for nitrate uptake by *M. rugosa* cv. Sapo (0.11 mM) was similar to that for *M. truncatula* cv. Parabinga (0.14 mM). In this study the equivalent V_{max} values (µmol nitrate taken up/h/ g root dry weight) were 52 and 72.

The *M. truncatula* cultivars showed a more pronounced growth response to the presence of 1 mM nitrate. At 14 days after inoculation it resulted in a 2.5-4.2 fold increase in total dry matter of *M. truncatula* cultivars but only a 1.6-2.1 fold increase in *M. rugosa* cultivars. The nitrate concentration in root tissues was higher than in shoots of all the medic cultivars tested, with overall values for *M. rugosa* cv. Sapo being lower than those in the *M. truncatula* cultivars. The level of nitrate reductase in root and shoot tissues of the medics was not related in any way to the uptake or accumulation of nitrate. About 90% of the nitrate taken up between 14-20 days after inoculation was assimilated while the equivalent value

between 27-34 day was 83-84%.

A comparison of three species with respect to nitrate effects on N_2 fixation, and nitrogenase activity, from 27 to 34 days after inoculation, showed again that *M. rugosa* cv. Sapo was more tolerant to nitrate than *M. polymorpha* cv. Serena. and *M. truncatula* cv. Parabinga and Sephi. In an N balance analysis, N_2 fixation in the presence of 1 mM nitrate was inhibited by 59% in Sapo and approximately 80% in the other medics. Measurements of the nitrogenase (AR) activity at the end of the seven day treatment with nitrate showed about 90% inhibition in all four medics.

When nitrate was withdrawn at 23 day, after a seven day treatment with 1 mM nitrate, there was a rapid recovery in the nitrogenase (AR) activity in five medic species studied. The AR activity per plant had reached that of the controls at 30 days. Nodule number remained constant during the period of nitrate treatment and in all medics, except *M. truncatula* cv. Parabinga, showed some increase after nitrate removal. Nodule number in *M. scutellata* cv. Kelson doubled in the seven day period after nitrate removal.

This study has shown variation in the sensitivity of different species of *Medicago* to the effect of nitrate ions on initial nodulation. A major factor in determining the response appeared to be the rate of nitrate uptake by the plants. The variation among species also provides scope for further studies to understand the physiological bases for tolerance of the symbiotic processes to nitrate ions and the opportunity for selection for such tolerance within annual medics.

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LIST OF ABBREVIATIONS

AR	Acetylene reduction
ATP	adenosine triphosphate
cv(s).	cultivar(s)
d	day (s)
DAI	days after inoculation
EDTA	ethylenediaminetetraacetic acid
Fd	ferredoxin
GOGAT	Glutamate synthase
GS	Glutamine synthetase
h	hour
L	litre
LSD	least significant difference
M	molar
mg	milligram
min	minute
mL	millilitre
mM	millimolar
nmol	nanomole
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NR	nitrate reductase
sec	second
S.E.	standard error of the mean
µg	microgram (s)
µmol	micromole
℃	degrees of Centigrade (Celsius)

CHAPTER 1

GENERAL INTRODUCTION AND AIMS

1.1. General Introduction

Legumes that have developed a symbiotic association with nitrogen fixing prokaryotes are able to fix atmospheric N_2 and can also use combined nitrogen in the soil. Throughout history, legumes have been used in rotations to replenish soil N reserves, but the benefits of legumes to following crops depend on the balance between N_2 fixation and the utilisation of soil mineral N. Nitrate in small amounts is sometimes beneficial in early stages of growth as 'starter N' which accelerates the formation of nodules and onset of N_2 fixation, but the potential of a legume to nodulate and fix N_2 is generally decreased by the presence of moderate or high levels of soil nitrate. To maximise the N benefit from a pasture legume, it is necessary to identify and develop pasture legumes which are capable of nodulating and fixing N_2 in the presence of nitrate in the soil. Of the several forms of nitrogen taken up by plants, nitrate is the one most usually present in the agricultural soils and is one of the most potent inhibitors to both nodule formation and function. Selection of a legume able to fix N_2 in the presence of nitrate would appear to be a feasible goal in attempting to maximise exploitation of symbiotic N_2 fixation.

There have been several studies of the inhibitory effects of nitrate on nodulation and nitrogen fixation in both pasture and grain legume species. The effect of nitrate varies with the species and cultivar of legume crops (Allos and Bartholomew 1959, Harper and Gibson 1984, Gibson and Harper 1985), strain of *Rhizobium* (McNeil 1982) and growth conditions of the plants (Ralston and Imsande 1983). A number of hypotheses have been proposed to explain the mechanism of nitrate inhibition on symbiosis including carbohydrate deprivation, nitrite toxicity, feedback of reduced nitrogenous compounds and decreased O_2 diffusion into the nodules. However, the actual mechanism by which nodulation and N_2 fixation are influenced by nitrate is still a subject of debate.

Pasture legumes play a vital role in Australian farming systems. The N requirement for wheat production in southern Australia is largely satisfied by N_2 fixed biologically by annual medics and subterranean clover (Greenland 1971, Puckridge and French 1983, McDonald 1989). Species of annual medics (*Medicago* spp.) grown as self-regenerating pastures are the most important N_2-fixers on the alkaline soils of the wheat belt of southern Australia. Seed germinates when the first rains fall in autumn or early winter. Often at this time nitrate concentration in the surface soil is high because moisture and temperature conditions are favourable for the mineralisation of soil organic matter, although the actual amount depends on the history of land use, soil type, rainfall and temperature (Leeper and Uren 1993). In a soil with high concentrations of nitrate, the N_2 fixed by the pasture legume might be low and the potential benefit of these plants in increasing soil mineral nitrogen might not be realised. Although many studies have been done on the sensitivity of nodulation and N_2 fixation to nitrate with pasture and grain legumes, little information is available on the sensitivity of nodulation and N_2 fixation of annual medics to nitrate. Studies with one cultivar of each of three species of annual medics (*M. murex*, *M. polymorpha* and *M. truncatula*) have demonstrated some variation in the sensitivity of nodulation of medics to nitrate (Ewing and Robson 1990). Harper and Gibson (1984) in their experiment with a range of tropical and temperate legumes in hydroponic culture, showed that nodulation of *M. truncatula* was the most sensitive when compared with a range of other legumes in the presence of 1 and 4 mM nitrate.

In the current study, the sensitivity of nodulation and N_2 fixation to nitrate in annual medics was examined in two ways: by continuous exposure of the plants to nitrate from planting until harvest and by exposing nodulated plants previously grown under N-free conditions to nitrate for a short period. Interaction between nitrate assimilation and N_2 fixation was examined by determination of nitrate uptake, nitrate assimilation and accumulation in the plants. These factors were related to the variation among medics in their sensitivity of nodulation and N_2 fixation. The increment of total nitrogen content of the whole plant with

time was used to estimate N_2 fixation while the acetylene reduction (AR) assay was used for comparative purposes.

1.2. Aims

The main aims of the current study were:

(a) to identify annual medics (*Medicago* spp.) which are capable of nodulating and fixing N_2 in the presence of moderate levels of nitrate.

(b) to investigate the basis of such tolerance through studies of the relationship between

(i) nitrate availability and initiation and development of nodules

(ii) nitrate assimilation and N_2 fixation

(iii) nitrate uptake and accumulation and N_2 fixation.

CHAPTER 2

REVIEW OF LITERATURE

A broad outline of the nitrogen nutrition of legume plants is presented, including sources of soil nitrogen for legumes, the growth responses of legume plants to combined nitrogen, and the effects on the processes of nodulation and N_2 fixation. As N_2 fixation has been measured in the present study by analysis of nitrate uptake and nitrogen content of the plants, the uptake and assimilation of nitrate are then assessed. Possible mechanisms involved in the inhibition of N_2 fixation by nitrate are also reviewed. Attention is focused on the variation in the sensitivity of nodulation and N_2 fixation of legume plants to nitrate.

2.1. Legume-*Rhizobium* Symbiosis

Despite the fact that more than 78% of the atmosphere consists of N_2 gas, most plants are not able to use it directly. Legume plants however, can utilise not only combined nitrogen in soils but through their symbiotic association with *Rhizobium* or *Bradyrhizobium*, they are able to convert atmospheric N_2 into a plant useable-form. The process of N_2 fixation has attracted considerable attention during this century, due to its economic importance and an increased awareness of environmental problems associated with the use of nitrogen fertilisers. Very significant progress has been made in the areas of physiology, biochemistry, genetics and the molecular biology of nodule formation and N_2 fixation. In addition, attempts have been made to induce nodules on non-legume hosts, such as wheat, rice and maize, to provide their own nitrogen needs. However, more research is required to elucidate the molecular mechanisms that regulate existing symbiotic associations before attempting to develop these complex symbioses. Benefits of the *Rhizobium*-legume symbiosis for agricultural production can be substantial and further investigations are required to optimise them.

2.2. Nitrogen Nutrition of Legume Plants

Nitrogen is required by plants because it is a constituent of amino and nucleic acids, proteins, peptides, chlorophyll and alkaloids (Bergmann 1992, Mengel 1992). Nitrogen constitutes 0.5-5 per cent of total plant dry matter (Larsson *et al.* 1992). A deficiency of nitrogen is a major constraint to plant growth because of inhibition of chloroplast and chlorophyll synthesis and impaired synthesis of enzymes involved in the many metabolic processes contributing to plant growth.

Biologically-fixed N_2 and soil inorganic nitrogen are the main sources of nitrogen for the growth of legume plants. Nitrogen fixation however constitutes a major input of nitrogen into the metabolic pathways of those plants which have developed symbiotic relationship with N_2-fixing prokaryotes through the formation of root nodules. Nitrogen is reduced to ammonia in the nodules where the N_2-fixing enzyme complex, nitrogenase, is located. It appears that even under favourable conditions for nodule establishment that N_2 fixation is not capable of providing the entire nitrogen requirements of a legume (see Section 2.3).

It is widely held that both nodulation and N_2 fixation by legumes are inhibited by mineral nitrogen and that N_2 fixation as a source of nitrogen is only utilised when the supply of combined nitrogen is not adequate to fulfil the requirements of plants (Silsbury *et al.* 1986). Therefore plants may benefit from the complementary operation of both N_2 fixation and combined nitrogen assimilation (Bergersen *et al.* 1985, Brockwell *et al.* 1985, Silsbury 1987).

2.2.1. Sources of Soil Nitrogen for Legumes

Nitrate and ammonia in the soil represent major sources of nitrogen for plants. Nitrate is usually of greatest importance because plants of agronomic significance obtain the bulk of their nitrogen from the soil as nitrate (Haynes 1986). Nitrate is formed from the activities of soil micro-organisms on soil organic matter or ammonia-based fertilisers and from the action

of lightning in the air (Addiscott 1990). Availability of nitrate in the soil however, fluctuates during the growing season depending on climatic conditions, soil properties and soil microbial activity (Haynes 1986). Ammonia can be released into the soil either as a result of the decomposition of amino acids, amides, and protein in dead plants, animals and micro-organisms or from animal excreta and ammoniacal fertilisers (Haynes 1986). When the soil is supplied with ammonium ions, under favourable soil moisture and aeration and temperature conditions, soil micro-organisms nitrify the ammonium to nitrate (Haynes, 1986). Ammonium is the major form of nitrogen available to legumes when conditions are unfavourable for the nitrification process (Haynes 1986).

Most legume crops are probably exposed to mixtures of the two forms of N throughout their life cycle, but the ratio of ammonium to nitrate is variable because of nitrification by the soil organisms. The quantity of these two ions presented to the roots of legume plants depends on the amounts released from soil organic nitrogen and fertiliser and on the balance that exists among factors affecting nitrogen mineralisation, immobilisation and losses from the soil (Haynes 1986).

2.3. Growth Responses of Legume Plants to Combined Nitrogen

Several investigators have shown that legumes assimilating nitrate or ammonium grow better than those dependent upon N_2 fixation (Sprent and Thomas 1984, Davidson and Robson 1986, Silsbury *et al.* 1986). The improved growth is manifested through the effect of combined nitrogen on various plant attributes such as leaf area, leaf number and nitrogen content of roots and shoots. Increases in leaf area may be due to increased number of leaves and/or to increased leaf size (Bouma 1970). Thus, the potential photosynthesis of nitrate-fed plants is increased. Bouma (1970) showed that nitrate-fed subterranean clover (*Trifolium subterraneum*) plants had 20% greater leaf area than nodulated plants and the latter contained much less total N than did nitrate-fed plants of the same age. Silsbury (1984) reported that %N was 1.8 times higher in nitrate-fed than N_2-fixing plants of subterranean clover.

In legume plants, an early supply of nitrate or ammonium may result in rapid initial plant growth and leaf area development (Mahon and Child 1979). Gibson (1974) argued that better growth of nitrate-fed plants is due to an earlier and better start to growth compared to nodulated plants receiving no nitrate. The process of infection by rhizobia and establishment of the N_2-fixing apparatus results in growth retardation when the host is grown without mineral nitrogen (Silsbury 1984). Nodulated seedlings without access to inorganic nitrogen usually have less leaf area and smaller root systems than non-nodulated seedlings because about 10% of their seed nitrogen resources are used for nodule formation (Pate and Layzell 1990).

Growth responses of well-nodulated plants to nitrate vary between legume species and time of application. Supplying 5, 10 and 20 mM nitrate for 8 days to faba bean and pea with established symbioses had no effect on shoot and root growth (Chalifour and Nelson 1988b) whereas 10 mM nitrate for 5 days significantly increased leaf, stem and root weights of soybean (Vessey *et al.* 1988a). Fishbeck and Phillips (1981) found that N fertilisation increased dry matter yield and N concentrations of lucerne (*M. sativa*) during the first two regrowth cycles after cutting whereas application after further cutting showed no benefit. The growth response to applied N in the later growth phases of grain legumes has been associated with a decrease in the amount of nitrogen fixed during this period due to nodule senescence (Weil and Ohlrogge 1972) or to competition between nodules and seeds for assimilates (Bhangoo and Albritton 1976).

2.3.1. Energy requirements of symbiotic nitrogen fixation and combined nitrogen assimilation

It has often been reported that legumes use combined nitrogen in preference to fixed N, leading to speculation that the energy requirements for N_2 fixation may be higher than those for the assimilation of combined nitrogen. Energy requirements for symbiotic N_2 fixation by nodulated legumes and for plants assimilating combined nitrogen have been compared by assessing their growth rate (Gibson 1966), measuring root and nodule respiration (Ryle *et*

al. 1979a) and determining the net assimilation rate (Broughton 1979). Minchin and Pate (1973) showed that 5.9 mg of carbon was respired for each mg of N_2 fixed in nodulated plants of pea, while nitrate-supplied plants respired 6.2 mg carbon for each mg of nitrate-nitrogen reduced. Pate *et al.* (1979) estimated CO_2 loss per unit nitrogen assimilated in white lupin roots as 10.2 mgC/mgN by the nodulated root and 8.1 mgC/mgN by the non-nodulated nitrate-fed roots while Neves *et al.* (1981) estimated C consumption by nodulated and non-nodulated cowpea root as 8.0 and 4.5 mgC/mgN assimilated respectively. Silsbury (1977), using carbon dioxide efflux in the dark, found that the energy requirement for symbiotic N_2 fixation in subterranean clover was greater than for those plants assimilating mineral nitrogen; 810 mg CO_2 was used for the synthesis of each gram dry weight by nodulated plants while the corresponding figure for nitrate-supplied plants was 510 mg. Ryle *et al.* (1979b) compared growth, photosynthesis, shoot and root respiration of three legumes when fixing N_2 and when utilising nitrate; soybean cv. Fishby V, cowpea cv. K2809 and white clover cv. Blanca. They concluded that the source of nitrogen had no effect on the rate of photosynthesis per unit area of leaf, or on the rate of respiration of shoot tissue, while the rate of root respiration per unit root weight of nodulated plants during N_2 fixation was twice that of nitrate-fed plants . Plants fixing N_2 respired 11-13% more of their photosynthate. Veau *et al.* (1990) reported that soybean plants dependent solely on N_2 fixation as the nitrogen source compared with nitrogen-fixing plants supplemented with NH_4NO_3, had a similar net CO_2 fixation rate per unit leaf area but a significantly lower protein/carbohydrate ratio in the leaves. Overall, the evidence is fairly convincing that symbiotic N_2 fixation is metabolically more expensive than the assimilation of nitrate or ammonium. However after the establishment phase and especially at high light flux density, Silsbury (1984) found the same growth rate in N_2 fixing and nitrate assimilating swards of subterranean clover.

Nitrogen fixation and combined nitrogen assimilation can be complementary in satisfying the nitrogen requirements of legumes (Silsbury *et al.* 1986, Silsbury 1987). They can operate in different stages of the life cycle of the plant, or both operate at the same time. In

the first case, young seedlings of legume plants find their initial nitrogen requirements through soil mineral nitrogen before the establishment of the N_2 fixation apparatus. After nodules have been established, fixation can provide the plant's nitrogen requirements up to early pod filling (Jensen 1987a). Later, as mentioned above, combined nitrogen may be necessary to fulfil the nitrogen demands of the plant. Alternatively, nitrogenase activity and combined nitrogen assimilation may simultaneously contribute to the nitrogen economy of the legume plants. Silsbury (1987) showed that the rate of nitrate assimilation was inversely related to the rate of N_2 fixation in subterranean clover. Likewise, Davidson and Robson (1986) found that white clover can rapidly switch on and off its N_2-fixing system in response to changes in nitrate availability, and in so doing make use of both sources of nitrogen by direct substitution of one source for the other to maintain high nitrogen content in its tissues. Silsbury *et al.* (1986) showed with nodulated subterranean clover and 0.5 to 7.5 mM nitrate supplied over a period of 3-7 d, there was a decrease in the acetylene reduction rates which was proportional to the level of nitrate supplied. As the nitrate reductase (NR) activity increased, nitrogenase activity decreased. At relatively low levels of nitrate (0.5 to 2.0 mM) nitrogenase activity was not completely suppressed and both nitrogenase and nitrate reductase contributed to the nitrogen economy of the subterranean clover sward. The results of above experiments suggest that legume plants can use both N_2 fixation and combined nitrogen to fulfil their nitrogen requirements.

2.4. Nodule Formation and Functioning

Bacteria of the genus Rhizobiaceae associate symbiotically with roots of leguminous plants to fix nitrogen. In the process of the symbiotic association, the host plant and the rhizobia have mechanisms that allow specific *Rhizobium* strains to penetrate and develop nodules. Specific contact between the *Rhizobium* and the host plant is required for recognition.

The establishment of an effective nodule occurs in a series of events consisting of colonisation of rhizosphere by rhizobia, attachment of the rhizobia to the root hairs, curling of the root hair and formation of infection threads within the root hairs. Growth of the

infection thread then occurs towards the pericycle of the root, later branching and releasing the rhizobia into the plant cytoplasm. The rhizobia become enclosed by a membrane of plant origin and are subsequently referred to as bacteroids. The development of rhizobia into bacteroids is paralleled by the production of leghaemoglobin and the final appearance of the fully developed and functioning root nodules. A summary of the current understanding of the communication between *Rhizobium* and its host plant is given in Fig. 2.1.

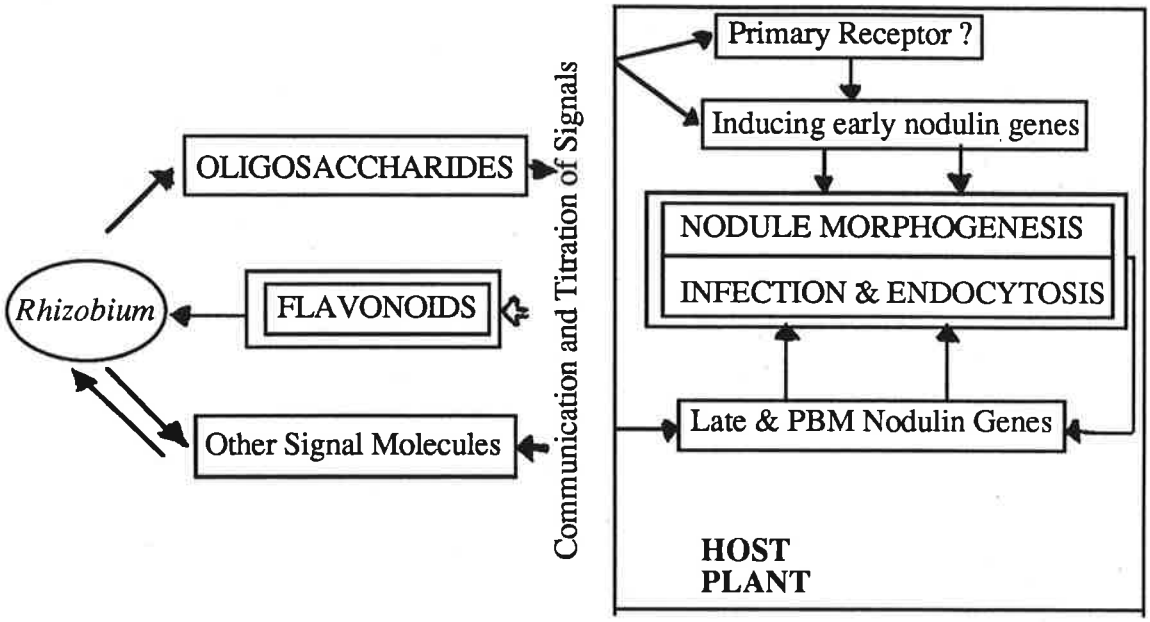


Fig. 2.1. Possible signals involved in the induction of nodulin genes and development of the root nodule (From Verma and Miao 1992).

2.4.1. Attraction and multiplication of rhizobia

Rhizobia are soil bacteria that can enter into a complex multi step interaction with a specific range of legume species that can result in symbiosis (Djordjevic *et al.* 1987, Long 1989). Rhizobial motility, chemotaxis and electrotaxis are important factors in establishing the initial chemical and physical contact between the rhizobia and the host root (Miller *et al.* 1986, Caetano-Anolles *et al.* 1988). Attraction and multiplication of rhizobia require the participation of the legume root exudates (Sprent and Minchin 1985). It has been shown that the exudation of flavonoids and iso-flavonoids from the root hair cells (Fig. 2.1) play

an important role in chemotaxis (Caetano-Anolles *et al.* 1988) and regulates the expression of the *nod* genes in the rhizosphere (Maxwell *et al.* 1989, Peters and Long 1988). Cho and Harper (1991) showed that hypernodulating soybean had higher root concentration of isoflavonoid than the Williams parent when inoculated with *Bradyrhizobium japonicum.* Products of the *nod* genes are involved in the synthesis of bacterial lipo-oligosaccharides, which induce nodulation-related processes in root cells of the host plant (Verma 1992). Root exudates and sloughed off cells provide the nutrient requirement of rhizobia for multiplication (Sprent 1989).

2.4.2. Rhizobial adsorption and root hair curling

When rhizobia come close to the legume root, they attach themselves to root hair tips, to epidermal cell junctions and/or to wound sites (Kijne *et al.* 1991). Interaction of host lectins and the surface polysaccharide of bacteria has been proposed by Dazzo and Gardiol (1984) to explain the attachment of *Rhizobium* to root surfaces. The site of lectin accumulation has been localised in the root hair tip of legume species (Dazzo and Hubbell 1975). Fimbriae (pili) and cellulose fibrils may be involved in the attachment process (Sprent and Sprent 1990). Smit *et al.* (1989) discovered a rhizobial surface protein, designated as rhicadhesin, which is probably involved in binding bacteria to the root hairs. Curling of the root hair occurs after attachment of homologous rhizobia to root hairs (Newcomb *et al.* 1979) and may result from a redirection of hair growth by rhizobia (Batenburg *et al.* 1986).

2.4.3. Infection and nodule initiation

Rhizobia may infect roots through the root hairs, wounds or cracks and through intercellular spaces in the epidermis (Sprent and Sprent 1990). In medics, infection takes place on new, emerging root hairs (Dart and Pate 1959). Infection thread initiation coincides with an arrest of tip growth of the root hair (Kijne 1992). Nodule initiation then begins with stimulation of cell division in the root cortex which occurs prior to the entry of rhizobia into the host (Verma 1992). These divisions lead to a proliferation of root tissue, eventually forming a

mature root nodule. Each individual cell or a limited number of cells may be infected by a branch of the infection thread (Sprent and Sprent 1990). In the inner cortex cells, the bacteria are released into the cytoplasm. Each bacterium is enclosed within a membrane called the peribacteroid membrane, to form a vesicle (Newcomb 1981, Sprent and Minchin 1985). The vesicle is important in the development of an effective legume-*Rhizobium* N_2 fixing symbiosis.

There are two types of nodules; indeterminate and determinate. In indeterminate nodules, each cell is invaded by a branch of infection thread, while in determinate nodules bacteroids continue to divide in parallel with host cells (Sprent and Thomas 1984). Indeterminate nodules possess an open type of vascular system and determinate nodule have a closed one (Sprent 1980).

2.4.4. Nitrogenase synthesis

Nitrogenase synthesis occurs after bacteria are released from infection threads (Sprent and Sprent 1990) and is paralleled by a switching on of the glutamine synthetase and glutamate synthase pathway (Lea *et al.* 1992). Low oxygen condition is required for nitrogenase synthesis (Adams and Chelm 1988) and high concentration of ammonium and availability of external nitrogen repress synthesis of this enzyme (Hom *et al.* 1980).

Nitrogenase is sensitive to oxygen and must be maintained under anaerobic conditions to prevent inactivation. In legumes, nodule resistance to oxygen diffusion plays an important role in the protection of nitrogenase (Tjepkema 1979). Sheehy *et al.* (1983) suggested that a diffusion barrier might control oxygen flux into the nodule (see Section 2.7.2). Leghaemoglobin, which conducts oxygen to the bacteroids at a low and constant concentration, makes a significant contribution to creating micro-aerobic conditions within the bacteroid and to maximising respiratory activity (Bergersen 1984). Nitrate has been shown to decrease the synthesis of leghaemoglobin in the nodule (Bisseling *et al.* 1978, Chen and Phillips 1977). Becana and Sprent (1989) compared the total leghaemoglobin

content in the nodules of several legume plants supplied with 20 mM nitrate for 7 days. The greatest decrease occurred in *Pisum*, *Trifolium* and *Phaseolus*; the level in *Vigna unguiculata*, *V. radiata* and *Glycine* being less affected while that in *Lupinus* remained constant.

2.4.5. Nitrogen Fixation

Nitrogenase is comprised of two component proteins, an MoFe-protein or dinitrogenase and an Fe-protein or dinitrogenase reductase (Burris *et al.* 1980). Nitrogenase reduces N_2 to ammonia. N_2 reduction by nitrogenase involves the reduction of Fe-protein by electron carriers such as ferredoxin or flavodoxin, the ATP dependent transfer of single electrons from Fe-protein to MoFe-protein and electron and proton transfer to the N_2 (Rees *et al.* 1993).

The N_2 fixation reaction of nitrogenase is:

$$N_2 + 12ATP + 6e^- + 8H^+ \longrightarrow 2NH_4^+ + 12ADP + 12P_i$$

Associated with this reaction is the production of hydrogen

$$4ATP + 2e^- + 2H^+ \longrightarrow H_2 + 4ADP + 4P_i$$

2.4.6. Relationship between acetylene reduction and N_2 fixation

The nitrogenase enzyme is not specific and it can reduce a considerable number of substrates including acetylene. The enzyme reduces acetylene by a two-electron step to ethylene. In other words, the enzyme is unable to distinguish between a C≡C and an N≡N triple-bond. The acetylene reduction technique has been widely used to estimate relative rates of N_2 fixation by legumes in glasshouse and field conditions, with the assumption that the relationship between acetylene reduction and N_2 fixation is constant. This method has been criticised by several workers (Minchin *et al.* 1983, Witty and Minchin 1988) as underestimating the actual rates of C_2H_4 production due to C_2H_2 induced decline in C_2H_4 production. The decline in C_2H_4 production has been found to be influenced by genotype (Tjepkema *et al.* 1988), *Rhizobium* strain (Skot *et al.* 1986), plant age (Witty *et al.* 1983,

Herdina and Silsbury 1990a), plant disturbance before assay (Minchin *et al.* 1986b), shoot excision (Herdina and Silsbury 1990a, Hansen *et al.* 1987) and nodule detachment (Trinick *et al.* 1976, Hudd *et al.* 1980). Environmental factors such as assay temperature and light also influence nitrogenase activity (Crall and Heichel 1982).

The acetylene reduction method should therefore be used with care. In the use of acetylene reduction method the reduction of acetylene requires 2 electrons per mole acetylene and occurs at a lower reduction stage than that of N_2 which requires 6 electrons (Burgess 1985).

$$N_2 + 6H^+ + 6\,e^- \longrightarrow 2NH_3$$

$$C_2H_2 + 2H^+ + 2\,e^- \longrightarrow C_2H_4$$

When nitrogenase is exposed to N_2, some electrons are directed to the reduction of protons to produce hydrogen (Evans *et al.* 1980). In the nodules of many leguminous species, 30-60% of the electron flux through nitrogenase may be lost as H_2. In contrast, when the enzyme is exposed to 10% acetylene, all electrons are used to reduce this substrate. Accordingly, the conversion factor of C_2H_2: N_2 varies according to the amount of hydrogen evolved and the flux of electrons to acetylene. Schubert and Evans (1976) concluded that hydrogen evolution was a major factor influencing the efficiency of N_2 fixation. They reported that at least one mole of H_2 is evolved per mole N_2 fixed and defined the relative efficiency of electron transfer to N_2 via nitrogenase as:

$$\text{Relative efficiency} = 1 - \frac{H_2 \text{ evolved}}{C_2H_4 \text{ produced}}$$

Relative efficiency has been found to vary between legume species and to be affected by a range of factors (Schubert and Evans 1976). External factors known to exert some influence on relative efficiency are nitrate and O_2 (Drevon *et al.* 1982), light (Silsbury 1981), temperature (Bertelsen 1985) and CO_2 (Edie 1983). It has been reported however that some symbioses can recycle H_2 (Schubert *et al.* 1977, Nelson and Salminen 1982). This factor further complicates the conversion factor.

There are important limitations to the use of the acetylene reduction assay to measure N_2 fixation. Witty and Minchin (1988) enumerated a number of factors which influence the acetylene reduction assay, but it is a convenient procedure for comparative studies on nitrogenase activity of nodulated plants (Herdina and Silsbury 1990a). Vessey (1994) has recently presented a well argued defence of the acetylene reduction assay for determining relative differences in nitrogenase activity of nodulated legumes.

2.4.7. Products of nitrogen fixation

The first stable product of N_2 fixation is ammonia (Bergersen 1965, Kennedy 1966). This is translocated out of the bacteroid into the cytosol of cells of the host plant (Ohyama and Kumazawa 1980). In the cytosol, ammonia is converted into glutamine and glutamate via the combined activity of glutamine synthetase (GS) and glutamate synthase (GOGAT) (Vance 1991).

The reactions for these two enzymes are:

$$NH_4^+ + \text{L- glutamate} + ATP \xrightarrow{\textit{Glutamine synthetase}} \text{L-glutamine} + ADP + P_i$$

$$\alpha\text{-Ketoglutarate} + \text{L-glutamine} + NAD(P)H + H^+ \xrightarrow{\textit{Glutamate synthase}} 2\text{L-glutamate} + NAD(P)^+$$

During nodule development, GS and GOGAT activities increase in parallel with nitrogenase activity. Nodule GS and GOGAT comprise up to 2 and 0.5 % of the total nodule soluble protein respectively (Vance 1991).

Nitrogen fixing plants may be classified as amide or ureide exporters based on the composition of the xylem sap (Sprent and Embrapa 1980). Temperate legumes usually export amides, whereas ureides predominate in tropical legumes.

2.5. Uptake and Assimilation of Nitrate

Nitrate uptake is the first step in the process of nitrate assimilation and may involve both active and passive components (Ullrich 1992). The active process is under metabolic control and requires ATP (Clarkson 1986). This explains why nitrate uptake is inhibited by low temperature (Neyra and Hageman 1975) and anaerobic conditions (Lee 1979). Nitrate uptake by roots is mediated by specific carrier proteins in the plasma membrane (Jackson *et al.* 1973, Dhugga *et al.* 1988). Net nitrate uptake is the balance of influx and efflux of nitrate (Morgan *et al.* 1973).

Ammonium has been shown to restrict net nitrate uptake by plant species (MacKown *et al.* 1982). The exact mechanism(s) of ammonium inhibition of nitrate uptake is not clear. Jackson (1978) reported that the endogenous level of cytoplasmic ammonium in root tissue inhibits nitrate uptake, whereas Doddema and Otten (1979) suggested that the amino acid accumulation in the roots during ammonium nutrition inhibits nitrate uptake.

Nitrate uptake rate by plants is dependent on the nitrogen demand for growth (Clarkson 1986, Rodgers and Barneix 1988), and is controlled by the level of nitrogen compounds in plant tissue (Lee and Rudge 1986, Muller and Touraine 1992), by carbohydrate availability (Mengel and Viro 1978, Arnozis *et al.* 1988) and by negative feedback by tissue nitrate (Breteler and Nissen 1982, Jackson *et al.* 1986). Plants that contain adequate concentrations of carbohydrates are able to take up nitrate at a faster rate than comparable plants containing lower concentrations of carbohydrates (Pilbeam and Kirkby 1990). A relationship between soluble sugar concentration in root tissue of wheat seedlings and uptake and reduction of nitrate was found by Champigny and Talouizte (1986). Using cowpea, Sasakawa and LaRue (1986) studied the uptake of nitrate both in the light and in the dark and concluded that low carbohydrate availability during the dark period had a negative effect on nitrate uptake. Recently, Touraine *et al.* (1992) investigated the relationship between nitrate uptake and transport of malate to the root of soybean and concluded that supplying malate to the roots, either by addition to the external solution or by artificially increasing transport from

the shoot in the phloem, resulted in a net increase of nitrate uptake rate. Muller and Touraine (1992) found that amino acids present in the root tissues exert a negative feedback on nitrate uptake and amino acids transported from the shoot control nitrate uptake by the root.

The energy requirement for nitrate uptake decreases as the external concentration of nitrate increases, because the electrochemical gradient between the cell and external nitrate is decreased accordingly (Pilbeam and Kirkby 1990). Clarkson and Hanson (1980) calculated the energy cost for the uptake of one mole of nitrate from 1.5 mM nitrate solution to be 3 times more than that from a 15 mM nitrate solution.

The amount of nitrate taken up by a plant varies with the stage of development (Streeter 1972a, b) and temperature, being depressed by low temperatures (Clarkson and Warner 1979). Nitrate uptake also increases with decrease in pH (Rao and Rains 1976). Other factors which control the amount of nitrate uptake are partitioning of absorbed nitrate in the cell, transport to the vacuole and to xylem vessels, efflux back across the plasmalemma (Jackson *et al.* 1986) and a feed-back effect on uptake (Siddiqi *et al.* 1989).

There are two main pools of nitrate in a plant cell. The first is a cytosolic metabolic pool which appears to be responsible for controlling the level of synthesis of nitrate reductase (NR). The second pool is a storage pool, mainly in the vacuole (Granstedt and Huffaker 1982, Hocking *et al.* 1984).

2.5.1. Nitrate reduction in legumes

Nitrate reductase (NR) and nitrite reductase (NiR) are substrate inducible enzymes found in the root and shoot of all plants including legumes, supplied with nitrate (Redinbaugh and Campbell 1981). Oghoghorie and Pate (1971) suggested that the advantage of nitrate assimilation over N_2 fixation is probably due to a more widespread distribution of NR sites in the plant and the rapid induction of the enzyme by nitrate. NR is NADH dependent and is found in the cytosol of the cell (Beevers and Hageman 1983). The reaction is as follows:

$$NO_3^- + NADH^+ + H^+ \longrightarrow NO_2^- + NAD^+ + H_2O$$

The NR enzyme of higher plants contains FAD, a heme and molybdopterin cofactor which are involved in the transfer of electrons from NADH to nitrate. In soybean and a few other legumes two constitutive NRs have been characterised. One of these is NAD(P)H bispecific. An evolution of NO_X by soybean leaves is associated with one of these constitutive enzymes (Dean and Harper 1986). Andrews *et al.* (1990) showed that this constitutive NR is present in leaves of the bean (*Phaseolus vulgaris*), and soybean (*Glycine max*) and roots and shoots of *Lotus uliginosis* but was absent from all tissues of a wide range of other legumes tested including the Trifoleae.

Further reduction of the nitrite produced is mediated by the ferredoxin-dependent NiR, which is located in the chloroplast or root plastid. NiR is present in excess of NR and nitrite does not accumulate under aerobic conditions. Its reaction is as follows:

$$NO_2^- + 8H^+ + 6Fd_{red} \longrightarrow NH_4^+ + 6Fd_{ox} + 2H_2O$$

Nitrate taken up by legume roots can be assimilated via NR and NiR enzymes in the roots (Beevers and Hageman 1980). In nodulated plants a fraction of nitrate is also reduced within the nodules (see Section 2.5.2).

The extent to which different plant organs participate in nitrate reduction may vary with the plant species (Harper and Gibson 1984, Wallace 1986), their development stage (Pate 1980) and also with the concentration of nitrate in the growth medium (Hervas *et al.* 1991). At low nitrate concentrations, legumes of temperate origin have between a third and a half of the total NR in their root tissues, but at high nitrate concentrations, nearly all the NR is found in the shoot (Wallace 1986). Legumes of tropical origin have most of their NR in the shoot regardless of nitrate concentration (Wallace 1986).

Because there is a competition between NR and nitrogenase activity for reductant and photosynthate (see Section 2.7.3), some investigators have studied the relationship between

NR activity and legume/*Rhizobium* symbiosis. For example, Harper and Gibson (1984) reported that *Glycine max* L. (soybean), *Lablabs purpureus* (Dc.) Urb.(lablab bean) and *Macroptilium atropurpureum* L. (siratro) had greater NR activity in the leaves than in the roots; *Trifolium subterraneum* L. (subterranean clover) had similar activity in leaves and roots and *Medicago truncatula* Gaertn. (Barrel medic), *Cicer arietinum* L.(chickpea) and *Lupinus angustifolius* L. (lupin) had a higher proportion of NR in the roots, relative to the leaves. Harper and Gibson concluded that the site of NR was not related to ability of the plant species to undergo the initial nodulation phase in the presence of nitrate.

Carroll and Gresshoff (1983) and Silsbury *et al.* (1986) showed an inverse relationship between NR and nitrogenase activity in white clover (*Trifolium repens*) and subterranean clover.

2.5.2. Nitrate reductase in legume nodules

Root nodules have the capacity to reduce nitrate in both the bacteroids and cytosol fractions (Randall *et al.* 1978, Manhart and Wong 1980, Stephens and Neyra 1983, Ligero *et al.* 1987b). Bacteroids from soybean, lucerne and cowpea nodules, but not those from peas express a constitutive NR (Becana and Sprent 1987). The bacteroid NR is not active with NADPH (Heckmann and Drevon 1988). Most legume nodules appear to have a nodule cytosol NR (active with NADH) but, unlike the root enzyme, the synthesis is largely constitutive and the presence of nitrate is even reported to decrease its activity (Caba *et al.* 1990, Hunter 1983). Becana and Sprent (1987) concluded that in soybean and lucerne most (79%) of the potential nitrate reducing activity of the nodules was in the bacteroid. However in contrast to the observation of others (see above), Serraj *at al.* (1992) produced evidence that the nodule cytosol NR in soybean was induced by nitrate (3 mM) and in its presence 73% of the nodule NR was in cytosol.

Hunter (1983) measured the nitrate concentration and NR in the roots and nodules of soybean. The concentration of nitrate in the root was 20-fold greater than within nodules

for the plants supplied with 3 mM nitrate. Nodule NR activity was much higher than that of the root and he estimated that 90% of nodule NR was of bacteroid origin. Ohyama (1983) found that after a 10 h feeding of $^{15}NO_3$, only 0.4% of the recovered ^{15}N was in nodules relative to 36% in leaves and 36% in roots. Giannakis *et al.* (1988) demonstrated that nitrate is prevented from entering the bacteroid zone and is restricted to the nodule cortex. A further study, Becana *et al.* (1989) on soybean showed that after 3 d of treatment with high nitrate (10-20 mM) nitrate does reach the bacteroids. Thus even where there is a high level of NR activity in the bacteroid it would appear to contribute little to the reduction of nitrate by the plant.

2.5.3. Studies with bacteroid mutants

When subterranean clover was inoculated with NR deficient mutants of *Rhizobium trifolii* no decrease in inhibitory effects of nitrate was observed, suggesting that bacteroid NR was not involved in the inhibition (Gibson and Pagan 1977). Streeter (1985) also showed that *Bradyrhizobium japonicum* mutants (76CR6) lacking NR are no more capable of symbiotic N_2 fixation in 6.4 mM nitrate treatment than the wild type (61A76), suggesting that nitrite produced by the bacteroid NR was not the cause of the nitrate inhibition of nitrogenase.

2.5.4. Studies with legumes possessing mutant NR

A wide range of NR-deficient mutants has been isolated in *Pisum sativum* (Warner and Muehlbauer 1982, Jacobsen 1984, Feenstra and Jacobsen 1980), *Glycine max* L. (Nelson *et al.* 1983). The NR-deficient mutant lines are of particular interest, since they can be used to study the interaction of N_2 fixation and nitrate assimilation. Feenstra *et al.* (1982) used the E1 mutant of pea, which possesses 20% of the NR of the wild type but has normal nitrate uptake, to study the effect of nitrate and ammonium on nitrogenase activity. Ammonium inhibited acetylene reduction to the same extent in both the wild type and the E1 mutant, but when supplied with 4 mM nitrate, acetylene reduction by the E1 mutant was not inhibited to the same extent as that of wild type, suggesting that the inhibitory effect of nitrate on N_2 fixation was due in part to the products of nitrate reduction. Vigue and Warner

(1987) reported that treatment with 15 mM nitrate for 8 d inhibited N_2 fixation in three NR-deficient pea mutants (A300, A317 and A314) to the same extent as in the wild type. It has subsequently been reported that these pea mutants still maintained considerable ability to reduce nitrate but in a 2 d treatments with 5 mM nitrate, nitrogenase activity was less affected in the nitrate reductase-deficient mutant (Walsh and Carroll 1992).

2.6. Effects of Nitrate on Nodulation Process

In the next two sections a detailed examination is presented of all the ways in which nitrate may interfere with the nodulation or N_2 fixation process. The promotive effect of low levels of nitrate is also considered. There has been much research in the interaction of nitrate assimilation and N_2 fixation (Munns 1977, Rigaud 1981, Gibson and Jordan 1983, Streeter 1988) and in spite of several hypotheses proposed to explain the inhibition, the actual mechanism is still elusive.

2.6.1. Promotive Effects of Nitrate on Nodulation and Nitrogen Fixation

The promotive effects of nitrate on the symbiotic association usually take place during the early stages of growth, and are expressed in accelerated growth, enhanced nodule formation and improved N_2 fixation. This is the so-called 'starter N' effect. Some legumes may suffer a period of N-stress during early vegetative growth (Phillips *et al.* 1981, Mahon and Child 1979). This occurs after the nitrogen content of the seed has been exhausted and before the establishment of effective symbiosis (Oghoghrie and Pate 1971). In such cases applications of small amounts of N fertiliser may be beneficial for the plants, especially where it does not inhibit nodulation. Legumes with high seed N content (e.g. pea) do not suffer from a period of 'N-hunger' following the exhaustion of cotyledons (Sprent and Minchin 1983).

The characteristic difference between different strains of *Rhizobium* in the speed with which they establish effective nodules on the roots is an important factor in determining the requirement for starter N. When *Rhizobium* strain-host plant combinations which rapidly form effective nodules are chosen, requirements for starter N can be reduced. Hungria *et al.*

(1991) suggested the possibility of avoiding symptoms of N deficiency in nodulated bean through inoculation with 'precocious' strains in which the nitrogenase had an initial higher efficiency of utilisation of electrons for reduction of N_2 and there was an earlier induction in the enzymes involved in ammonium assimilation.

Environmental conditions such as temperature, light and moisture affect the requirement for starter N. Usually legumes grown at high temperatures become N deficient, whereas those at lower temperature do not (Jones *et al.* 1981). This is due to the rate of growth; at a low temperatures, nodule formation is able to keep pace with plant growth, whereas at high temperatures, this does not occur.

2.6.2. Inhibitory Effects of Nitrate on Nodulation

Nitrate has been shown to inhibit the infection of the root hairs by *Rhizobium,* nodule development and nitrogenase activity (Streeter 1988). The subject has been investigated in the following ways:

(i) Identification of legume species and cultivars that are tolerant to nitrate. Natural variation occurs among legume species and cultivars in their symbiotic tolerance to nitrate (Harper and Gibson 1984, Hardarson *et al.* 1984, Gibson and Harper 1985, Buttery and Dirks 1987, Park and Buttery 1989, Ewing and Robson 1990).

(ii) Investigating the mechanisms of the inhibition of nodulation and N_2 fixation by nitrate. This approach has been pursued by numerous investigators for many decades, yet according to Streeter (1988) 'in spite of a steadily growing body of scientific literature which reflects some innovative and thorough research, we do not seem to be closer to understanding or alleviating the inhibitions than we were in 1916'.

(iii) Mutagenesis. This has been used to generate phenotypes forming high numbers of nodules in the presence of nitrate (Carroll *et al.* 1985). Some of these, with a reduced ability to utilise nitrate, have been selected (Carroll and Gresshoff 1986) for use in breeding programs and physiological studies.

2.6.2.1. Inhibitory effects of nitrate on the infection process

Nitrate has been shown to affect four aspects of the infection process. It decreases root hair deformation, the binding of *Rhizobium* to root hairs, lowers the number of infection threads and increases the number of aborted infections (Dazzo and Brill 1978, Truchet and Dazzo 1982). Lafreniere *et al.* (1984) showed that the addition of 5 or 16 mM nitrate decreased the number of rhizobia (*Rhizobium meliloti*, strain A_2) adhering to the roots of lucerne seedlings and addition of 18 mM nitrate to the roots completely inhibited accumulation of *R. meliloti* cells on root hairs of lucerne (Truchet and Dazzo 1982). An early proposal on the mechanism of the nitrate inhibition was that nitrite produced by bacterial reduction of nitrate caused destruction of indole-3-acetic acid (IAA) and interfered with nodule development (Tanner and Anderson 1964). However the inhibition occurred in plants nodulated with NR deficient mutants of rhizobia (Gibson and Pagan 1977). Dazzo and Hrabak (1982) showed that nitrate did not inhibit binding of rhizobia to root hairs but reduced the number of lectin binding sites, indicating a plant-mediated effect. Dazzo *et al.* (1984) observed that hydroxyproline increased 70 to 100% in white clover roots exposed to nitrate and suggested that increase of a hydroxyproline-rich protein in root hair cell walls reduces infection.

It is generally considered that the initial signalling between the host plant and *Rhizobium* involves the exudation of flavonoids and isoflavonoids from roots which induce the expression of bacterial nodulation genes (Fig. 2.1). In a study with nitrate tolerant-hypernodulating mutants of soybean and the parent line Williams, Cho and Harper (1991) showed that in the presence of 5 mM nitrate, the mutant had higher root isoflavonoid concentrations. There was a positive relationship between root isoflavonoid concentration and nodule number. Differential tolerance of nodulation between legume species could be due to the different levels of isoflavonoid production in the roots.

Nitrate induces ethylene biosynthesis in uninoculated and inoculated legume roots (Ligero *et al.* 1987a), thereby inhibiting nodulation by preventing cell division (Apelbaum and Burg 1972), root extension and lateral root initiation (Feldman 1984). Ligero *et al* (1991) showed

that the inhibitory effect of nitrate on the nodulation of lucerne can be eliminated by the addition of aminoethoxyvinylglycine (an inhibitor of ethylene synthesis). It is therefore possible that differences between legume species in their tolerance of nodulation to nitrate is related to ethylene biosynthesis in the root, although how this effect is mediated is not understood.

2.6.2.2. Inhibitory Effects of Nitrate on Nodule Growth

Addition of nitrate to the rooting medium of legumes can inhibit nodule growth and nodule activity (Gibson and Pagan 1977, Latimore *et al.* 1977, Houwaard 1980, Vessey *et al.* 1988a). Dart and Mercer (1965) showed that addition of 10.7 mM ammonium nitrate for 2, 3 and 8 days to the rooting medium of *Medicago truncatula* Gaertn. and *Trifolium subterraneum* L. affected the fine structure of nodules in both plants, but the disorganisation of nodule meristem for *M. truncatula* was more rapid and severe than that for *T. subterraneum.*

2.7. Possible Mechanisms of Nitrate Inhibition of Nitrogen Fixation

The effect of nitrate on N_2 fixation of well-nodulated legume plants is diphasic (Carroll and Gresshoff 1983). In the first phase, nitrate treatment leads to rapid inhibition of nodule nitrogenase activity during the first 1-2 days. This inhibition corresponds to an increase in the activity of NR and can be partially alleviated by increasing photosynthate supply. In the second phase, a complete inhibition of nitrogenase activity occurs after 4-6 d of nitrate application. This inhibition corresponds to the suppression of bacteroid N_2 fixation (Schuller *et al.* 1986).

The effect of nitrate on nitrogenase activity may be either a direct inhibitory effect on the symbiotic and catalytic system or an indirect effect on associated plant metabolism. The following effects of nitrate will be considered below; direct inhibitory effects including nitrite toxicity, decreased oxygen diffusion and leghaemoglobin content and indirect effects

including carbohydrate deprivation, as a result of nitrate assimilation, plus feed-back effects consequent on nitrate assimilation.

2.7.1. Nitrite toxicity

Among the hypotheses proposed to explain the depressive effect of nitrate on nitrogen fixation, a role for nitrite has been suggested (Rigaud 1976). Nitrite inhibits nitrogenase activity when added to the purified enzyme (Trinchant and Rigaud 1980) or bacteroid *in vitro* (Kennedy *et al.* 1975, Rigaud and Puppo 1977) or when supplied to detached nodules (Kamberger 1977). Nitrite inhibition of nitrogenase is probably due to the binding of nitrite to the Mo-Fe component (Trinchant and Rigaud 1980) or to the deoxygenation and oxidation of leghaemoglobin as found *in vitro,* resulting in the formation of ferric leghaemoglobin (Riguad and Puppo 1977). Burris (1977) suggested that deficiency in the supply of oxygen, rather than the presence of N_2O, inhibited the utilisation of N_2 by legume plants. Trinchant and Rigaud (1982) observed that the reduction in nitrogenase activity and respiration of bacteroids isolated from nitrate treated *Phaseolus* nodules was mimicked by the application of nitrite to bacteroids from untreated plants. They suggested reduced bacteroid respiration as a possible cause of the effect of nitrate on nitrogenase activity.

Nitrite has been detected in nodule extracts of several legumes (Stephens and Neyra 1983, Becana *et al.* 1985, Streeter 1985, Wasfi and Prioul 1986) and has been attributed to the high level of bacteroid NR present (Chalifour and Nelson 1988b) and negligible NiR (Giannakis *et al.* 1988). However, some legumes do not have a bacteroid NR (Chalifour and Nelson 1988b) and mutants are available (Gibson and Pagan 1977) in which it is absent, yet nitrate is still inhibitory in these plants. Indeed, Sprent *et al.* (1987) showed that nitrate does not enter the bacteroid region and thus accumulation of nitrite in nodules of plants supplied with nitrate was an artefact of tissue maceration (Giannakis *et al.* 1988). There is an NR enzyme in the nodule cytosol (see Section 2.5.2) but also an accompanying NiR enzyme (Becana and Sprent 1987). Thus, as in other parts of the plant, nitrite would not normally accumulate. Kanayama *et al.* (1990) did however detect nitrite in the cytosol

of soybean nodules after a 12 h treatment with 10 mM nitrate and suggested that its reaction with leghaemoglobin, and interference in the oxygen supply to the bacteroid, caused the inhibition of nitrogenase activity observed.

2.7.2. Increased resistance of the oxygen diffusion barrier

Organisms that fix N_2 require oxygen for ATP production but need to protect nitrogenase against inactivation by direct contact with oxygen (see Section 2.4.4). Both the Fe-protein and MoFe-protein are extremely sensitive to oxygen, and have a half-life of 45 seconds and 10 min at atmospheric oxygen tension respectively (Gallon 1992). In N_2-fixing legume nodules, oxygen supply to the bacteroid zone is restricted by cell layers which are thought to act as a barrier to oxygen diffusion (Tjepkema and Yocum 1974, Witty *et al.* 1984, Parsons and Day 1990). The existence of a barrier to oxygen diffusion in the inner cortex of legume nodules has been demonstrated by gas exchange studies (Hunt *et al.* 1988), micro-electrode studies (Tjepkema and Yokum 1974, Witty *et al.* 1987), anatomical studies (Dakora and Atkins 1989, Parsons and Day 1990) and studies of *in situ* leghaemoglobin oxygenation (Layzell *et al.* 1990). Webb and Sheehy (1991) proposed that oxygen diffusion into the infected region of the nodule involves a pathway that is partially air-filled and partially water-filled. The air-filled region of the pathway consists of intercellular air spaces. Oxygen diffuses more rapidly through the air-filled portion of the pathway than through the water-filled portion, so alteration in the relative proportion of these regions alters the rate of diffusion of oxygen to the bacteroids. An alternative mechanism proposed by James *et al.* (1991) suggests that the intercellular air-space becomes filled with water or glycoprotein. Nodule specific proteins (nodulins) that are produced during the early stages of nodule development may have a role in limiting oxygen diffusion to the infected cells (Hirsch 1992).

Nitrate has been shown to increase oxygen diffusion resistance in both determinate and indeterminate nodules (Minchin *et al.* 1986a). It appears that a supply of photosynthate to the nodule is necessary to ensure a low resistance to O_2 (Vessey *et al.* 1988b). It could be,

as Vessey and Waterer (1992) have pointed out that the nitrate inhibitory effect was indirect and resulted from carbohydrate deprivation of the nodules following its utilisation for nitrate assimilation elsewhere in the plant.

Schuller *et al.* (1986), Silsbury *et al.* (1986) and Silsbury (1987) showed a lag period of several days between the supply of nitrate and the inhibition of nitrogenase activity. However, an increase in the resistance of the diffusion barrier to nitrate occurs after 24 h of nitrate application (Witty and Minchin 1990). If the decline in acetylene reduction was due to an increase in the resistance of the diffusion barrier, a much faster response would have been obtained. This hypothesis therefore does not completely explain inhibition of N_2 fixation by nitrate.

2.7.3. Carbohydrate deprivation

Many investigators have demonstrated that nitrogenase activity depends on photosynthate supply to the nodule. However, when nitrate is supplied to nodulated plants, there is a diversion of photosynthetically-derived energy and reductant from the nodule to the sites of nitrate assimilation in the plant (Oghoghorie and Pate 1971). The carbohydrate deprivation hypothesis attributes the decline in nitrogenase activity, when nodulated plants are supplied with nitrate, to the diversion of carbohydrate. Ta *et al.* (1990) demonstrated that the N_2 fixation process in legume root nodules relies heavily on the supply of fresh photosynthate from the shoot for nodule function and growth. The requirements of reductant for nitrogenase activity and carbon skeletons for the incorporation of fixed ammonia in the shoot are provided by photosynthate from the host plant. Many studies have provided data to suggest that decreases in nitrogenase activity have been associated with a number of treatments involving interruptions in photosynthate supply to nodules. These include extended dark treatment (Minchin *et al* 1985), defoliation (Hartwig *et al.* 1987) and stem girdling (Vessey *et al.* 1988b).

Latimore *et al.* (1977) showed that addition of nitrate to soybean plants resulted in a changed distribution pattern of photosynthate, with less carbohydrate reaching the root nodules. The same phenomenon has also been reported for pea and subterranean clover plants with the addition of nitrate (Small and Leonard 1969). In a simple and interesting experiment, Vessey *et al.* (1988a) showed that within 5 days of exposure of nodulated soybean to 10 mM nitrate, the relative growth rate of nodules declined whereas the relative growth rate of leaves, stems and roots increased. The increase in relative growth rate of nitrate-fed plants was 1.37 times that of control plants. This indicates that photosynthate partitioning was altered within the nitrate-treated plant such that less photosynthate was allocated to nodules. Gibson (1976) showed a decline in the rate of ^{14}C translocated to soybean nodules 24 h after supplying 7 mM nitrate to the plants. The proportion of ^{14}C in the nodules decreased as the duration of the nitrate treatment increased. Reductions in sugar and starch content of nodules has been reported with nitrate addition to pea plants (Nelson and Edie 1988, Taylor *et al.* 1988, Walsh and Carroll 1992) and to field bean (Wasfi and Prioul 1986). Nelson and Edie (1991) showed that pea nodules which contained low carbohydrate reserves and large organic acid pools had a greater inhibition of N_2 fixation in the presence of 5 mM NH_4NO_3 than the nodules with high carbohydrate reserves. Walsh and Carroll (1992) demonstrated that addition of 5 mM nitrate for two days decreased nodule starch level by 74% in pea, supporting the suggestion that carbohydrate deprivation may inhibit N_2 fixation. Vessey *et al.* (1988a) and Taylor *et al.* (1988) proposed that the pool size of nodule starch is an indicator of the carbohydrate status of nodule metabolism. In contrast, Wasfi and Prioul (1986) concluded that the nitrate inhibition of nodule function was not caused by a change in photosynthate supply to nodules. Using a steady-state $^{14}CO_2$ feeding technique and measuring ^{14}C partitioning to nodules, they found an increase in the ^{14}C partitioned to nodules after 24 h of nitrate treatment even though the measured rate of acetylene reduction was significantly lower. Nelson and Edie (1988) showed that the concentrations of malate and succinate increased in pea nodules after 7 days of exposure to nitrate and Streeter (1987) found increases in malate, succinate, fumarate and citrate

concentrations in soybean nodules treated with 15 mM nitrate for 2 days. These results suggest that inhibition of N_2 fixation by nitrate is not due solely to carbohydrate deprivation.

2.7.4. Feed-back mechanism

Silsbury *et al.* (1986) considered that the inhibitory effect of nitrate on N_2 fixation was due to a feed back effect of the reduced nitrogen compounds results from nitrate assimilation. Silsbury and his students used several approaches to test this hypothesis. When they treated subterranean clover plants with 15 mM nitrate for 5 days, nitrogenase activity declined by up to 80%. After nitrate was removed, acetylene reduction activity was restored by 7 days to a level slightly above the control. Oti-Boateng and Silsbury (1993) found, in *Vicia faba* cv. Fiord, that inhibition of nitrogenase activity following the application of nitrate and asparagine, was due to an increase in the pool of soluble N in the plant. Gibson (1976) reported that when a nitrate treatment, which caused N_2 fixation to decline, was withdrawn after continuous treatment for either 6 or 10 days, nitrogenase activity completely recovered within 4 days. Similarly, Streeter (1981) found only a partial recovery 4 days after removing a nitrate treatment, but after 7 days acetylene reduction had returned to its former activity. Oti-Boateng *et al.* (1994) in a debudding experiment found that plant soluble nitrogen increased due to the inability of the plant to utilise this nitrogen for growth and this caused nitrogenase activity to decline.

Other approaches to test the feed-back mechanism have been to decrease the number of nodules or to defoliate the plant. Herdina and Silsbury (1990b) showed that 5 d after faba bean (*Vicia faba* cv. Fiord) was deprived of 50% of its nodules, nitrogenase activity per plant was again at the level recorded before nodule removal. This was due to an increase in specific nodule activity. Hartwig and Nosberger (1993) defoliated white clover to reduce the plant N sink strength and concluded that demand for N by the plant regulates nitrogenase activity. Fujita *et al.* (1988, 1991) found that reduction of nitrogenase activity following pod removal or defoliation in soybean could be attributed to a corresponding reduced

requirement for N. All of the above results indicate that the plant demand for N is involved in the regulation of nitrogenase activity.

2.8. Variation in the Sensitivity of Nodulation and Nitrogen Fixation to Nitrate in Legumes

Nodulation and N_2 fixation are, in general, inhibited when legumes are supplied mineral nitrogen, but variation among legume species for these attributes in the presence of nitrate and other forms of mineral nitrogen has been reported (Allos and Bartholomew 1959, Gibson and Nutman 1960, Harper and Gibson 1984, Hardarson *et al.* 1984, Buttery and Dirks 1987, Chalifour and Nelson 1988a, Ewing and Robson 1990). There is also variation within legume species in the tolerance of nodulation and N_2 fixation to nitrate both in naturally existing legume species and cultivars (Hardarson *et al.* 1984, Gibson and Harper 1985, Bett and Herridge 1987, Buttery and Dirks 1987, Murphy 1988, Park and Buttery 1989) and in plants obtained through mutagenesis (Jacobsen and Feenstra 1984, Carroll *et al.* 1985). Nitrate tolerance could be a useful character to select to enhance nodulation and possibly N_2 fixation in soils containing nitrate.

2.8.1. Natural variation between legume species in the sensitivity of nodulation and nitrogen fixation to nitrate

Many workers have attempted to screen for nitrate tolerant plants and strains of *Rhizobium*. Comparison of the variation in nitrate tolerance reported in the various studies is difficult due to variations in culture media, legume species and strains of *Rhizobium* used and the criteria applied to determine nitrate tolerance. Despite these difficulties, there is sufficient evidence to indicate that variation exists.

In a comparison between legume species, Allos and Bartholomew (1959) showed that the effect of ammonium addition on N_2 fixation depended on the growth rate of the plant. Symbioses involving faster-growing species growing in sand culture with the same initial levels of N (soybean, lucerne and sweet clover) were more tolerant to combined nitrogen

than slow growing species (Ladino clover and birdsfoot trifoil). Harper and Gibson (1984) compared the nitrate sensitivity of eight legumes and found that *Glycine max* and *Medicago truncatula* were more sensitive than *Trifolium subterraneum* and *Pisum sativum* in terms of nodulation and nitrogenase activity in the presence of 1 and 4 mM nitrate. Chalifour and Nelson (1987) found that nitrate was less inhibitory to infection and initial nodule development in faba bean than in the pea. Faba bean is capable of symbiotically-fixing more N_2 than the pea when plants are grown on increasing levels of combined nitrogen, an effect attributed to a more limited capacity for nitrate uptake in beans than in peas (Chalifour and Nelson 1988a). Cowie *et al.* (1990) compared the relative effect of 8 mM nitrate in soil on the nodulation of lupin, chickpeas and peas. The ratio of nodule growth (numbers and weights) for lupin, chickpeas and peas was reduced to 40, 46 and 68% of that of controls (grown with 2 mM nitrate).

2.8.2. Natural variation within legume cultivars in the sensitivity of nodulation and nitrogen fixation to nitrate

Murphy (1988) compared the nodule formation of three *Trifolium repens* cultivars in symbiosis with two *Rhizobium trifolii* strains and found that in the presence of 8.4 mM nitrate, nodulation was reduced by 20-50% in the symbiosis with one and by more than 90% with the other. Jensen (1987b) used the acetylene reduction (AR) assay to compare the sensitivity of nitrogenase in 6 cultivars of pea to two levels of nitrate. The purple flowered cultivars retained 70-80% of their nitrogenase activity after the application of 15 mM nitrate for 7 d, whereas cultivars with white flowers retained only 30-40%. It was suggested that sensitivity was correlated with a higher level of nitrate accumulation in the nodules but a fully replicated study was not undertaken. Differences in the sensitivity of nodulation and N_2 fixation were found between 16 cultivars of common bean (*Phaseolus vulgaris* L.) grown in the presence of 0, 3.5 and 10.5 mM combined nitrogen as nitrate and ammonium (Park and Buttery 1989). Four cultivars showed nitrate tolerant characteristics as measured by nodule dry weight, visual nodulation score and nodulation index. Buttery and Dirks (1987) examined the effect of 6 mM nitrate on nodule fresh weight and nitrogenase activity

(AR activity) of 10 cultivars of soybean, each inoculated with 12 strains of *Bradyrhizobium japonicum* . They found differences in the degree of tolerance between cultivars, but little effect of bacterial strain. This conclusion is similar to that made by Gibson and Harper (1985) who examined nitrate effects on cultivars of *G. max* and strains of *Bradyrhizobium japonicum* in hydroponics, and confirmed the earlier report of McNeil (1982) that there are greater differences between hosts than strains of *Bradyrhizobium.* Thus, variation among strains of *Rhizobium* in their tolerance of the inhibitory effect of nitrate on nodulation is limited and most studies on tolerance to nitrate have focused on the response of host plant.

Bett and Herridge (1987) screened 489 diverse genotypes of soybean in pots and found four genotypes of Korean origin that displayed higher than average levels of symbiotic activity in the presence of nitrate. A subsequent comparison of two of these lines, Korean 466 and Korean 468 with the wild-type cv. Bragg, in a soil with 20 kg nitrate/ha, showed they had up to a 17-fold increase in nodule weight and 20 fold increase in N_2 fixation with comparable growth relative to cv. Bragg (Herridge and Bett 1988). Hardarson *et al.* (1984) concluded that there was a large difference between cultivars of soybeans in their ability to fix N_2 in the presence of 20 and 100 kgN/ha, as assessed by a ^{15}N technique and the AR assay. Serraj *et al.* (1992) also showed differences between soybean cultivars in the tolerance of nodulation and nitrogenase activity to nitrate. The cultivars which maintained high rates of AR activity in the presence of nitrate had low induction of nodule cytosolic nitrate reductase.

2.8.3. Variation in mutants in the sensitivity of nodulation and nitrogen fixation to nitrate

Since Gibson and Pagan (1977) suggested that tolerance to nitrate was more dependent on host plant than the *Rhizobium* strain used, mutagenesis has been used to isolate mutants whose nodulation is not affected by exogenous nitrate. Supernodulating soybean, pea and common bean genotypes which produce high numbers of nodules in the presence of high

levels of nitrate have been isolated (Jacobsen 1984, Carroll *et al.* 1985, Park and Buttery 1988).

Carroll *et al.* (1985) compared supernodulating soybeans with a wild-type (Bragg) in the presence of 5 mM nitrate. NR activities of both genotypes were found to be similar, suggesting that the affected gene in the supernodulating line is concerned with the regulation of nodule development and not with nitrate assimilation. Day *et al.* (1989) concluded that the inhibitory effect of nitrate on nodule initiation and development depended on an interaction between nitrate and an autoregulatory signal originating in the shoots. In a field evaluation of the hypernodulating mutants of soybean, Wu and Harper (1991) confirmed that they were more tolerant to added N fertiliser (Urea) but also concluded that with respect to the wild-type cultivar 'Williams' they were inferior agronomically.

Jacobsen and Feenstra (1984) selected a pea mutant which nodulated efficiently in the presence of nitrate. The mutant formed more than 250 nodules per plant in the absence or presence of nitrate (15 mM) while in wild-type (cv. Rondo), nodule number decreased from about 59 to 17 per plant. The mutants had similar nitrogenase activity (5 µmol C_2H_4/h/plant) with or without nitrate; in the wild-type however nitrate lowered activity from 2 to 0.2 µmol C_2H_4/h/plant. In a comparison between the nodulation of common bean (*Phaseolus vulgaris* L. cv. Rico) and its supernodulating mutants (RBS15), nodulation in RBS15 was two fold that of Rico in the presence of 0.5 mM nitrate and up to 15 times the amount of the Rico at 6.0 mM nitrate (Hansen *et al.* 1992a).

The degree of nitrate tolerance appears to be a stable genetic character, implying some underlying physiological or biochemical differences between nitrate tolerant and nitrate sensitive legumes. Nodulation of soybean mutants and supernodulating *Phaseolus vulgaris* was found to be tolerant of nitrate. However, contrary to the results with peas (see above), nitrogenase activity was inhibited in the both supernodulating mutants (Caetano-Anolles and Gresshoff 1991, Hansen *et al.* 1992b, c).

2.8.4. Variation in the Sensitivity of Nodulation and Nitrogen Fixation to Nitrate in Annual *Medicago* species

Nodulation and N_2 fixation in annual *Medicago* species are believed to be less tolerant to nitrate than those of most other legumes (Harper and Gibson 1984), but there is evidence for variation in the sensitivity of nodulation of the medics to nitrate (Ewing and Robson 1990).

In a preliminary study, the late Dr. J. H. Silsbury compared the sensitivity of nitrogenase activity (AR) of six species of *Medicago* to nitrate (Fig. 2.2). Swards were allowed to establish a closed canopy (40 d) and were supplied with 0, 1, 2.5 and 5 mM nitrate for a further 7 days. Clear differences were observed, with the *M. rugosa* species being more tolerant to 1 and 2.5 mM nitrate in terms of AR activity than the remaining species. *M. littoralis* cv. Harbinger was the most sensitive, with the other cultivars showing intermediate degrees of tolerance (*M. truncatula* cv. Borung > *M. polymorpha* cv. Circle Valley > *M. polymorpha* cv. Serena > *M. scutellata* cv. Commercial > *M. tornata* cv. Tornafield). At 5 mM nitrate, AR activity of the medics was inhibited to between 68 and 79% of that of control plants except in Tornafield and Harbinger where AR was inhibited by 90 and 99% respectively.

These preliminary observations, albeit deriving from different experiments, indicated a high degree of variation between annual *Medicago* species in their ability to maintain nitrogenase activity in the presence of nitrate. They provided the initial rationale for undertaking the research reported in this thesis.

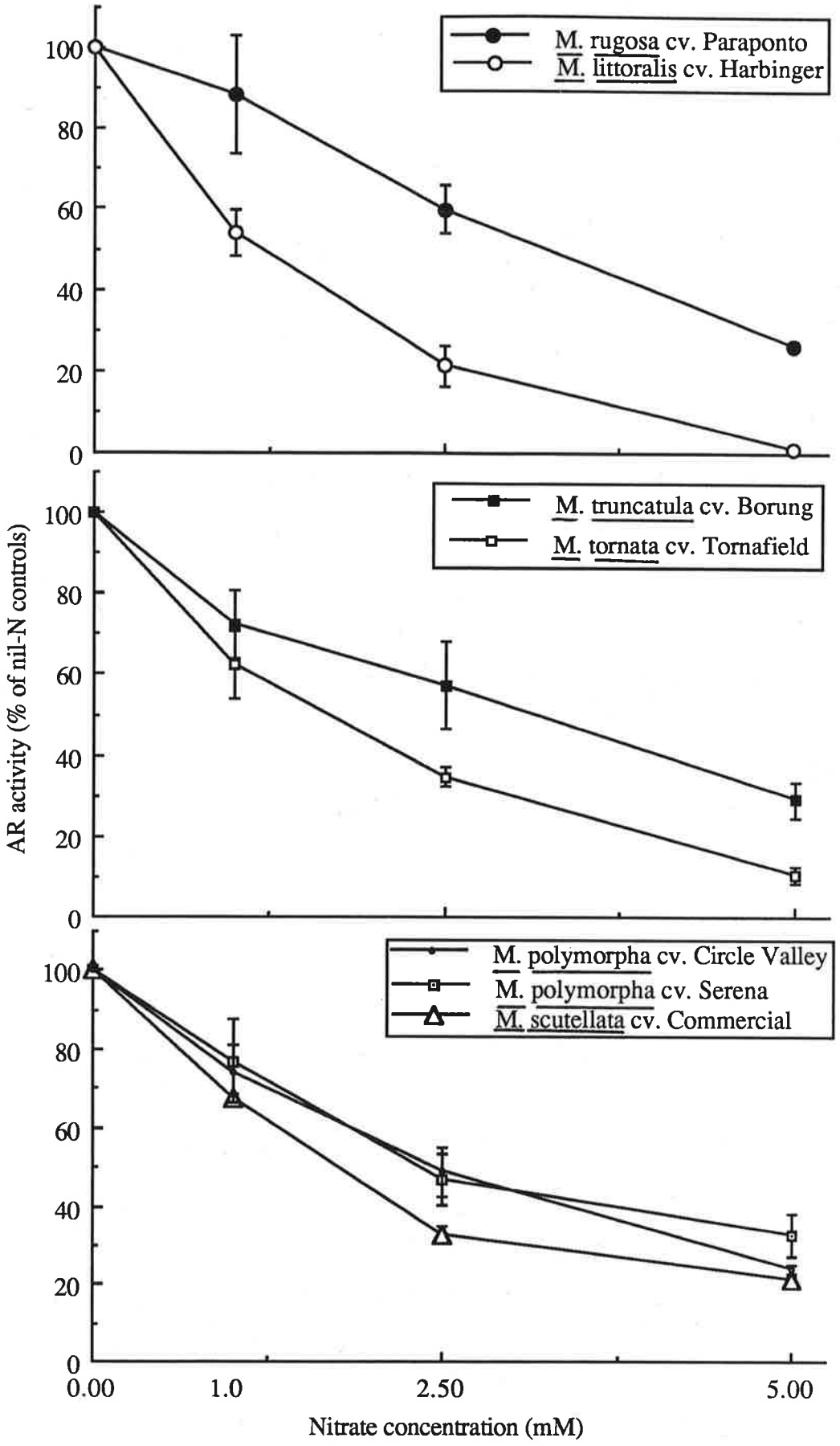
2.9. Conclusion

The above review suggests that variation does exist between legume species and legume/*Rhizobium* combinations in the sensitivity of nodulation to nitrate. The mechanism(s) by which nitrate inhibits nodulation and N_2 fixation is not yet completely understood (Streeter 1988). In the presence of high levels of nitrate however, both nodulation and N_2 fixation are sensitive to nitrate. While the basis for this variation is not

clear, the factor(s) responsible for it appear to reside in the host plant although there are a few reports of variation among strains of *Rhizobium* in their tolerance to nitrate (Senaratne *et al.* 1987). Chalifour and Nelson (1987) and Harper and Gibson (1984) have reported that tolerance of nodulation and N_2 fixation to nitrate between legume species was associated with more limited capacity for nitrate uptake which supports the view that plant factors may be responsible for the variation between legume species in the sensitivity of nodulation and N_2 fixation to nitrate.

Fig. 2.2. Influence of nitrate on nitrogenase activity in six annual medic species.

The plants were inoculated with *Rhizobium meliloti* strain CC169 and grown for 40 d without combined nitrogen and then exposed to 0.0, 1.0, 2.5 and 5.0 mM nitrate for 7d. Swards were grown at 550 ±70 μmol quanta/m^2/s and 20°C. Data were collected in separate experiments (Jan-Sep 1984). The nitrogenase activity (μmol C_2H_4/h/pot) for the control plants (0.0 mM nitrate treatment) were as follows: Paraponto, 17.2; Harbinger, 16.5; Borung, 16.5; Tornafield, 12.7; Circle Valley, 11.1; Serena, 14.6; Commercial, 28.1. Each point represents the mean of four replicates while bars indicate ±S.E.


M. rugosa cv. Paraponto
M. littoralis cv. Harbinger
M. truncatula cv. Borung
M. tornata cv. Tornafield
M. polymorpha cv. Circle Valley
M. polymorpha cv. Serena
M. scutellata cv. Commercial
AR activity (% of nil-N controls)
0
20
40
60
80
100
0.00
1.0
2.50
5.00
Nitrate concentration (mM)

CHAPTER 3

MATERIALS AND GENERAL METHODS

3.1. Plant materials

The following *Medicago* species and cultivars were used: Lucerne (*Medicago sativa* L. cv. Hunter River), Barrel medic (*Medicago truncatula* Gaertn. cvs. Caliph, Borung, Cyprus, Jemalong, Sephi, Parabinga, Paraggio and Mogul), Gamma medic (*Medicago rugosa* Desr. cvs. Paraponto, Paragosa and Sapo), Strand medic (*Medicago littoralis* Rhode. cvs. Harbinger and Harbinger AR), Burr medic (*Medicago polymorpha* L. cvs. Serena, Santiago and Circle Valley), Murex medic (*Medicago murex* cv. Zodiac), Snail medic (*Medicago scutellata* L. cv. Kelson), Disc medic (*Medicago tornata* (L.) Miller cv. Tornafield). Subterranean clover (*Trifolium subterraneum* L. cv. Woogenellup) was used in two experiments. Apart from *M. truncatula* cvs. Caliph and Mogul which were kindly supplied by J. H. Howie (South Australia Research and Development Institute, Adelaide) all other medic and subterranean clover seeds were obtained from the Waite Agricultural Research Institute, University of Adelaide. Further details on the origin of the above material and seed characteristic are given in Appendix 1.

3.2. Growth Conditions

The plants were grown in a growth room equipped with high pressure sodium 'GE Lucalox' lamps (Nela Park, Cleveland, USA) providing a photosynthetic photon flux density of 570 ±50 µmol quanta/m^2/s measured at the level of the leaf canopy (LicoR meter LI-170, Lambda Instrument Corporation, Lincoln, Nebraska). The growth room was set at 18°C and 16°C for a 12h light and 12 h dark period respectively.

3.3. Plant Culture

Seeds of medics (*Medicago* spp.) and subterranean clover (*Trifolium subterraneum*) were

graded to achieve uniform size and surface sterilised by immersing in 95% (v/v) ethanol for 10 sec followed by 0.2% (w/v) $HgCl_2$ for 3 min and then washed thoroughly with sterile water.

When grown in sand, seeds were sown in square plastic pots of 12.2 x 12.2 x 10 cm with perforated bottoms. The rooting medium was coarse, washed river sand with a water holding capacity of 11% (w/w). An excess of seed was sown and seedlings thinned at 2 d after germination to achieve the population required for each experiment.

For the hydroponic studies, seedlings were germinated as above and were removed from the sand 4 d after sowing and transferred to modified centrifuge microtubes (Micro Test Tube 3810, Eppendorf, Germany). The seedlings were held in place by sterile cotton wool and the microtubes placed in the lid of 12.2 x 12.2 x 10 cm pots that contained 1.5 L nutrient solution within a plastic bag (Plates 1 and 2). At least 2-3 cm of the roots were below the surface of the nutrient solution in the pots. Aeration rate is specified in individual experiments.

3.3.1. Inoculation

Three strains of *Rhizobium meliloti* WSM540, CC169 and WSM826 were used as inoculants. These were kindly supplied by Dr. A. H. Gibson, Division of Plant Industry, Canberra. The *Rhizobium* strains were grown either as a liquid culture in yeast mannitol broth (K_2HPO_4, 0.5 g; $MgSO_4$. $7H_2O$, 0.2 g; NaCl, 0.1 g; mannitol, 10 g; Difco yeast extract (DIFCO LABORATORIES, Detroit Michigan, USA), 0.4 g and deionised water 900 mL) or on yeast mannitol agar (above broth plus 1.66% w/v Difco agar). Incubation took place on a shaker at 25°C. The *Rhizobium* strains were harvested after 4 d. Four-day-old cultures of yeast mannitol broth or yeast mannitol agar were suspended in sterile nutrient solution and used for inoculation.

In some of the initial experiments with sand as a rooting medium, seeds were inoculated with the commercial peat inoculant for strain CC169 (Nodulaide, Group A; Agricultural

Plate 1. Experimental arrangement for study of medics in hydroponic culture.

Plate 2. Plants of *M. truncatula* cv. Borung grown hydroponically for 22 d with the *Rhizobium meliloti* strains shown.

Laboratories Pty Ltd, N.S.W., Australia) or for strain WSM540 (Nitri-life, Group A; Inoculant Service, Victoria, Australia).

3.3.2. Nutrient Solution

N-free nutrient solution for hydroponic culture contained: $MgSO_4.7H_2O$ (1 mM); KH_2PO_4 (0.25 mM); H_3BO_3 (46.2 μM); $MnCl_2.4H_2O$ (9.14 μM); $ZnSO_4.7\ H_2O$ (0.76 μM); $Na_2MoO_4.2H_2O$ (0.5 μM); $CuSO_4.2H_2O$ (0.32 μM); ethylenediaminetetraacetic acid (EDTA) (0.78 mM); $FeSO_4.7H_2O$ (0.71 mM); $CaSO_4.2H_2O$ (2.50 mM) and K_2SO_4 (1.25 mM). When KNO_3 and $Ca\ (NO_3)_2.4H_2O$, were added, the level of K_2SO_4 and $CaSO_4.2H_2O$ were adjusted to maintain the same balance of K^+ and Ca^{2+}. Full details on the stock solution of nutrients used plus the composition of nutrient solution for sand culture are given in Appendix 2. The pH of the nutrient solution was adjusted to 6.5 with 1 M potassium hydroxide.

3.4. Determination of Total Nitrogen in Plant Tissues

To estimate the total N in plant materials grown without nitrate, one Kjeldahl Catalyst Tablet (each tablet contained 1.0 g Na_2SO_4, and the equivalent of 0.01 g of selenium, Ajax Chemicals, N.S.W. Australia), and 7.5 mL of concentrated H_2SO_4 were added to a 250 mg dry plant sample in a 50 mL test tube and digested for 1 h on an aluminium heating block pre-heated to 380°C. The digest was diluted with 25 mL distilled water, and the ammonia in the digest was determined with the KJELTEC Auto 1030 Analyser (Hoganas, Sweden).

Estimation of the total nitrogen in plant tissues containing nitrate by the conventional Kjeldahl method has been found to be unsuitable for total N determination (Silvertooth and Westerman 1988). Under such conditions nitrate is converted to ammonia by pretreating the samples with 3% (w/v) salicylic acid-H_2SO_4 and sodium thiosulphate (Eastin 1978, Bergersen 1980). The nitro compounds formed by the reaction of salicylic acid with nitrate in an acid medium are then reduced to corresponding amino compounds by heating the mixture with sodium thiosulphate.

The procedure for the reduction of nitrate to ammonia was as follows: 250 mg plant material, a Kjeldahl Catalyst Tablet (low selenium), 7.5 mL of salicylic acid-H_2SO_4 mixture (20 g of salicylic acid per 600 mL of concentrated H_2SO_4) and 0.75 g of sodium thiosulfate were placed into a 50 mL digestion tube and left for 1h to ensure the completion of the reaction. The samples were digested as for samples without nitrate. Recovery of added nitrate by this method ranged from 96.4-99.9% for shoot and root tissues respectively (Table 3.1).

3.5. Nitrate Measurement

Nitrate was determined in tissue samples by enzymatic reduction (*E. coli* nitrate reductase) of nitrate to nitrite and the measurement of the latter with a colorimetric reagent (McNamara *et al.* 1971). A direct absorbance procedure (A_{210nm})was used to measure nitrate in solution.

3.5.1. Preparation of tissue extracts

Plant samples were dried at 85°C for 24 h and ground to pass a 40 mesh screen. 60 mg of ground tissue was suspended in 40 ml boiling water and mixed. After cooling (approximately 30 min), the extract was filtered through Whatman No.1 filter paper.

3.5.1.1. Assay procedure

The assay mixture contained 0.5 ml of 0.1 mM K phosphate buffer (pH 7.5), 0.5 mL of 0.4 M sodium formate, *E. coli* extract (usually 0.1 mL), 0.1 mL plant extract containing less than 100 nmol nitrate and H_2O in a final volume of 2 mL. After incubation at 45°C for 4 h the reaction was stopped by the addition of 1 mL of 1% sulphanilamide in 1.5 N HCl and rapidly mixed with 1 mL of 0.01% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride. After 10 min, 2 mL water was added and the mixture centrifuged for 15 min. The absorbance of the pink diazo dye compound in the supernatant was determined at 540 nm and the reading converted to amounts of nitrate using the calibration graph shown in Fig. 3.1A.

Table 3.1. Recovery of nitrate from plant tissues in total nitrogen assay.

250 mg dry shoot and root samples of *M. littoralis* cv. Harbinger were used. 3.8 mg N as KNO_3, 7.5 mL of 3% (w/v) salicylic acid-H_2SO_4 and 750 mg sodium thiosulphate were added into each digestion tube. The samples were digested in an aluminium block at 380°C for 1 h. After the addition of 25 mL H_2O, the total nitrogen was determined by Auto Analyser. Values are averages of 4 samples.

Sample	Total N before nitrate addition (mg)	N as nitrate added (mg)	Total N after nitrate addition (mg)	% recovery
Shoot	7.81	3.8	11.2	96.4
Root	8.40	3.8	12.2	99.9

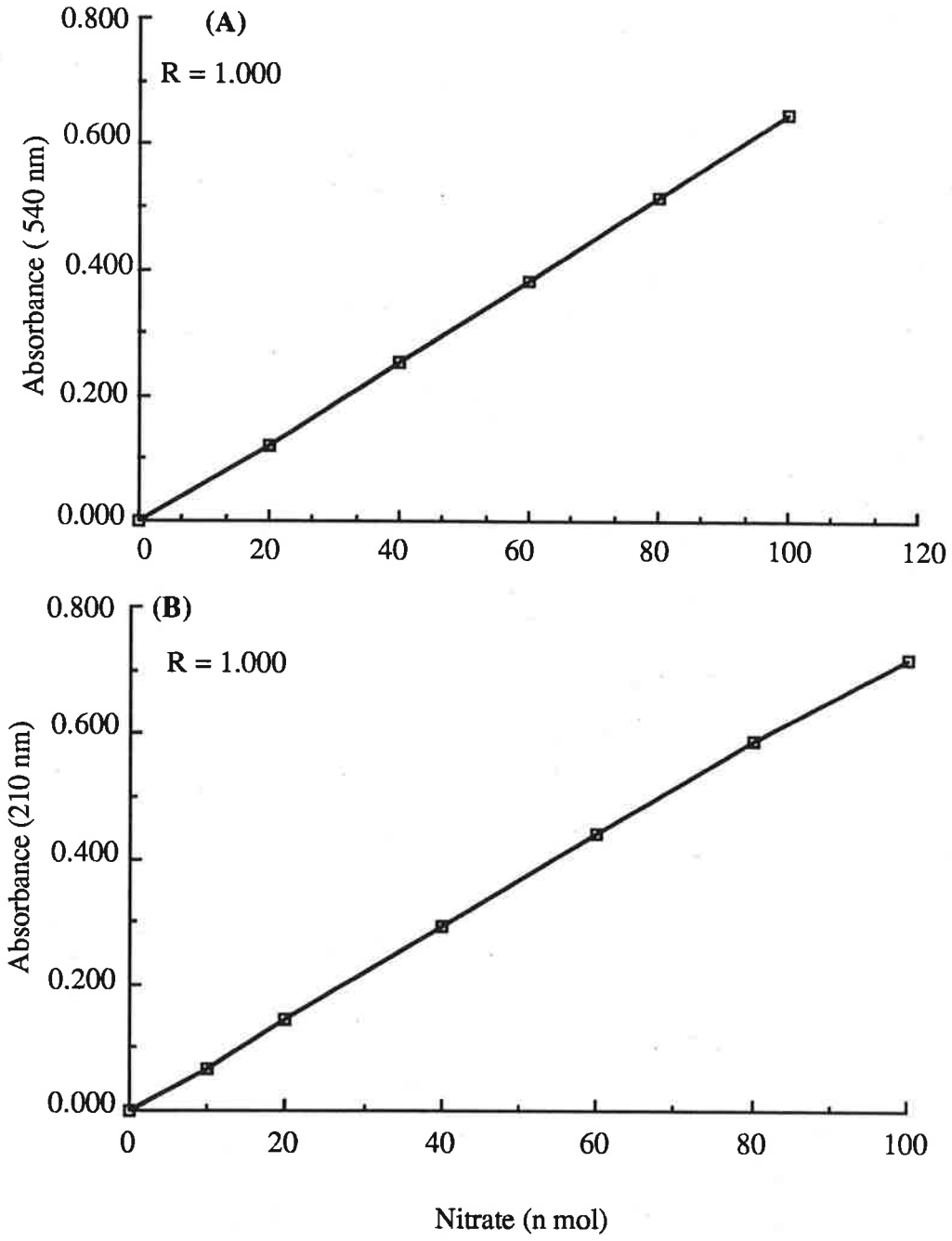


Fig. 3.1. Calibration graph for estimation of nitrate by *E. coli* nitrate reductase procedure (A) and for the estimation of nitrate in nutrient solution by ultraviolet spectrophotometry method (B).

In a test on 0.5 mM nitrate added to a medic shoot and root sample, the recovery was 99.7 and 95.0% respectively.

3.5.1.2. Culture of *E. coli* and preparation of nitrate reductase sample

The growth medium for *E. coli* contained the following compounds (g/L): Bactopeptone, 10; Beef Extract, 1; Yeast Extract, 3; Casein Hydrolysate, 3; K_2HPO_4, 1; KNO_3, 1. These were autoclaved at 120°C for 20 min. A 50% (w/v) solution of glucose was autoclaved separately and added to give a final sugar content of 2% (w/v).

A 500 mL starter-culture was inoculated from an *E. coli* slope, flushed with N_2 and grown at 37°C on a flat-bed shaker for approximately 8-12 hours. This was then transferred to 8 L of medium in a 10 L bottle which was maintained under anaerobic conditions with a trickle of N_2 and the culture grown at 37°C with gentle stirring. The pH of the culture was maintained at 7 by using a pH stat unit with 1L of 2.5N KOH in the reservoir (Radiometer Prop. Band 0.1). The growth of the culture was followed by monitoring its absorbance at 660 nm and harvested when the stationary phase of growth was attained, approximately 12 hours. The cells were harvested at 4°C using the Sorvall continuous flow system (20000_g) and flow rate adjusted to 150 mL/min. Approximately 50 g moist weight of cells were obtained.

The cells were transferred to a 250 mL centrifuge bottle and washed twice with cold 1% (w/v) NaCl. After each wash they were centrifuged at 3300_g for 10 min and re-suspended by glass rod and magnetic stirrer with the centrifuge bottle maintained on ice. The cells were re-suspended in 4 mL/g moist weight of cold 0.1 M K-phosphate pH 7.5 and disrupted by passing them twice through a French Pressure Cell (Aminco Instrument Co., Maryland, USA) operating at 18000 psi, and then centrifuged at 3300_g for 20 min. The supernatant was re-centrifuged at 36900_g for 1 h. The resulting pellet was re-suspended in 2 mL/g original moist weight of cold 0.1 M K-phosphate, pH 7.5, using a glass homogeniser.

Aliquots of the extract were stored under N_2 in sealed containers at 0-4°C. Under these

conditions good enzyme activity was maintained for up to 12 months.

3.5.2. Estimation of Nitrate in Nutrient Solution

Ultraviolet spectrophotometry (Cawse 1967) was used to estimate the concentration of nitrate in nutrient solution. One mL of nutrient solution containing nitrate was diluted to 10 mL by nutrient solution containing no nitrate. The absorbance of the samples was measured on a spectrophotometer at 210 nm. Nitrate content of the samples was estimated using the calibration graph (Fig. 3.1B).

Nitrate uptake of the plants was calculated as follows:

$$\text{nitrate uptake} = V_1C_1 - V_2C_2$$

where V_1 and V_2 are the initial and final volumes of nutrient solution and C_1 and C_2 are the initial and final nitrate concentration of nutrient solution respectively. The volume of nutrient solution was calculated by measuring the weight of the solution.

3.6. Acetylene Reduction (AR) Assay of Nitrogenase Activity

3.6.1. The AR assay procedure used

The closed system used in the present study was that of Herdina and Silsbury (1990a). Plants were removed from the pots at 9.00 h (4 h after the start of the light period) and excess solution blotted from the nodulated roots. The plants were sealed in a glass jar (details given in individual experiments) equipped with a screw-type lid penetrated by a suba-seal. Acetylene was injected into the jar, with a hypodermic syringe to achieve a concentration of 10% (v/v) acetylene. An extra needle stuck through the suba-seal allowed excess air to escape. Triplicate samples of gas were removed after incubation for 10 and 40 min and injected into a gas chromatograph (Varian, Model 3400) equipped with a flame ionisation detector and a column of Porapak N, 80-100 mesh range. The temperatures of the column, detector and injector were 55°C, 50°C and 150°C respectively. Nitrogen was used as a carrier gas.

Two standards of known concentration of C_2H_4 were made up in 10% (v/v) C_2H_2 in air at 20°C and analysed to calibrate the chromatograph. The chromatograph output for the unknown gas samples was compared with that of the standard to estimate ethylene concentration in the samples. Ethylene production was calculated as follows:

$$\mu\text{mol } C_2H_4/h/\text{plant} = \frac{PsVs}{PcVc} \times C \times \frac{273}{T\,(22.414)}$$

where:

Ps and Pc are the reading of ethylene peak of gas sample and pure ethylene control respectively. Vs and Vc are the gas volumes in the sample and control jars respectively, C is the concentration of pure ethylene, T is the incubation temperature (20°C) and 22.414 the volume occupied by one µmol of gas at 0°C.

3.6.2. Evaluation of the AR assay procedure used

The closed system of AR assay was criticised by Minchin *et al.* (1983) because cumulative ethylene production underestimated actual rates and produced low apparent values for nitrogenase activity (see Section 2.4.6). The critical consideration in the application of the AR assay in a closed system is the acetylene induced decline in nitrogenase activity. To verify this, a study on *Medicago rugosa* cv. Sapo and *M. truncatula* cv. Parabinga, grown hydroponically for 14 days after inoculation, was undertaken (Fig. 3.2). Ten plants of each cultivar were placed separately in a glass jar of 500 mL capacity. The plants were assayed for ethylene production after incubation in 10% acetylene at 5 min intervals for a total of 60 min. The rate of ethylene production was linear with time, and there was no evidence of a decline in the rate of ethylene production in the closed system.

Disturbance of the plants is also claimed to affect nitrogenase activity (Minchin *et al.* 1986). This was investigated by assaying undisturbed and disturbed plants. Small swards of *M. rugosa* cv. Paraponto were grown under controlled conditions without mineral nitrogen. At

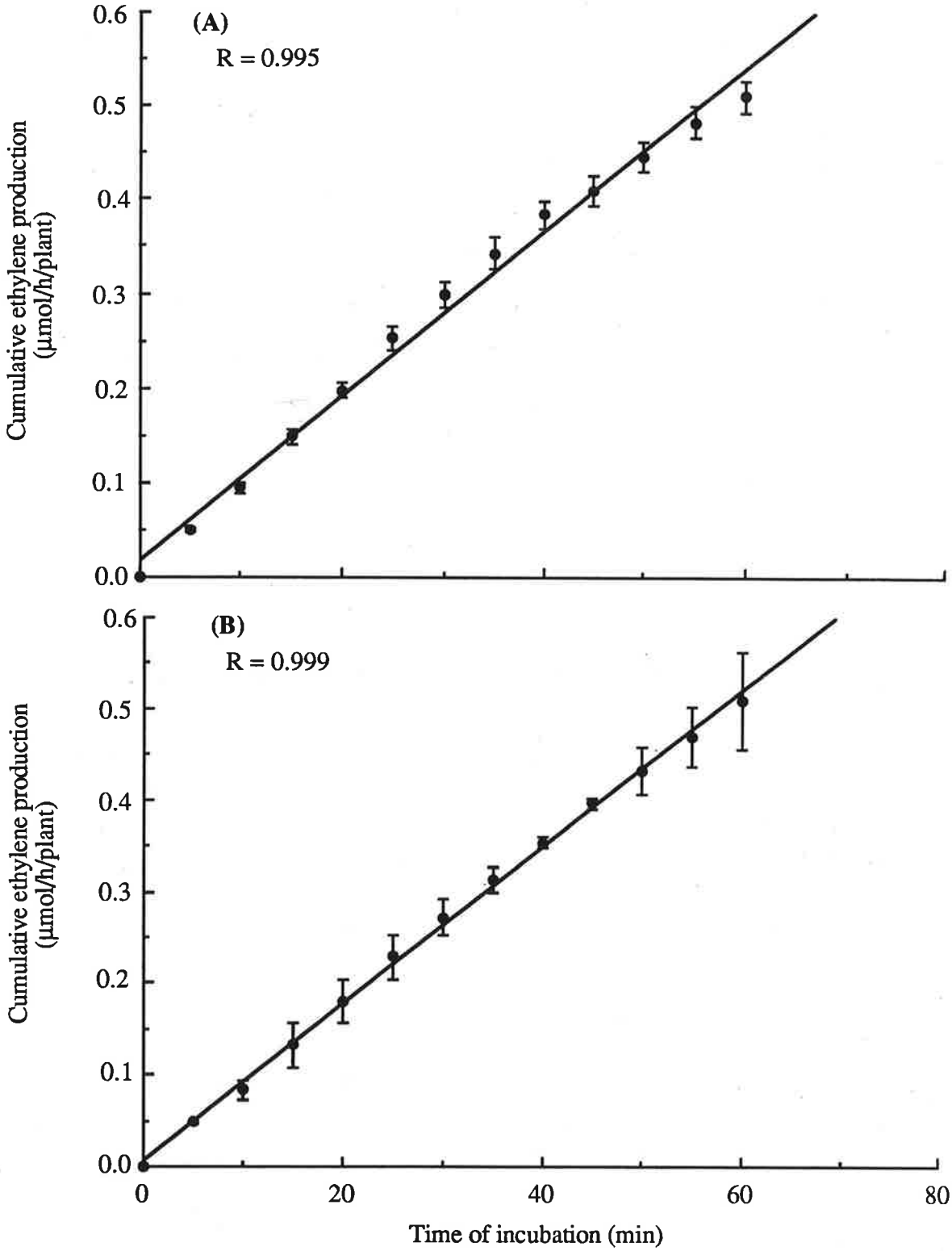


Fig. 3.2. Relationship between cumulative ethylene production and time of incubation.

M. rugosa cv. Sapo **(A)** and *M. truncatula* cv. Parabinga **(B)** assayed in a closed system. Each point represents the mean of four replicates while bars indicate ±S.E.

45 d after inoculation, the plants were divided into two groups of 5 pots and assayed. One group was assayed as described earlier. Samples of air from the jar were removed after 10 and 40 min incubation for estimation of ethylene production. The second group was assayed as undisturbed plants in a 4.0 L modified sealed pressure cooker. 10% of the air in the cooker was replaced with acetylene and gas samples withdrawn for injection into a gas chromatograph after 10 and 40 min. Standards of known C_2H_4 concentration were made up in 10% C_2H_2 in air at 20°C for both methods. Results showed that nitrogenase activity (µmol C_2H_4/h/pot) of undisturbed plants (12.62±0.34) was similar to that of disturbed plants (11.98±0.64).

It was concluded that the closed system could be used in comparative estimates on nitrogenase activity.

3.7. Nitrate Reductase Assay of Plant Tissue

Nitrate reduction in plant tissue was determined colorimetrically from the rate of nitrite production. Shoot and root samples were extracted immediately after harvest with chilled extraction medium (4°C). The extraction medium contained 0.2 M Tris-HCl, pH 8.5 and 0.05 M K-phosphate, pH 8.5, containing 5 mM EDTA, casein (Hammarsten, BDH Chemicals Ltd Pool, England) 1% (w/v) and 1 mM cysteine (Wallace 1986). Samples cut finely with scissors were macerated in a chilled mortar with a pestle in 3 volumes of extraction medium per 1 g fresh weight of sample. The extracts were centrifuged at 15000g for 10 min at 4°C.

3.7.1. Assay procedure

The incubation mixture was 0.5 mL of 0.1 mM K-phosphate pH 7.5, 0.1 mL 0.05 M KNO_3, 0.3 mL H_2O, 0.1 mL NADH (1.5 mg/mL in 25 mM K-phosphate pH 7.5) and 0.05 or 0.1 mL plant extract which gave a total volume of 1 mL. Incubation was for 30 min at 25°C. The reaction was stopped and excess NADH oxidised by adding 0.1 mL of a mixture of 0.1 mM phenazine methosulfate (PMS) and 4 mM potassium ferricyanide. 1 mL of 1%

sulphanilamide in 1.5 M HCl and 1 mL 0.02 (w/v) N-(1-naphtyl) ethylenediamine dihydrochloride were added to determine nitrite. The samples were centrifuged at 3500g on a bench centrifuge for 15 min. Nitrite concentration was determined by measuring the absorbance at 540 nm for each sample.

3.8. Dry Weight and Relative Growth Rate

Root, nodule and shoot samples were dried separately in a forced draught oven at 80°C to a constant weight for dry matter determinations. Relative growth rate (RGR) was calculated from the formula : $RGR = (\ln W_2 - \ln W_1)/(T_2 - T_1)$ where W_1 and W_2 are total plant dry weight at T_2 and T_1 respectively.

3.9. Statistical Analysis

All data were subjected to analysis of variance. Differences among treatment means were separated by the least significant differences (LSD) test (Steel and Torrie 1960).

CHAPTER 4

SENSITIVITY OF NITROGENASE ACTIVITY TO NITRATE IN ANNUAL *MEDICAGO* SPECIES

4.1. Introduction

In a preliminary study by Silsbury (see Section 2.8.4) variation in the sensitivity of nitrogenase activity (AR assay) to nitrate in annual *Medicago* species was observed. The nitrogenase activity of *M. rugosa* cv. Paraponto was two and three times more tolerant to the application of 1 and 2.5 mM nitrate than *M. littoralis* cv. Hårbinger. Other annual medics examined had intermediate degrees of tolerance to nitrate. The two main experiments in this chapter were undertaken to verify or otherwise refute the preliminary study referred to above. In well nodulated plants of four annual medics examined however only a low variability was detected in the sensitivity of the nitrogenase to nitrate. Therefore the final experiment included the perennial *Medicago sativa* and *Trifolium subterraneum*, which have been reported to have marked differences in the tolerance to nitrate (Harper and Gibson 1984).

4.2. EXPERIMENT 1: Variation in the sensitivity of nitrogenase activity to nitrate in *M. littoralis* cv. Harbinger and *M. rugosa* cv. Paraponto.

4.2.1. Materials and Methods

Medicago littoralis cv. Harbinger and *Medicago rugosa* cv. Paraponto were inoculated with *Rhizobium meliloti* strain WSM540 as described in Section 3.3.1 and grown in sand in a growth room as described in Section 3.2. After emergence the plants were thinned to 49 per pot, and the pots flushed with minus nitrate nutrient solution every day. Plants in each pot were raised as a sward and the canopy restrained to the surface area of the pot by a wire mesh (Plate 3). At 42 d after emergence, five nitrate treatments (0, 1, 2.5, 5 and 10 mM)

Plate 3. Plants of *M. rugosa* cv. Paraponto grown in sand for 35 d. Wire mesh was used to confine the canopy to the surface area of the pot. This type of well-nodulated material was used in the experiments described in Chapter 4.

were introduced. Assays for nitrogenase activity (AR) were made at 2, 4 and 6 d after nitrate application as described in Section 3.6.1. At each assay, plants were removed from the pots by gently washing away the sand with water. The whole plants were then placed in 1.08 L jars and sealed for measurement of AR activity. Shoot and root nitrate concentrations and dry weight were determined as described in Sections 3.5. and 3.8. respectively. The experiment was a split-split plot design with the two medics as whole plots, five concentrations of nitrate as sub plots and three treatment periods as sub-sub plots. All treatments were replicated four times. Analysis of variance was performed on AR, shoot nitrate and root nitrate data. Because the assumptions of the analysis of variance were not met (ie. constant variance), transformation of the data was necessary. A logarithmic transformation was applied, which in the case of AR was $\log_e$ (AR +1). Shoot and root nitrate measurements responded well to a square root transformation, thus $\sqrt{\text{root } NO_3^- + 0.5}$ and $\sqrt{\text{shoot } NO_3^- + 0.5}$ were used.

4.2.2. Results

When analysed at 2, 4 and 6 d after nitrate application, the average total dry weight values of the plants were as follows: *M. littoralis* cv. Harbinger (g/pot ±S.E) 4.27±0.87, 4.63±0.81 and 5.34±0.10; *M. rugosa* cv. Paraponto 5.15±0.13, 5.75±0.14 and 6.65±0.12 respectively. There was no significant alteration in dry weight in response to nitrate treatments. Nitrogenase activity in both medics was inhibited by all nitrate concentrations (Fig. 4.1). The inhibition was proportional to nitrate concentration and exposure time. By 4 d, nitrogenase activity had been completely inhibited by 5 and 10 mM nitrate in Harbinger, while it persisted at a low level in Paraponto. It had been completely inhibited by 5 and 10 mM in both the cultivars by 6 d.

The nitrate concentration in the plant tissues (Table 4.1) was proportional to the external nitrate concentration and period of application and was higher in the root than shoot of both species. In the plants supplied with nutrient solution to which no nitrate was added, low levels of nitrate (3-9 µmol/g dry weight) were detected. This was due to nitrate in the rain

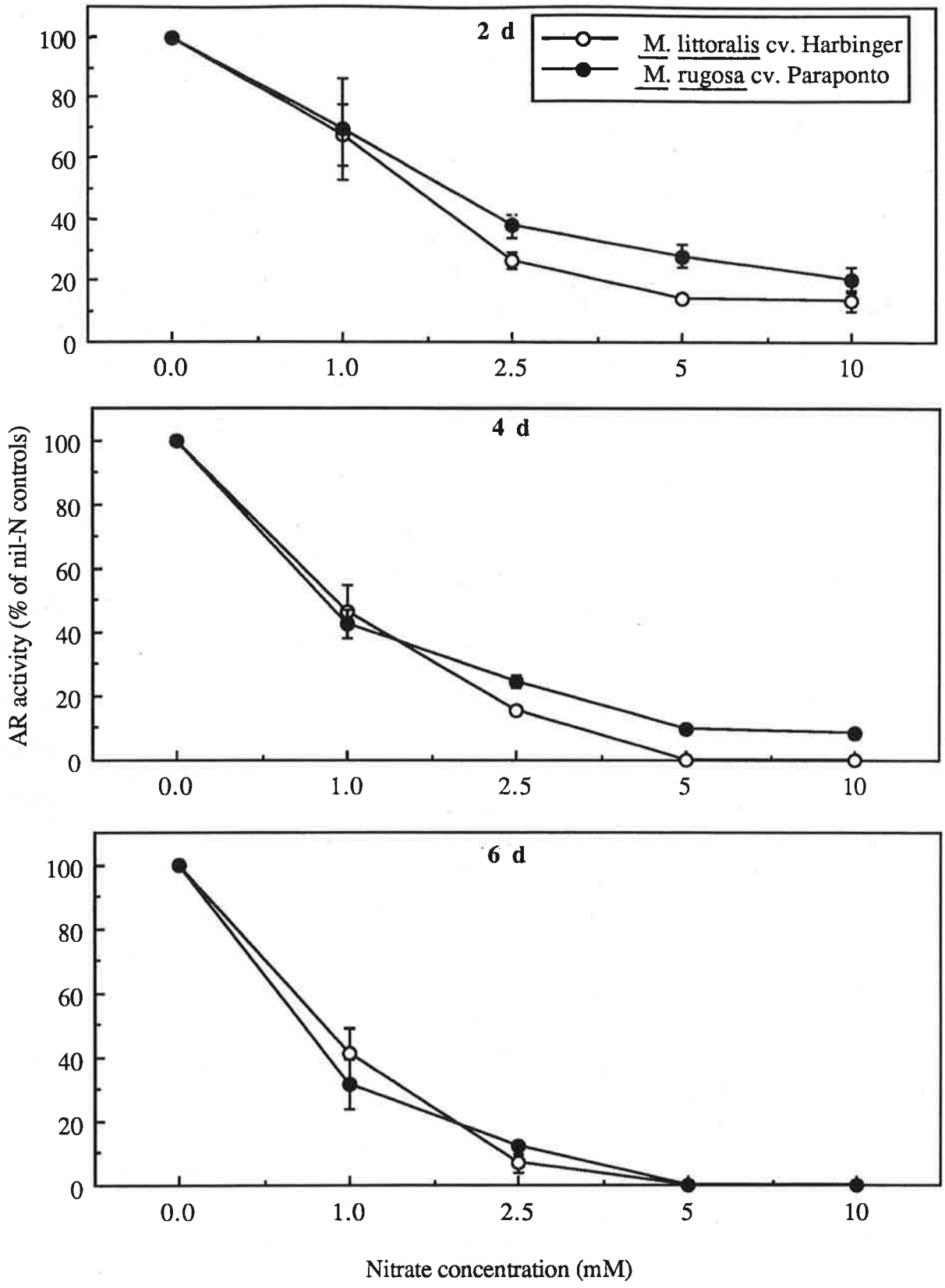

2 d
4 d
6 d
M. littoralis cv. Harbinger
M. rugosa cv. Paraponto
AR activity (% of nil-N controls)
Nitrate concentration (mM)
0
20
40
60
80
100
0.0
1.0
2.5
5
10

Table 4.1. Nitrate concentration in the shoots and roots of *M. littoralis* cv. Harbinger and *M. rugosa* cv. Paraponto.

Plants were grown and supplied with nitrate as indicated in Fig 4.1. Data are expressed as means of four replications with mean of transformed data (see Section 4.2.1) in parentheses.

Nitrate treatment (mM)	Shoot nitrate (µmol/g dry weight)			Root nitrate (µmol/g dry weight)		
	2 d	4 d	6 d	2 d	4 d	6 d
			Harbinger			
0	9 (3.0)	7 (2.7)	8 (3.0)	4 (2.1)	4 (2.1)	5 (2.5)
1	92 (9.6)	107 (10.4)	142 (11.9)	125 (11.2)	137 (11.9)	167 (12.9)
2.5	108 (10.4)	237 (15.4)	327 (18.1)	196 (14.0)	261 (16.2)	314 (17.7)
5	178 (13.3)	279 (16.7)	343 (18.5)	354 (18.8)	538 (23.2)	663 (25.7)
10	200 (14.1)	339 (18.4)	414 (20.4)	449 (21.1)	649 (25.5)	761 (27.6)
			Paraponto			
0	3 (1.8)	3 (1.9)	3 (1.9)	5 (2.4)	6 (2.6)	6 (2.6)
1	83 (9.1)	106 (10.3)	231 (15.2)	150 (12.2)	191 (13.8)	238 (15.4)
2.5	151 (12.3)	232 (15.2)	360 (19.0)	242 (15.6)	299 (17.3)	393 (19.8)
5	153 (12.4)	256 (16.0)	370 (19.2)	393 (19.8)	405 (20.1)	443 (21.0)
10	156 (12.5)	263 (16.2)	429 (20.7)	418 (20.5)	446 (20.9)	468 (21.6)
LSD[a] 5%		(1.14)			(0.30)	
1%		(1.53)			(0.39)	

[a]LSD based on transformed data for medic x day x nitrate interactions.

water (0.007 mM) used for preparation of the nutrient solution. Silsbury *et al.* (1986) also found low levels of nitrate (1-2 µmol/g fresh weight) in subterranean clover not supplied with nitrate. The rain water used was from an open dam at the Waite Agricultural Research Institute. In subsequent experiments all nutrient solutions were prepared with Reverse Osmosis treated water.

There was a good inverse relationship between nitrate concentration of the root and shoot with nitrogenase activity in both *M. rugosa* cv. Sapo and *M. littoralis* cv. Harbinger (Fig. 4.2). In both species, the higher the concentration of nitrate in plant tissue, the higher the inhibition of nitrogenase activity.

4.3. EXPERIMENT 2: Variation in the sensitivity of nitrogenase activity to nitrate in *M. littoralis* cv. Harbinger, *M. truncatula* cv. Borung and *M. tornata* cv. Tornafield

In Experiment 1 the original Silsbury observation that nitrogenase activity in *M. rugosa* cv. Paraponto was more tolerant to nitrate than in *M. littoralis* cv. Harbinger was not confirmed. In this experiment Harbinger was compared with two other medic species, *M. truncatula* cv. Borung a tolerant species and *M. tornata* cv. Tornafield a sensitive species in the original Silsbury study (Fig. 2.2). The *Rhizobium meliloti* strain CC169 was used as in the Silsbury study.

4.3.1. Materials and Methods

M. littoralis cv. Harbinger, *M. truncatula* cv. Borung and *M. tornata* cv. Tornafield were inoculated with the *Rhizobium meliloti* strain CC169 and grown with minus nitrate nutrient solution in sand for 37 d. Only one harvest, 4 d after application of 1, 2.5, and 5 mM nitrate was made. All other procedures were the same as described in Experiment 1 (Section 4.2.1), except that the plants were grown for 20 d in a glasshouse under natural irradiance (July 1991 and mean daily minimum and maximum temperatures ranging from 10.2 and

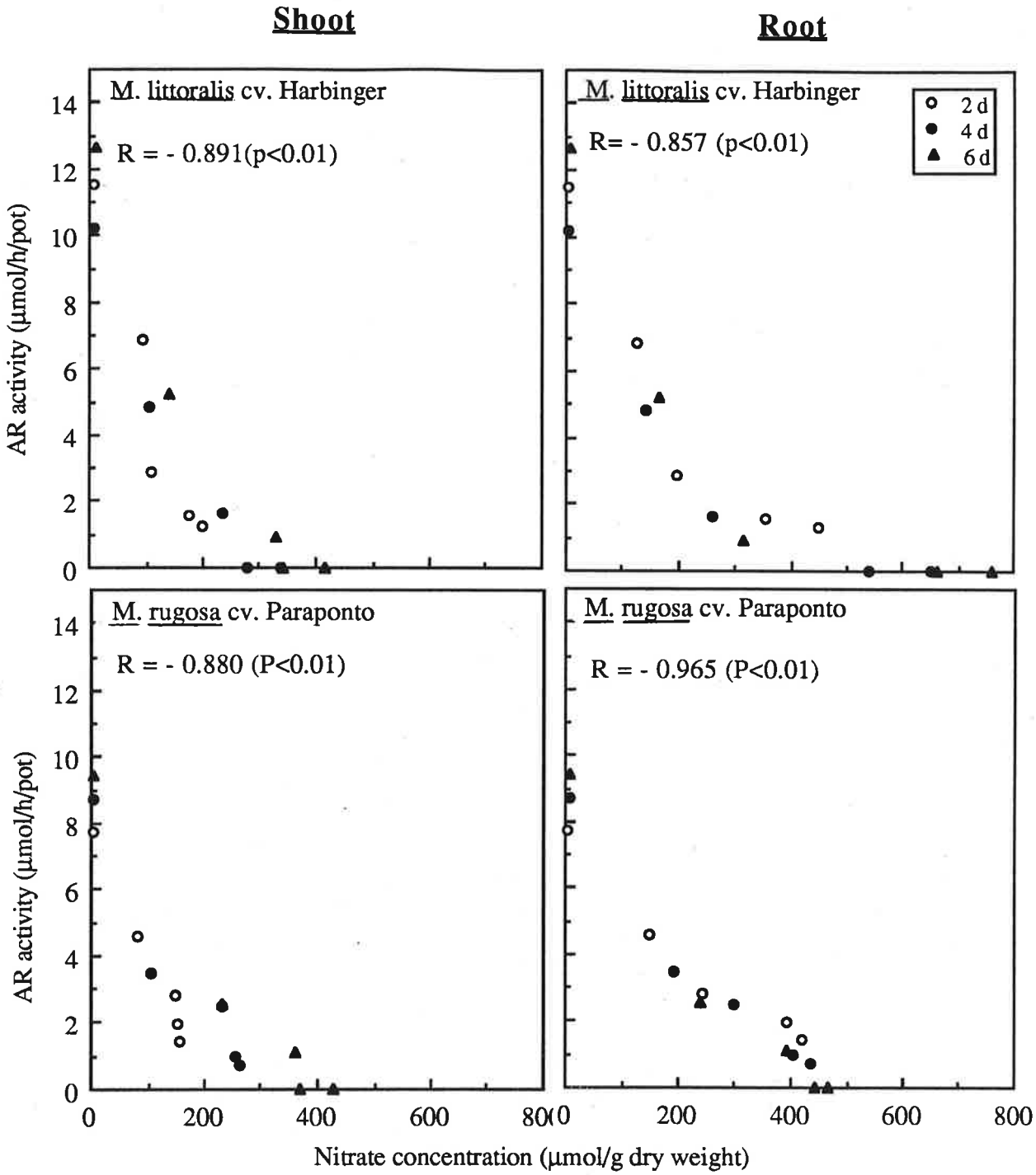


Fig. 4.2. Relationship between nitrogenase activity and nitrate concentration in medic tissues.

Plants were grown and supplied with nitrate as indicated in Fig. 4.1.

18.6°C) before being transferred to the growth room. A factorial experiment using a completely randomised block design with four replications was used.

4.3.2. Results

When harvested at 41 d, the total dry weights (g/pot ±S.E) of *M. truncatula* cv. Borung (6.85±0.15) and *M. tornata* cv. Tornafield (6.92±0.12) were almost twice that of *M. littoralis* cv. Harbinger (4.03±0.14). The 4 d nitrate treatment did not result in any significant increase in dry weight over the nil controls. The nitrogenase activities per pot (see legend to Fig 4.3) were fairly similar but Harbinger (4.9 μmol/h/g dry weight) had more than double the AR activity per g dry weight than the other two medics (2.7 for Borung and 2.1 for Tornafield). At 1 mM nitrate, nitrogenase activity of Harbinger was unaffected, but that of Borung and Tornafield were inhibited by 21 and 29% respectively of that of control plants (Fig. 4.3). At 2.5 mM nitrate, inhibition of nitrogenase activity in all species was 38-49% of that of the control. With 5 mM nitrate, nitrogenase activity in all species was inhibited to about 76% of that of the control plants.

4.4. EXPERIMENT 3: Variation in the sensitivity of nitrogenase activity to nitrate in *M. littoralis, M. sativa* and *Trifolium subterraneum.*

In Experiment 1 and 2 no major species difference was found in the tolerance of nitrogenase activity in well nodulated annual medics to nitrate. This experiment was undertaken to see how the sensitivity of the annual medic, *M. littoralis* cv. Harbinger compared with the perennial medic, *M. sativa* cv. Hunter River and subterranean clover, *Trifolium subterraneum* cv. Woogenellup. The latter was shown to be more tolerant to nitrate than a cultivar of *M. truncatula* by Harper and Gibson (1984), albeit in an experimental system different from that used here.

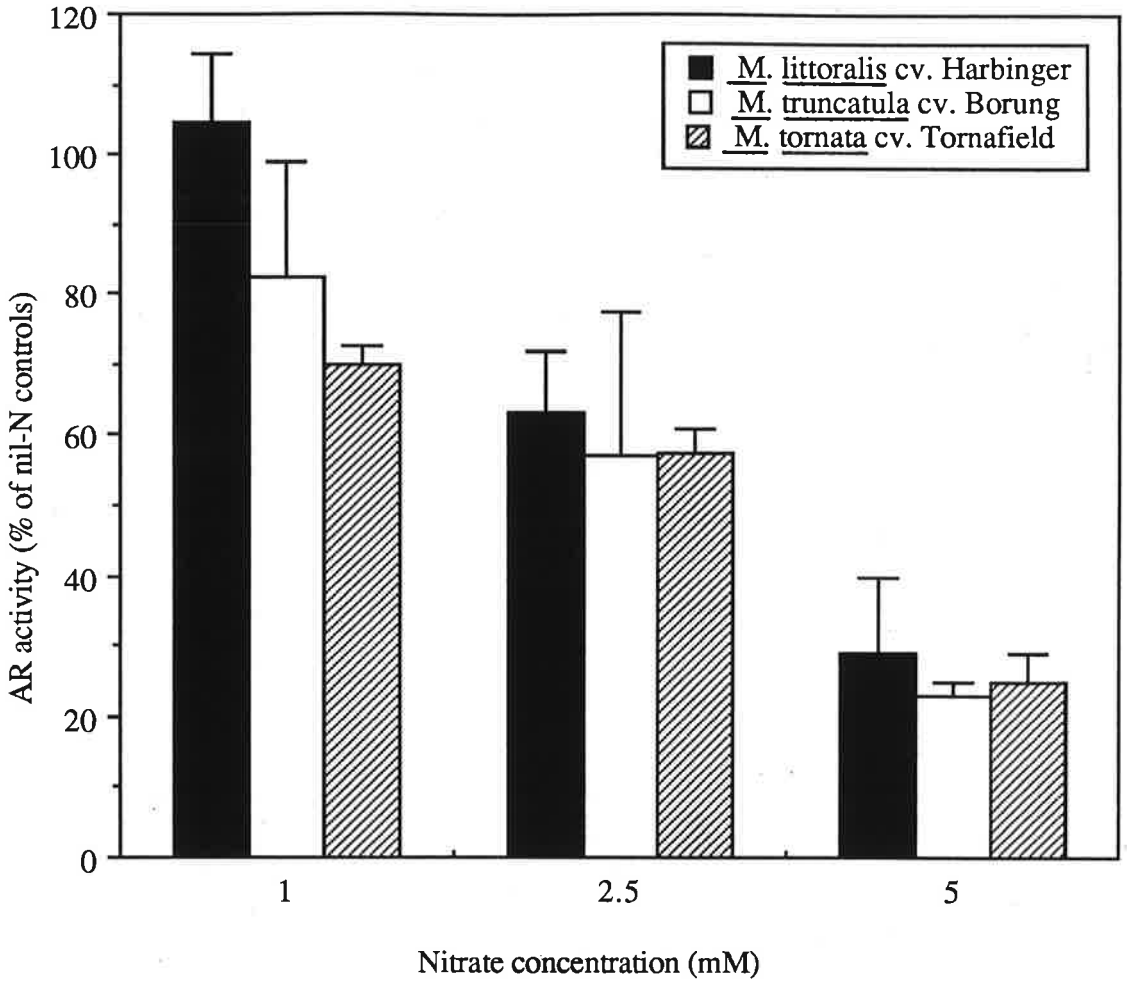


Fig. 4.3. Influence of nitrate supply on nitrogenase activity in medics.

M. littoralis cv. Harbinger, *M. truncatula* cv. Borung and *M. tornata* cv. Tornafield were inoculated with *R. meliloti* strain CC169, grown 37 d without combined nitrogen and exposed to four levels of nitrate for a further 4 d. The AR activity (µmol/h/pot) for the plants supplied with the minus nitrate nutrient solution were as follows: *M. littoralis*, 19.8; *M. truncatula*, 18.2 and *M. tornata* 14.3. Values represent the mean of four replicates while the bars indicate ±S.E.

4.4.1. Materials and Methods

Medicago littoralis cv. Harbinger, *Medicago sativa.* cv Hunter River and *Trifolium subterraneum* cv. Woogenellup plants were grown as described in Experiment 1 (Section 4.2.1). Harbinger and Hunter River were inoculated with *Rhizobium meliloti* strain CC169 while Woogenellup was inoculated with *Rhizobium trifolii* strain WU95. Swards were grown for 40 d without combined N and supplied with 0, 1 and 2.5 mM nitrate for 4 d. AR activity of the swards was determined as described in Section 3.6.1. The experimental design and analysis were the same as for Experiment 2 (Section 4.3.1), except that six replications were used.

4.4.2. Results

The average values for total dry weight of the plants at 4 d of nitrate treatment with 0, 1 and 2.5 mM nitrate were (g/pot ±S.E): *M. littoralis* cv. Harbinger, 6.27±0.07; *M. sativa* cv. Hunter River, 5.47±0.09 and *T. subterraneum* cv. Woogenellup, 7.94±0.10. The nitrogenase activity (μmol C_2H_4/h/pot) values for the minus nitrate treatment are given in the legend to Fig. 4.4. When the nitrogenase activity is expressed as C_2H_4/h/g dry weight, the values for Harbinger, Hunter River and Woogenellup were 1.0, 5.1 and 1.8 respectively.

When 1 mM nitrate was supplied to swards of nodulated plants of Harbinger, Hunter River and Woogenellup, nitrogenase activity was inhibited by 15-21% in all cultivars (Fig. 4.4); at 2.5 mM nitrate, the inhibition was 42-54%. *M. sativa* had a significantly lower nitrate concentration than the other species, in both shoot and root, irrespective of the level nitrate supplied (Table 4.2). The nitrate concentrations in both shoot and root of all species was higher at 2.5 mM nitrate than 1 mM nitrate treatment.

4.5. Discussion

In the experiments described in this Chapter where nitrate was supplied to well nodulated plants no major variation in the sensitivity of the nitrogenase activity (AR) was detected in

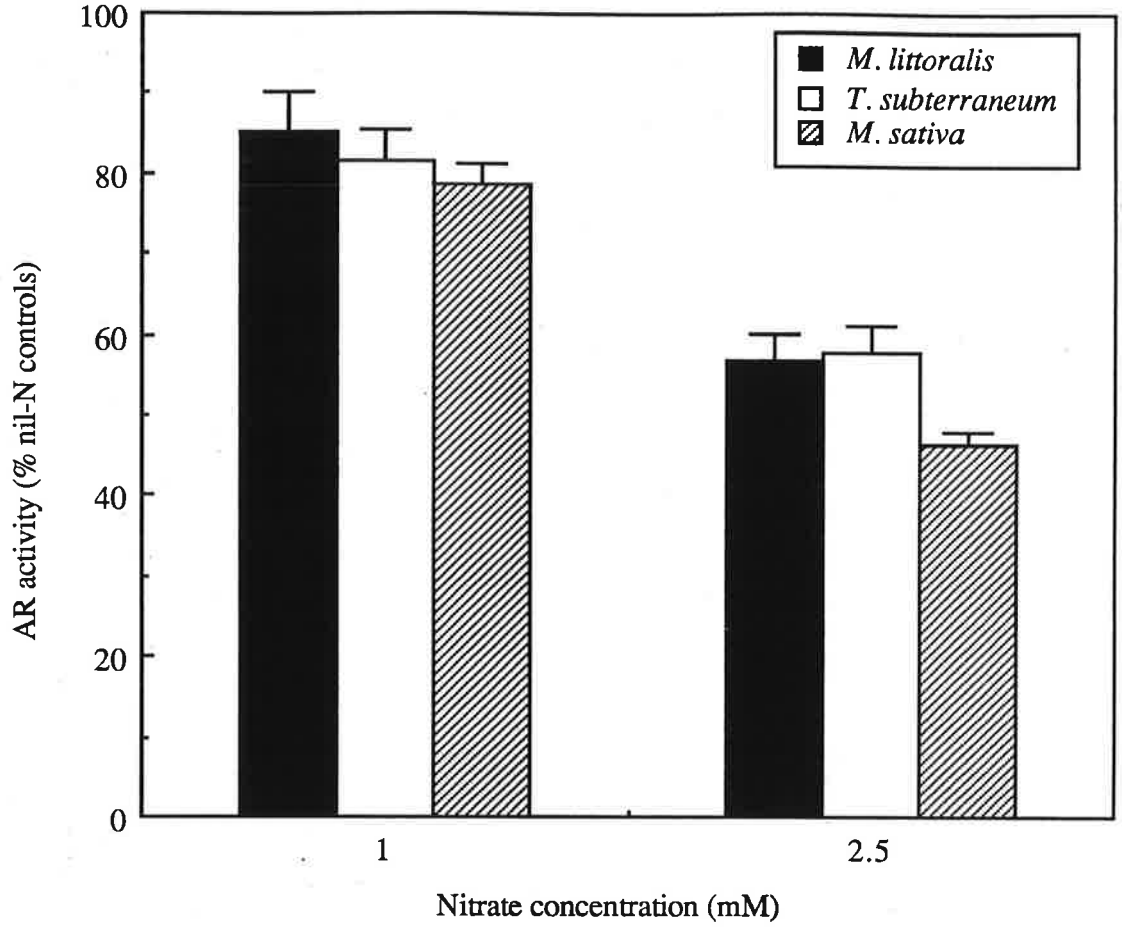


Fig. 4.4. Influence of nitrate supply on nitrogenase activity in an annual and perennial medic and in subterranean clover.

M. littoralis cv. Harbinger, *M. sativa* cv. Hunter River and *Trifolium subterraneum* cv. Woogenellup swards were grown for 40 days without combined nitrogen and exposed to three levels of nitrate for 4 days. The AR activity (μmol/h/pot) for the plants supplied with minus nitrate nutrient solution were as follows: *M. littoralis,* 11.0, *T. subterraneum*, 8.2 and *M. sativa* 27.6. Values represent the mean of six replicates while the bars indicate ± S.E.

Table 4.2. Nitrate concentration in the shoot and root of *M. littoralis*, *M. sativa* and *T. subterraneum*.

The plants were grown as described in Fig. 4.4 and nitrate data given as μmol/g dry weight.

Plant species	Shoot		Root	
	1 mM nitrate	2.5 mM nitrate	1 mM nitrate	2.5 mM nitrate
M. littoralis				
cv. Harbinger	59.1	143.1	82.8	220.3
M. sativa				
cv. Hunter River	50.1	96.5	72.9	149.7
T. subterraneum				
cv. Woogenellup	73.9	130.4	118.8	203.8
LSD[a] 5%		12.30		9.39
1%		16.75		12.75

[a]For nitrate x species interactions

four species of medic. Thus the preliminary findings of Silsbury (Section 2.8.4) were not confirmed. The discrepancy may be due to the use of oil dry (a fritted, calcined clay) as rooting medium by Silsbury and sand in the current study. Silsbury used the *Rhizobium meliloti* strain CC169. In the current study CC169 was used in Experiment 2 and WSM540 in Experiment 1, so the rhizobial strain did not appear to influence the degree of inhibition of nitrogenase. In the Silsbury study however all medics were not compared in the same experimental period (see Section 2.8.4).

In Experiment 3 a comparison was made of the relative sensitivity to nitrate of nitrogenase in 40 d old and well nodulated plants of *M. littoralis*, *M. sativa* and *T. subterraneum.* No major difference was found. This contrasts with the study of Harper and Gibson (1984) on hydroponically grown plants of a cultivar each of *M. truncatula* and *T. subterraneum* supplied with 1 and 4 mM nitrate for 17 d after inoculation. They found that nitrogenase activity (AR) in *T. subterraneum* increased approximately 1.8 fold in response to 1 mM nitrate whereas in the *M. truncatula* it declined to 15% of that of the controls. At 4 mM nitrate however nitrogenase activity in *T. subterraneum* was inhibited by 24% relative to controls and was completely inhibited in the *M. truncatula* . However in contrast to the current study, where nitrate was supplied to well nodulated plants for 4 d, the Harper and Gibson study was undertaken on plants supplied with nitrate at the time of inoculation and then monitored in a run down situation.

The conclusion from the present experiments is that the decline in nitrogenase activity after nitrate addition to plants with established symbioses was similar in the annual *Medicago* studied and *T. subterraneum.* In the studies of Harper and Gibson (1984) the effect of nitrate on nitrogenase activity may have been confounded by the earlier effects of nitrate on nodulation. Indeed they observed that the rate of nodule appearance in *T. subterraneum* was not markedly altered by nitrate but was decreased in *M. truncatula*, especially at 4 mM nitrate. Thus although no variation has been found in the tolerance of nodulated medics to nitrate, there may be differences in the sensitivity at the nodulation stage.

It has been shown in these studies (Fig. 4.2 and Table 4.2) that nitrogenase activity is negatively correlated with nitrate accumulation in the *M. rugosa* and *M. littoralis* tissue. As the concentration of nitrate increased in both shoot and root of *M. littoralis* and *M. rugosa* (Experiment 1) nitrogenase activity decreased. Silsbury *et al.* (1986) determined nitrogenase activity and the amount of nitrate in well nodulated *T. subterraneum* supplied with 0, 1 and 5 mM nitrate for up to 173 h and also found a negative correlation between nitrogenase activity and nitrate content (μmol/g fresh weight) of the shoots and roots. Jensen (1987b) also found an inverse relationship between specific nitrogenase activity and nitrate accumulation in pea nodules supplied with up to 15 mM nitrate for 7 d.

It can not be concluded from this data if it is the level of nitrate accumulated which results in nitrogenase inhibition or whether an associated high level of nitrate assimilation, and competition for photosynthate for example, causes the inhibition. This topic is investigated further in Chapter 7.

CHAPTER 5

ASSESSMENT OF *RHIZOBIUM MELILOTI* STRAINS ON THE GROWTH, NODULATION AND N_2 FIXATION OF ANNUAL MEDIC SPECIES AND THE INFLUENCE OF ROOTING MEDIUM

5.1. Introduction

In the experiments described in Chapter 4 on well-nodulated medic plants, no major variation in the sensitivity of nitrogenase activity (AR) to nitrate was found. However, variability in nodulation in the presence of nitrate among annual medic species (see Section 2.8.4) has been reported and furthermore, in a preliminary experiment carried out in hydroponic culture with three cultivars of *M. rugosa*, three cultivars of *M. truncatula* and *M. littoralis* cv. Harbinger, the nodulation in all cultivars of *M. rugosa* appeared to be relatively tolerant to nitrate (0.5-2.0 mM), whereas *M. truncatula* cultivars were sensitive (Appendix 3).

Little is known about the effect of rooting medium on growth and nodulation of annual medics. The advantage of hydroponically-grown plants is that it is easier to study the effect of nitrate on nodulation and N_2 fixation because (i) the nodulation can be readily followed without undue disturbance to plants and (ii) nitrate levels in the medium can be monitored easily. However it presents a very different medium to that in which plants normally grow.

In this Chapter, the effect of nitrate on the nodulation of three annual *Medicago* species is examined in sand and hydroponic culture, in two simultaneous experiments (Experiment 1A and 1B). An examination of *Rhizobium* strain effects, and the effect of nitrate on the nodulating ability, is examined within these experiments. The final experiment involves three strains of *R. meliloti*, as used in commercial inoculants. These were inoculated on to eight cultivars of *M. truncatula*, the principal species in this study, in order to determine whether host x strain specificity is a significant factor in the nodulation of it. In these studies it appeared that the *R. meliloti* strain CC169, currently recommended as the inoculant

for *M. truncatula* cultivars, was inferior to strain WSM540 (recommended for *M. truncatula* cultivars in Western Australia) and WSM826 (the Australian-wide lucerne inoculant strain).

5.2. EXPERIMENT 1A: Assessment of three strains of *Rhizobium meliloti* in symbiosis with three annual *Medicago* species grown in sand in the presence and absence of nitrate.

5.2.1. Materials and Methods

Seeds of *Medicago truncatula* cv. Borung, *M. rugosa* cv. Sapo and *M. polymorpha* cv. Serena were surface sterilised as described in Section 3.3 before being sown in sand. After emergence, 8 uniform seedlings of each species were selected per pot. Four days after sowing, 2.5 mL of a culture of approximately 10^9 cells/mL of either the *Rhizobium meliloti* strain WSM540, CC169 or WSM826 was added to each pot. The plants were watered twice daily with nutrient solution containing either 0 mM or 1 mM nitrate and grown in a growth room as described in Section 3.2. Twenty two days after inoculation (DAI) the plants were harvested and measurements taken on nodule number and dry weight, total dry weight and total nitrogen of the plants. Nitrate concentration in shoot samples was determined as described in Section 3.5. There were 4 replicates arranged in a split-plot design. Because the assumptions of the analysis of variance were not met, nodule number, nodule dry weight, total dry weight and total nitrogen data were transformed to Ln (x+1) prior to analysis using Genstat version 5.

5.2.2. Results

M. rugosa cv. Sapo and *M. polymorpha* cv. Serena grown in sand without added nitrate had a much larger number of nodules with WSM540 and CC169 than WSM826 (Table 5.1). Although the nodules formed by WSM826 were larger, with Sapo and Serena, total nodule weight was less than that found with the other strains. For *M. truncatula* cv. Borung the nodule parameters for three strains were similar. Statistical calculation of the various parameters showed highly significant ($P<0.01$) second order interactions between medic

Table 5.1. Effect of nitrate on nodule number and nodule dry weight in three medic species grown in sand.

M. rugosa cv. Sapo, *M. polymorpha* cv. Serena and *M. truncatula* cv. Borung were inoculated with three strains of *Rhizobium meliloti* (WSM540, CC169 and WSM826) and harvested 22 DAI. Data are expressed as means of four replicates with mean of the Ln (x+1) transformed data in parentheses.

	Treatments					
	0 mM nitrate			1 mM nitrate		
Medic cultivar	WSM540	CC169	WSM826	WSM540	CC169	WSM826
Nodule number/plant						
Sapo	15 (2.75)	17 (2.90)	3 (1.43)	14 (2.66)	11 (2.48)	5 (1.83)
Serena	31 (3.46)	28 (3.38)	5 (1.79)	21 (3.11)	22 (3.15)	12 (2.55)
Borung	14 (2.71)	14 (2.70)	16 (2.83)	11 (2.46)	7 (2.02)	11 (2.47)
LSD[a] 1% = (0.230)						
Total nodule dry weight (mg/plant)						
Sapo	1.5 (0.88)	1.6 (0.94)	1.0 (0.62)	1.4 (0.86)	1.2 (0.78)	1.1 (0.73)
Serena	2.8 (1.34)	2.5 (1.26)	1.0 (0.70)	2.8 (1.33)	2.4 (1.17)	2.5 (1.25)
Borung	1.7 (0.97)	1.3 (0.83)	1.9 (1.07)	1.3 (0.80)	0.8 (0.61)	1.0 (0.73)
LSD 1% = (0.230)						
Individual nodule dry weight (mg)						
Sapo	0.10	0.09	0.33	0.10	0.11	0.22
Serena	0.09	0.09	0.20	0.13	0.11	0.21
Borung	0.12	0.09	0.12	0.12	0.11	0.09
NS[b]						

[a]based on Ln (x+1) data for medic x *Rhizobium* x nitrate interactions

[b]Not significant

species, *Rhizobium* strains and nitrate. In the presence of 1 mM nitrate, nodule number in Sapo and Serena with WSM826 was enhanced relative to the nil-controls, but nitrate resulted in a decrease in nodule number in most of the remaining symbiotic combinations. In the presence of nitrate, nodulation of Borung was inhibited by 21% when inoculated with WSM540 and by 50% and 33% when inoculated with CC169 and WSM826 respectively.

In the absence of nitrate (Table 5.2), there were strong strain effects on total dry weight, with WSM826 significantly poorer than the other two strains on Serena, but significantly better than these strains on Borung. In Sapo, total dry weight was not affected by the *Rhizobium* strains used. With nil-nitrate, total N in Sapo and Serena inoculated with WSM540 and CC169 was higher than in those inoculated by WSM826. Although the number of nodules formed with WSM826 on Sapo and Serena was low, there was no corresponding decrease in total nitrogen or total dry weight per plant. Serena showed a greater growth response to nitrate (118-218% increase in dry weight) than Sapo (33-51%) or Borung (4-73%). These differences were also reflected in the total nitrogen values.

By deducting the seed nitrogen from total plant nitrogen it was possible to estimate the N_2 fixed by each medic/*R. meliloti* combination. This measure of N_2 fixation (expressed relative to nodule dry weight, Table 5.2) showed no differences between the medics when inoculated with different *Rhizobium* strains. The nitrate concentration in the shoot of *M. rugosa* cv. Sapo (Table 5.2) was lower than that in *M. truncatula* cv. Borung which in turn was lower than *M. polymorpha* cv. Serena.

Table 5.2. Effect of nitrate on dry matter, total nitrogen and shoot nitrate concentration in three medic species.

M. rugosa cv. Sapo, *M. polymorpha* cv. Serena and *M. truncatula* cv. Borung were grown in **sand** as described in Table 5.1. N_2 fixation was expressed as the amount of N_2 fixation per mg nodule dry weight. Data are expressed as means of four replicates with mean of the Ln (x+1) transformed data in parentheses.

	Treatments					
	0 mM nitrate			1 mM nitrate		
Medic cultivar	WSM540	CC169	WSM826	WSM540	CC169	WSM826
Total dry weight (mg/plant)						
Sapo	53 (3.99)	56 (4.04)	56 (4.04)	80 (4.39)	80 (4.38)	75 (4.33)
Serena	69 (4.25)	61 (4.13)	44 (3.81)	151 (5.02)	134 (4.90)	140 (4.94)
Borung	59 (4.09)	49 (3.91)	75 (4.33)	84 (4.44)	85 (4.46)	78 (4.37)
LSD[a] 1% = (0.138)						
Total nitrogen (mg/plant)						
Sapo	1.8 (1.00)	1.8 (1.02)	1.1 (0.76)	2.7 (1.30)	2.6 (1.33)	2.6 (1.26)
Serena	2.7 (1.32)	2.3 (1.20)	1.1 (0.72)	6.5 (2.01)	5.7 (1.90)	6.2 (2.03)
Borung	1.8 (1.03)	1.8 (1.02)	2.5 (1.26)	3.3 (1.45)	3.1 (1.43)	3.3 (1.45)
LSD 1% = (0.087)						
N_2 fixation (mg N_2 fixed/mg nodule dry weight)						
Sapo	0.88	0.83	0.63	-	-	-
Serena	0.87	0.81	0.63	-	-	-
Borung	0.90	1.18	1.17	-	-	-
NS[b]						
Shoot nitrate concentration (µmol/g shoot dry weight)						
Sapo	-	-	-	89	79	73
Serena	-	-	-	259	272	310
Borung	-	-	-	165	134	134
LSD 1% = 50.21						

[a]based on Ln (x+1) data for medic x *Rhizobium* x nitrate interactions.
[b]Not significant

5.3. EXPERIMENT 1B: Assessment of three strains of *Rhizobium meliloti* in symbiosis with three annual *Medicago* species in the presence and absence of nitrate under hydroponic culture.

5.3.1. Materials and Methods

This experiment was conducted simultaneously with Experiment 1A under the same experimental conditions, inoculation procedures and nitrate treatments. Four d old seedlings of *M. truncatula* cv. Borung, *M. rugosa* cv. Sapo, and *M. polymorpha* cv. Serena were transferred to 2.5 L pots containing hydroponic solution as described in Section 3.3. Nutrient solutions in each pot were renewed every other day from 5 until 12 DAI and then daily until the end of the experiment. The aeration rate in each pot was 1.0 L/min.

5.3.2. Results

In the absence of nitrate, *M. rugosa* cv. Sapo and *M. polymorpha* cv. Serena inoculated with WSM540, again had the highest nodule number and total nodule mass, but with *M. truncatula* cv. Borung, nodule numbers which were much lower and were similar with the three strains. Strain WSM826 nodulated Sapo relatively poorly compared to CC169, but these two strains produced a similar number of nodules, and nodule mass, on Serena (Table 5.3). In the presence of 1 mM nitrate, the nodulation in Sapo was inhibited by 50, 30 and 67% when inoculated with WSM540, CC169 and WSM826 respectively. For Serena, the inhibition in nodule number was 60, 53 and 94% for the three strains, and for Borung the inhibition was 89, 88 and 63%. In the presence of nitrate, there were very few nodules on *M. truncatula* cv. Borung, and nodulation by strain WSM826 was severely retarded on Sapo and Serena. Analysis of the data showed highly significant ($P<0.01$) second order interactions between nitrate, medic species and *Rhizobium* strains for all parameters except nodule dry weight, and for this parameter, the three first order interactions between the three variables were all significant ($P<0.01$).

Table 5.3. Effects of nitrate on nodule numbers and nodule dry weight in three annual medic species grown in hydroponics.

M. rugosa, *M. polymorpha* and *M. truncatula* were inoculated with three strains of *Rhizobium meliloti* (WSM540, CC169 and WSM826) and harvested 22 DAI. Data are expressed as means of four replicates with mean of the Ln (x+1) transformed data in parentheses.

	Treatments					
	0 mM nitrate			1 mM nitrate		
Medic cultivar	WSM540	CC169	WSM826	WSM540	CC169	WSM826
Nodule number/plant						
Sapo	36 (3.61)	23 (3.14)	9 (2.31)	18 (2.90)	16 (2.83)	3 (1.29)
Serena	25 (3.24)	19 (3.01)	17 (2.86)	10 (2.39)	9 (2.31)	1 (0.69)
Borung	9 (2.33)	8 (2.14)	8 (2.22)	1 (0.66)	1 (0.90)	3 (1.49)
LSD[a] 1% = (0.294)						
Total nodule dry weight (mg/plant)						
Sapo	2.7 (1.30)	1.9 (1.08)	1.0 (0.72)	1.7 (0.98)	1.6 (0.94)	0.3 (0.24)
Serena	2.8 (1.33)	1.7 (0.98)	1.6 (0.96)	0.9 (0.62)	0.8 (0.56)	0.1 (0.03)
Borung	1.4 (0.89)	1.0 (0.71)	1.3 (0.83)	0.2 (0.18)	0.2 (0.09)	0.3 (0.30)
LSD 1% = (0.178)						
Individual nodule dry weight (mg)						
Sapo	0.08	0.08	0.11	0.09	0.10	0.10
Serena	0.11	0.09	0.09	0.09	0.09	0.10
Borung	0.16	0.13	0.16	0.20	0.20	0.10
NS[b]						

[a]based on Ln (x+1) data for medic x *Rhizobium* x nitrate interactions

[b]Not significant

With 0 mM nitrate, analysis of the total dry weight and total nitrogen values indicated that *R. meliloti* strain WSM540 > CC169 > WSM826 for Sapo and Serena (Table 5.4) whereas with Borung, CC169 was inferior to the other strains. Values for N_2 fixed/mg nodule dry weight were relatively similar for all the medic/*R. meliloti* strains (Table 5.4) and were generally lower than in the sand grown material (Table 5.2).

Sapo, Serena and Borung grown in the presence of nitrate had 3.8, 4.0 and 5.0 fold more total dry matter respectively than the corresponding plants grown without nitrate. The nitrate concentration in the shoot of Sapo inoculated with the three *Rhizobium* strains was about 50% of that in Serena and Borung. When the data for the hydroponic plants are compared with those for sand grown material, *M. truncatula* cv. Borung had only about half the nodule number in hydroponics (Table 5.3) than in sand (Table 5.1). The reverse was the case for *M. rugosa* cv. Sapo i.e. this medic nodulated better under hydroponic culture. For *M. polymorpha* cv. Serena and *R. meliloti* strain WSM826 nodulation in hydroponics was 3 fold that in sand grown material.

In terms of total dry weight and total nitrogen, and for plants grown in sand or hydroponics, WSM540 > CC169 > WSM826 for *M. polymorpha* cv. Serena (Table 5.2 and 5.4). This was also the case for *M. rugosa* cv. Sapo in hydroponics but when this species was grown in sand, plant growth was the same with the three *Rhizobium* strains. *M. truncatula* cv. Borung differed from the above in that WSM826 was considerably superior to CC169 and WSM540 in sand and WSM540 and WSM826 were superior to CC169 in hydroponics.

In the absence of nitrate, the growth of plants was usually less in hydroponics than in sand (Table 5.2 and 5.4) especially in *M. truncatula* cv. Borung. The opposite was the case in the presence of 1 mM nitrate especially for Sapo and Borung where the total dry matter of plants in hydroponics was at least double that of those grown in sand. The shoot nitrate concentration in hydroponic culture was 2-5 fold higher than that in plants grown in sand.

Table 5.4. Effects of nitrate on dry matter yields, total nitrogen and shoot nitrate concentration in three medic species.

M. rugosa cv. Sapo, *M. polymorpha* cv. Serena and *M. truncatula* cv. Borung were grown in **hydroponics** as described in Table 5.3. N_2 fixation was expressed as the amount of N_2 fixed per mg nodule dry weight. Data are expressed as means of four replicates with mean of the Ln (x+1) transformed data in parentheses.

	Treatments					
	0 mM nitrate			1 mM nitrate		
Medic cultivar	WSM540	CC169	WSM826	WSM540	CC169	WSM826
Total dry weight (mg/plant)						
Sapo	59 (4.09)	51 (3.95)	41 (3.73)	203 (5.32)	183 (5.21)	186 (5.23)
Serena	60 (4.11)	46 (3.83)	34 (3.54)	208 (5.34)	160 (5.08)	192 (5.26)
Borung	38 (3.66)	28 (3.37)	37 (3.64)	184 (5.22)	168 (5.13)	161 (5.09)
LSD[a] 1% = (0.134)						
Total nitrogen (mg/plant)						
Sapo	2.0 (1.09)	1.7 (1.01)	1.1 (0.78)	9.1 (2.30)	8.7 (2.27)	8.5 (2.25)
Serena	2.2 (1.17)	1.8 (1.02)	1.1 (0.76)	10.3 (2.42)	8.3 (2.23)	9.4 (2.36)
Borung	1.2 (0.80)	0.9 (0.66)	1.3 (0.80)	8.8 (2.27)	9.0 (2.24)	8.4 (2.19)
LSD 1% = (0.110)						
N_2 fixation (mg N_2 fixed/mg nodule dry weight)						
Sapo	0.57	0.61	0.63	-	-	-
Serena	0.69	0.90	0.52	-	-	-
Borung	0.67	0.64	0.80	-	-	-
NS[b]						
Shoot nitrate concentration (µmol/g shoot dry weight)						
Sapo	-	-	-	345	395	380
Serena	-	-	-	720	777	703
Borung	-	-	-	653	659	605
LSD 1% = 88.25						

[a]based on Ln (x+1) data for medic x *Rhizobium* x nitrate interactions
[b]Not significant

A comparison of the effect of 1 mM nitrate on the nodule number and nodule dry weight for hydroponic and sand grown plants is shown in Table 5.5. The reduction in nodulation was greater under hydroponics than in sand culture. In hydroponics with 1 mM nitrate, Sapo produced 54% of the nodules formed in the absence of nitrate, while Serena (33%) and Borung (20%) showed greater inhibition. In sand, however, no significant differences were found between the medic species and the reduction in nodule number in the presence of nitrate was markedly less than in hydroponics. Nodule dry weight values showed the same degree of inhibition by nitrate in the three species as above, and similarly in sand less inhibition was again observed.

5.4. EXPERIMENT 2: The effects of cultivar and *Rhizobium* strain on the growth and N_2 fixation of *Medicago truncatula*.

The results of the previous experiments indicated that the *R. meliloti* strain recommended for *M. truncatula* cv. Borung, strain CC169, was not always the most effective strain with this cultivar. In addition it appeared that nodulation in *M. truncatula* may be especially sensitive to nitrate and that nodulation of Borung was generally poor compared to the other two medic species, especially in hydroponics. This experiment was done to ensure that the best host x *Rhizobium* strain combination for several *M. truncatula* cultivars would be employed in the remainder of this project.

5.4.1. Materials and Methods

Seeds of *M. truncatula,* cvs. Borung, Caliph, Cyprus, Mogul, Jemalong, Paraggio, Parabinga and Sephi were surface sterilised and sown as described in Section 3.3. The seedlings were thinned to a density of 12 plants per pot at emergence. The surface of the sand in each pot was covered with three mm white Alkathene beads to a depth of six mm to minimise evaporation and the possibility of splash contamination from pot to pot. Half-strength Hoagland nutrient solution, free of mineral nitrogen, (Appendix 2) was flushed through the pots every other day.

Table 5.5. Influence of nitrate on nodule number and nodule dry weight in three medic species grown in sand and hydroponics.

Data were taken from Experiments 1A and 1B (Tables 5.1 and 5.3) and averaged from three *R. meliloti* strains CC169, WSM540 and WSM826 for each medic cultivar. The values show the ratio of total nodule numbers and nodule dry weights at 1 and 0 mM nitrate.

Culture medium	Sapo[a]	Serena	Borung	LSD 5%
Nodule number/plant				
Hydroponics	0.54	0.33	0.20	0.19
Sand	0.86	0.86	0.66	NS[b]
Nodule dry weight (mg/plant)				
Hydroponics	0.64	0.30	0.19	0.21
Sand	0.90	1.22	0.63	0.56

[a]*M. rugosa* cv. Sapo, *M. polymorpha* cv. Serena and *M. truncatula* cv. Borung.
[b]Not significant

Plants of each of the 8 cultivars were inoculated with one of the 3 strains of *R. meliloti* (CC169, WSM540, and WSM826), or left uninoculated as controls. The pots were arranged in 4 replicate blocks. The treatments were replicated 4 times for each cultivar in each *Rhizobium* strain.

Twenty three DAI, blue-green aphids (*Acyrthosiphon kondoi* Shinji) attacked Borung and Mogul cultivars and these were sprayed with PIRIMOR® (0.05% w/v). This treatment controlled the aphids, but a loss of about 15% of the shoots occurred.

Thirty DAI, shoots of the plants were cut off at sand level, roots were washed free of sand and nodules counted. Total nitrogen and total dry weight were determined as described in Sections 3.4 and 3.8 respectively.

5.4.2. Results

At 30 DAI, there were more nodules on Caliph, Jemalong, Parabinga and Paraggio than the other medic cultivars tested (Fig. 5.1). In most cultivars there was only a small variation in nodule number with rhizobial strain and, except for Sephi, the largest number of nodules was obtained with WSM540 or WSM826. A low proportion (12%) of the uninoculated control plants developed a few small white nodules.

The influence of *Rhizobium* strains was more evident from plant dry weight data (Table 5.6). For Borung, Caliph, Jemalong, Parabinga and Sephi, there was significantly more shoot growth with WSM540 and WSM826 than with CC169. With Jemalong, WSM826 was superior to WSM540; with Cyprus, WSM540 was superior to WSM826 and CC169. In all cultivars root dry weights were lower with CC169 than with the other strains, although not always significantly ($P< 0.05$). Nodule dry weight/plant was also generally higher with WSM540 and WSM826 than with CC169, the increases with both strains being significant ($P< 0.05$) with Borung, Jemalong, Paraggio and Sephi. In all cases total nodule dry weight was least with CC169, with the effect being significant for Borung, Caliph, Jemalong, Parabinga and Sephi; except for Paraggio nodule dry weight was 20-60% higher

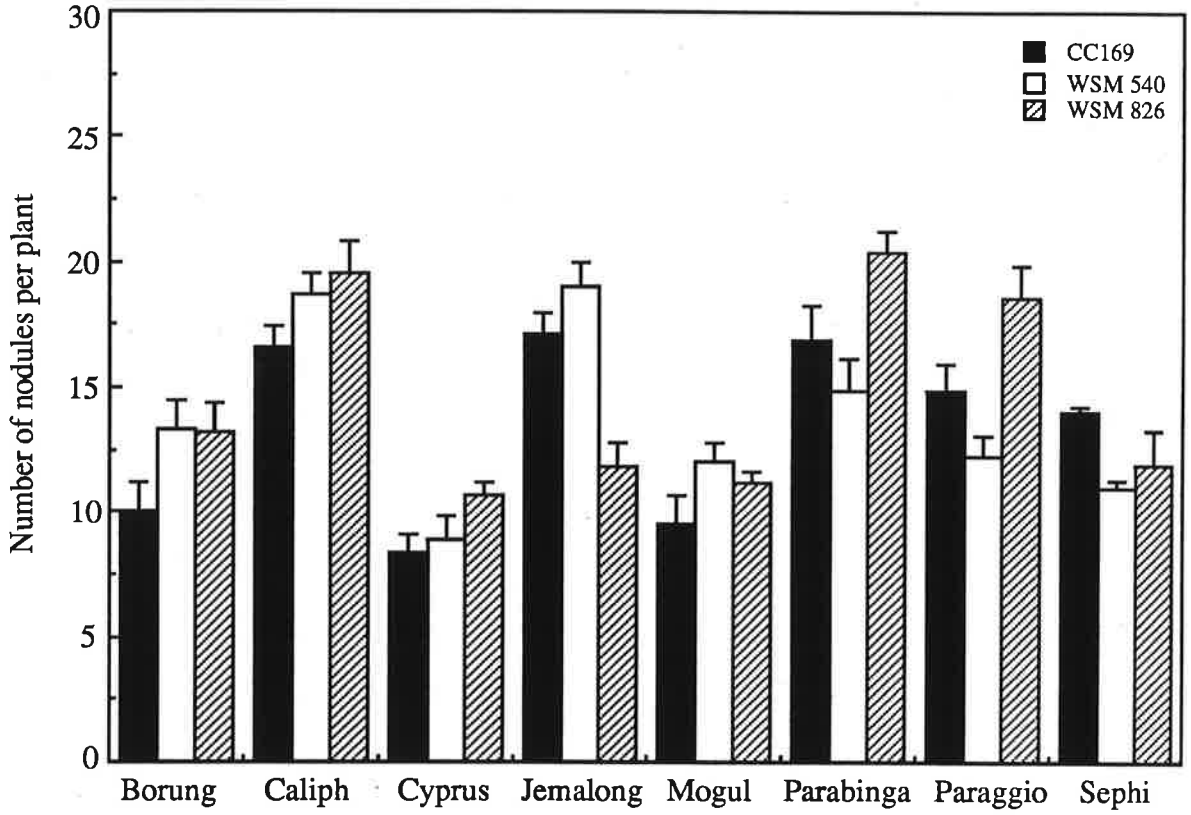


Fig. 5.1. Influence of three strains of *Rhizobium meliloti* on the nodulation of *M. truncatula* cultivars.

The plants were grown in sand for 30 DAI and supplied with minus-nitrate nutrient solution. Values represent the average number of nodules per plant while the bars indicate ±S.E.

Table 5.6. Cultivar and *Rhizobium* strain effects on dry matter production of *Medicago truncatula* cultivars.

The plants were grown in sand as described in Fig. 5.1. Data in the parentheses are dry weight of individual nodules.

Medic cultivar	*Rhizobium* strain	Nodule Dwt.[a] (mg/plant)	Shoot Dwt. (mg/plant)	Root Dwt. (mg/plant)	Total Dwt. (mg/plant)
Borung	WSM540	2.46 (0.18)	76.4	36.4	115.3
	CC169	1.69 (0.17)	54.7	27.0	83.4
	WSM826	2.76 (0.22)	75.1	35.8	113.7
Caliph	WSM540	3.64 (0.19)	125.5	47.5	176.6
	CC169	3.08 (0.19)	95.9	40.8	139.8
	WSM826	3.95 (0.20)	119.0	46.3	169.2
Cyprus	WSM540	3.44 (0.39)	90.4	40.2	134.0
	CC169	2.45 (0.30)	69.6	31.4	103.4
	WSM826	2.40 (0.22)	74.4	35.0	111.8
Jemalong	WSM540	2.56 (0.13)	99.9	48.5	151.0
	CC169	1.49 (0.09)	74.0	39.2	114.7
	WSM826	2.38 (0.21)	119.7	61.1	183.2
Mogul	WSM540	3.25 (0.27)	72.9	34.7	110.8
	CC169	2.00 (0.22)	55.7	28.5	86.2
	WSM826	2.32 (0.21)	69.6	36.2	108.1
Parabinga	WSM540	3.60 (0.24)	142.1	61.8	207.5
	CC169	2.95 (0.17)	98.3	45.8	147.0
	WSM826	3.62 (0.18)	131.9	58.6	194.1
Paraggio	WSM540	3.69 (0.31)	110.4	51.9	166.0
	CC169	2.93 (0.20)	102.4	49.6	154.9
	WSM826	4.10 (0.22)	111.7	54.6	170.4
Sephi	WSM540	4.23 (0.39)	141.6	64.3	210.1
	CC169	3.12 (0.22)	101.7	41.3	146.1
	WSM826	3.87 (0.33)	128.9	51.4	184.2
LSD 5%		0.39	15.4	4.8	12.1

[a]Dry weight

with either WSM540 or WSM826. The largest difference was found with Jemalong. The apparent overall poorer performance of Borung and Mogul could have been due to aphid damage (see Section 5.3.2).

All medic cultivars, except Cyprus and Paraggio, had significantly more (1.4 to 1.7 times) shoot N when inoculated with either WSM540 or WSM826 than when inoculated with CC169 (Table 5.7). The influence of WSM540 was greatest with Caliph, Mogul and Parabinga while Jemalong/WSM826 produced 70% more shoot nitrogen than Jemalong/CC169. These effects of *Rhizobium* strains were also reflected in root nitrogen data. Thus, all medic cultivars fixed more nitrogen when inoculated with either WSM540 or WSM826 than when nodulated with CC169. These increases were statistically significant ($P < 0.05$) except for Cyprus and Paraggio. N_2 fixation per mg nodule dry weight in Jemalong was approximately 1.4 times higher than that of the other cultivars. This was due to lower nodule dry weight in this cultivar compared with the others.

5.5. Discussion

It was established in this chapter that it will be possible to use hydroponics in this study of the role of nitrate in the inhibition of nodule formation. The advantages of hydroponic culture over sand culture are it is possible to (i) examine the root systems thoroughly for nodules, (ii) maintain the nitrate concentration and measure its uptake (iii) keep the concentration of the other nutrients constant, and (iv) minimise changes in pH. N_2 fixation per mg nodule dry weight in sand culture was approximately 35% higher than in hydroponics. This may be due to low oxygen level in the hydroponic system (Ferguson and Bond 1954).

Growth of the medics, *M. rugosa* cv. Sapo and *M. polymorpha* cv. Serena in hydroponic culture was generally comparable to that in sand. However growth and nodulation of *M. truncatula* cv. Borung in hydroponics was only about half that on sand. In hydroponic or sand culture some differences in the degree of effectiveness (measured by nodule formation

Table 5.7. Cultivar and *Rhizobium* strain effect on nitrogen accumulation in *Medicago truncatula* cultivars.

The plants were grown in sand as described in Fig. 5.1. N_2 fixation was calculated as plant N less seed N.

Medic cultivar	*Rhizobium* strain	Seed N (mg/seed)	Shoot N (mg/plant)	Root N (mg/plant)	N_2 fixation (mg/plant)	N_2 fixation (mg/mg NDW[a])
Borung	WSM540	0.26	2.92	1.02	3.68	1.50
	CC169		2.12	0.77	2.64	1.56
	WSM826		2.91	1.01	3.67	1.33
Caliph	WSM540	0.32	4.18	1.46	5.32	1.46
	CC169		3.02	1.19	3.89	1.26
	WSM826		3.83	1.44	4.96	1.26
Cyprus	WSM540	0.24	2.80	1.24	3.80	1.10
	CC169		2.44	0.95	3.15	1.29
	WSM826		2.59	1.07	3.42	1.43
Jemalong	WSM540	0.32	3.05	1.47	4.19	1.64
	CC169		2.21	1.07	2.97	1.99
	WSM826		3.75	1.69	5.12	2.15
Mogul	WSM540	0.30	2.92	1.10	3.73	1.15
	CC169		1.96	0.82	2.48	1.24
	WSM826		2.64	1.02	3.36	1.45
Parabinga	WSM540	0.34	4.55	1.83	6.04	1.63
	CC169		3.03	1.35	4.04	1.37
	WSM826		3.55	1.58	4.79	1.32
Paraggio	WSM540	0.38	3.84	1.42	4.88	1.32
	CC169		3.56	1.41	4.59	1.57
	WSM826		3.73	1.54	4.90	1.20
Sephi	WSM540	0.41	3.73	1.98	6.32	1.49
	CC169		3.23	1.16	3.98	1.28
	WSM826		4.15	1.59	5.33	1.38
LSD 5%			0.29	0.14	0.38	NS[b]

[a]Nodule dry weight
[b]Not significant

and total dry matter production) of the three *R. meliloti* strains were observed but these were not reproduced with both root media. Brockwell and Hely (1966) also reported that nodule formation, ability to fix N_2 and the level of fixation may be influenced by rooting medium. They compared agar culture and vermiculite and found that some species in association with *R. meliloti* grew and nodulated better in vermiculite than in an agar medium, while growth was similar in other species irrespective of media used.

When the effects of 1 mM nitrate on the nodulation of annual medic in hydroponic and sand cultures were compared, nodule number was reduced by about 20% in sand compared to 60% in hydroponic culture. In Experiment 1A, where plants were grown in 3 kg of sand with a water holding capacity of about 11% (w/w), the pots could hold 0.33 L of nutrient solution, while under hydroponic conditions the pots contained 2.5 L of nutrient solution. Nitrate availability to the plants in hydroponic culture was therefore about 7.5 times more than that in sand culture. This is reflected in the more pronounced effect of nitrate on nodulation and greater dry weight of the nitrate-supplied plants growing in hydroponic culture than in sand.

There were no marked or consistent differences between the three medics inoculated with the different *R. meliloti* strains in the inhibition of nodulation by nitrate. Nitrate significantly decreased nodule number in all host-*Rhizobium* combinations except Sapo and Serena when inoculated with WSM826 in sand culture, but in this case nodulation in the absence of nitrate was poor. Pate and Dart (1961) and Heichel and Vance (1979) reported significant differences in the ability of strains of *Rhizobium* to nodulate the host in the presence of moderate levels (0.3-1.1mM) of nitrate, but at higher levels (> 1.1 mM), these differences disappeared. McNeil (1982) also found that differences did exist in the ability of strains to nodulate the host in the presence of low levels of nitrate (0.2-2.0 mM). Under hydroponics, at 1 mM nitrate, all the medic species tested in the current study produced approximately similar dry matter irrespective of *Rhizobium* strains. This suggests that the effect of

Rhizobium strains is not very marked under conditions where nitrate is available in quantities which will not limit growth.

Nitrate had only a small effect on the individual nodule dry weight (Table 5.1 and 5.3). This suggests that nodule initiation is more sensitive to nitrate than subsequent stages of nodule development. Ralston and Imsande (1983) also reported that nitrate prevented some early stages in nodulation but the nodules that develop in the presence of nitrate were not retarded in their growth or activity.

The *Rhizobium meliloti* strains WSM540 and WSM826 were found to be more effective than the currently recommended strain CC169 in terms of N_2 fixation and growth with a range of cultivars of *M. truncatula*. For Jemalong, WSM826 was also considerably better than WSM540 for total dry weight and total nitrogen. Synman and Strijdom (1980) who tested five Australian cultivars of *M. truncatula* with a range of *R. meliloti* strains found that Jemalong had a higher degree of specificity. None of the *Rhizobium* strains evaluated in this experiment was originally collected from *M. truncatula* nodules. Strain CC169 was collected from nodules of *M. rugosa* Desr in South Australia, WSM540 from *M. murex.* in Sardinia (Howieson *et al.* 1988) and WSM826 from *M. littoralis* on Naxos Island, Greece (J. Howieson personal communication).

While nodule number is a very important consideration of symbiotic effectiveness it should be combined with total dry matter and total nitrogen in the evaluation of strains. Even when there is little variation in nodule numbers with different *Rhizobium* strains, differences in total dry matter and total nitrogen of the host plant could be significant.

CHAPTER 6

THE EFFECTS OF NITRATE ON NODULATION AND N_2 FIXATION OF ANNUAL MEDICS

6.1. Introduction

In the experiments described in Chapter 5, there was evidence for differences between medic cultivars with respect to the effects of nitrate on nodulation. Ewing and Robson (1990) also found variation in the sensitivity of nodulation to nitrate in a cultivar of each of three medic species. In the first five experiments described in this chapter, sixteen cultivars, representing seven annual medic species, were examined to determine the range of the nodulation response to nitrate, with some cultivars examined twice, or three times. In the fifth experiment, a species very susceptible to nitrate, *Medicago sativa* (Gibson and Nutman 1960), and a very tolerant species (*Trifolium subterraneum*) (Harper and Gibson 1984) were included for comparison.

By using hydroponic culture, a ready estimate of nitrate uptake was obtained, and the results were examined in relation to effects on nodulation, and in two instances, on nitrogenase activity (AR). To further help explain the observations, the kinetics of nitrate uptake was examined with selected medics, as were levels of nitrate reductase in tissues of different cultivars.

6.2. EXPERIMENT 1: A comparison of the response to nitrate of *M. rugosa* cv. Sapo and *M. truncatula* cvs. Caliph, Cyprus, Parabinga and Sephi.

6.2.1. Materials and Methods

M. rugosa cv. Sapo and *Medicago truncatula* cvs. Parabinga, Cyprus, Caliph, Sephi were grown as described in Section 3.3. Ten seedlings of each cultivar were transferred to hydroponic culture in 1.5 L pots with or without 1 mM nitrate. The seedlings were

inoculated with *Rhizobium meliloti* strain WSM540 as described in Section 3.3.1. During the first 11 DAI, the nitrate concentration in the nitrate treatment was maintained within 80% of the initial concentration by renewing the nutrient solutions every other day. From 11 to 20 DAI the nutrient solution was renewed every day. The aeration rate was 0.8 L/min. Four plants were marked in each of 3 replicated pots and used for the daily assessment of nodule numbers.

At 14 and 20 DAI, five plants per pot were harvested, partitioned into roots, shoots and nodules and dried at 80°C for 24 h as described in Section 3.8. Total nitrogen, nitrate concentration in the shoots and roots and nitrate uptake were determined as described in Sections 3.4, 3.5 and 3.5.2 respectively. From total nitrogen data, the level of N_2 fixation in the presence of nitrate was calculated by subtracting total nitrate-N taken up by the plants from total plant nitrogen.

The experiment was designed as a completely randomised factorial combination of two nitrate treatments (0 and 1 mM) and five cultivars of annual medic with three replicates. Before data analysis, parameters were averaged for 5 plants from each treatment in all replicates. Logarithmic transformation of the data for total dry weight and total nitrogen were used to obtain more homogeneity of variance among the minus-nitrate and plus-nitrate grown plants. Values for nodule number presented as means ±S.E. Least significant differences (LSD) were calculated at the $P < 0.05$ level.

6.2.2. Results

Without nitrate, the time for initial nodule appearance was 3-4 d for all cultivars (Fig. 6.1). With 1 mM nitrate, nodulation was delayed in all the *M. truncatula* cultivars (1 d for Caliph, 2 d for Cyprus and 3 d for Parabinga and Sephi), but not in *M. rugosa* cv. Sapo. With both minus and plus nitrate, nodule numbers on Sapo were higher than on the four *M. truncatula* cultivars. From 8 DAI, the rate of nodule production on all cultivars, with or without nitrate declined. Nitrate resulted in a greater reduction in nodule numbers, relative to the control at

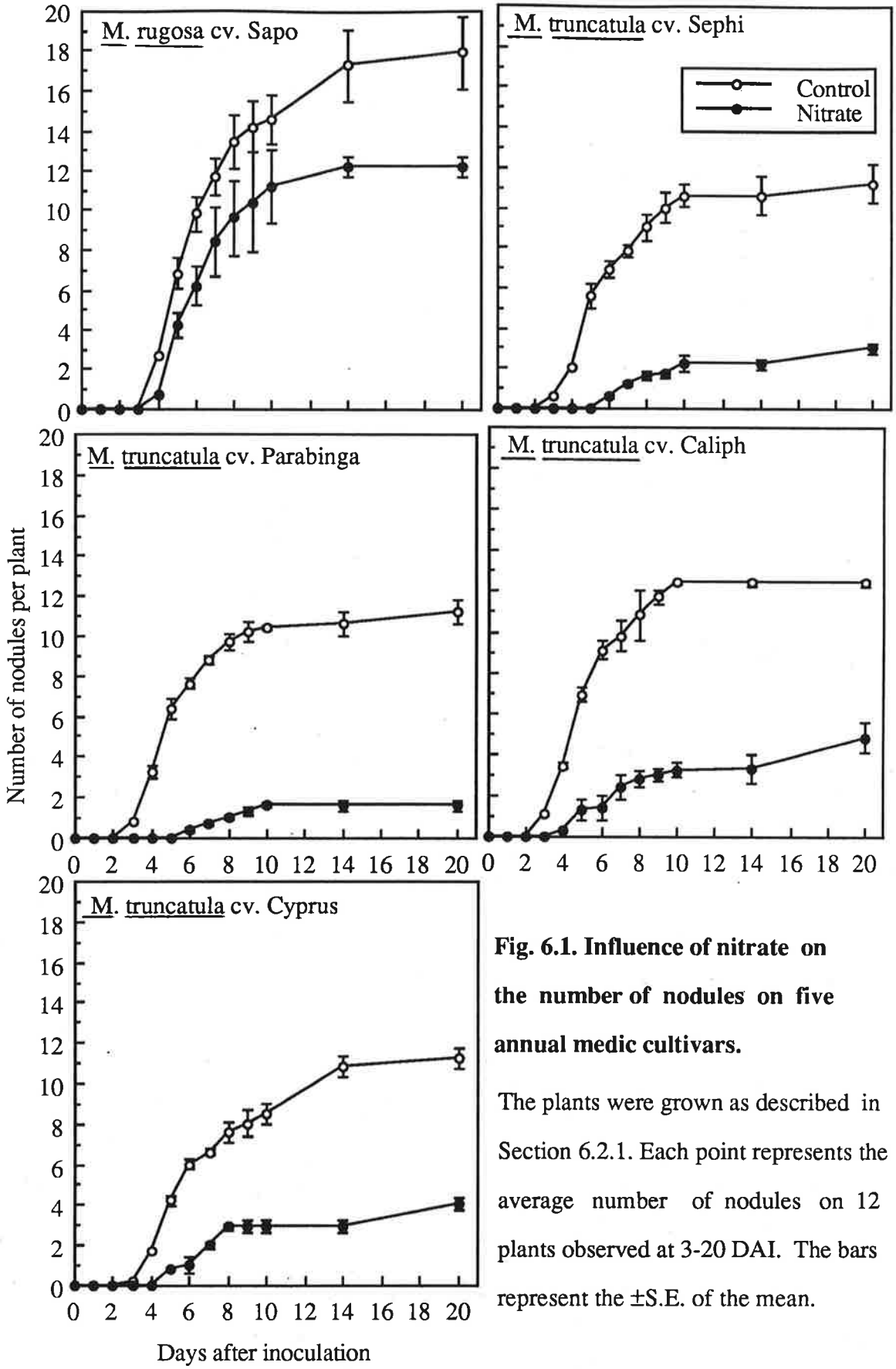


Fig. 6.1. Influence of nitrate on the number of nodules on five annual medic cultivars.

The plants were grown as described in Section 6.2.1. Each point represents the average number of nodules on 12 plants observed at 3-20 DAI. The bars represent the ±S.E. of the mean.

20 DAI, on the *M. truncatula* cultivars, (Parabinga 85%, Cyprus 73%, Caliph 63% and Sephi 79%) than on *M. rugosa* cv. Sapo (30%)

At 14 DAI (Table 6.1) the total dry weights of the medic cultivars grown in minus-nitrate nutrient solution ranged from 29 to 49 mg/plant. Total dry weights were significantly higher in plants grown with nitrate than those grown without. At 14 DAI, the increase over minus nitrate was approximately 2 times for Sapo, 2.5 for Caliph and Sephi, 3 for Cyprus and 4 times for Parabinga. The average shoot : root ratio for Sapo at 14 and 20 DAI was 2.6 in minus nitrate and 3.3 in 1 mM nitrate (data not shown). The corresponding average values for the *M. truncatula* cultivars were 3.1 and 4.0 respectively. The relative growth rate calculated from 14 to 20 DAI (Table 6.1) in nitrate-fed plants was higher than in nitrate-minus fed plants.

In the absence of nitrate, the individual nodule dry weight for *M. truncatula* cv. Sephi was higher than in the other medics tested in this experiment (Table 6.1). All the medics showed a 2-3 fold increase in nodule dry weight between 14-20 DAI. The presence of 1 mM nitrate had no effect on the nodule weight of *M. rugosa* cv. Sapo at 14 DAI and caused a relatively small decrease at 20 DAI. In all the *M. truncatula* cultivars the nodule weight for the nitrate-treated plants was inhibited: Caliph, 33%; Cyprus, 48%; Sephi, 61%; and Parabinga, 76% with reference to the control at 20 d.

From 14 to 20 DAI total nitrogen contents (Table 6.2) of the minus-nitrate plants doubled while those grown with nitrate increased by between 3.5 (Sapo, Cyprus and Caliph) and 4.3 times (Parabinga and Sephi). The overall % N for the medics grown in the absence of nitrate was 3.3. With 1 mM nitrate supplied this value was 4.2 in Sapo but 4.9 in the other medics.

Between 14 and 20 DAI, the uptake of nitrate per day per gram final root dry weight for Sapo was 0.098 mmol, while for the *M. truncatula* cultivars it was approximately 0.175. Nitrate concentrations in the root tissues were higher than those in the shoots (data not

Table 6.1. Influence of nitrate on dry matter, relative growth rate and nodule dry weight of five medic cultivars.

Five plants in each pot were harvested at 14 and 20 DAI. The dry weight data shown are means of determinations made on 15 plants. Means of the Ln transformed data for total dry weight are given in parentheses. Relative growth rate was determined between 14-20 DAI. LSD data are for medic x nitrate interactions.

Harvest time	Nitrate treat. (mM)	Medic cultivar[a]					LSD 5%
		Sapo	Sephi	Parabinga	Caliph	Cyprus	
Plant dry weight (mg)							
14 d	0	49 (3.9)	46 (3.8)	34 (3.5)	31 (3.4)	29 (3.4)	(0.23)
"	1	104 (4.6)	125 (4.8)	141 (4.9)	79 (4.4)	90 (4.5)	
20 d	0	92 (4.5)	101 (4.6)	77 (4.3)	74 (4.3)	68 (4.2)	(0.08)
"	1	320 (5.8)	461 (6.1)	565 (6.3)	269 (5.6)	327 (5.8)	
Dry weight ratio (+N/-N))							
14 d		2.1	2.7	4.2	2.5	3.1	0.66
20 d		3.5	4.5	7.4	3.7	4.8	0.59
Relative growth rate (mg/mg/day)							
	0	0.106	0.131	0.140	0.143	0.144	0.037
	1	0.189	0.218	0.232	0.207	0.216	
Nodule dry weight (mg/nodule)							
14 d	0	0.10	0.15	0.10	0.09	0.08	0.032
"	1	0.10	0.05	0.00	0.03	0.03	
20 d	0	0.24	0.36	0.25	0.21	0.23	0.094
"	1	0.20	0.14	0.06	0.14	0.12	

[a]*M. rugosa* cv. Sapo, *M. truncatula* cvs. Parabinga, Cyprus, Caliph and Sephi.

Table 6.2. Nitrogen content, nitrate uptake and nitrate assimilation by medics. Nitrate uptake was determined by its disappearance from the nutrient solution from 14 to 20 DAI. The nutrient solution was renewed as described in Section 6.2.1. Nitrate assimilation was determined by subtracting the final amount of nitrate in both shoots and roots from the total uptake of nitrate at the end of experiment (j= e-(g-f)-(i-h)). Means of the Ln transformed data for total nitrogen are given in parentheses. LSD data are for medic x nitrate interactions. Other details as in Table 6.1.

Harvest time	Nitrate treat. (mM)	Medic cultivar					LSD 5%
		Sapo	Sephi	Parabinga	Caliph	Cyprus	
Total nitrogen (mg/plant)							
14 d (a)	0	1.6 (0.5)	1.5 (0.4)	1.2 (0.2)	1.1 (0.1)	1.0 (0.0)	(0.22)
" (b)	1	4.2 (1.4)	5.7 (1.7)	6.9 (1.9)	3.9 (1.3)	4.4 (1.5)	
20 d (c)	0	2.9 (1.1)	3.0 (1.1)	2.6 (0.9)	2.4 (0.9)	2.1 (0.7)	(0.09)
" (d)	1	13.7 (2.6)	23.6 (3.2)	29.1 (3.4)	13.8 (2.6)	16.4 (2.8)	
Nitrate-N uptake[a] (mg/plant) (e)	1	7.35	17.49	22.29	9.18	11.70	2.53
Shoot nitrate-N (mg/plant)							
14 d (f)	1	0.19	0.72	0.74	0.36	0.42	0.13
20 d (g)	1	0.40	2.06	2.45	0.69	1.15	0.21
Root nitrate-N (mg/plant)							
14 d (h)	1	0.19	0.27	0.33	0.20	0.22	0.08
20 d (i)	1	0.50	1.19	1.75	0.57	0.75	0.24
Nitrate assimilation[a] (mg/plant) (j)	1	6.81	15.24	19.16	8.48	10.44	2.64

[a]14-20 DAI

shown) in all the medic cultivars tested and were considerably lower in Sapo than in the *M. truncatula* cultivars. Between 60-70% of the plant nitrate was found in the shoots of Parabinga, Cyprus, Caliph and Sephi, while in Sapo it was equally distributed between the shoot and root. It is shown in Table 6.2 that approximately 90% of the nitrate taken up by the medics between 14 and 20 DAI was assimilated.

A nitrogen balance study (Fig. 6.2) showed that Sapo, unlike the other medic cultivars, continued to fix N_2 in the presence of nitrate. Although the nodule numbers (12±0.4) and total nodule dry weight (2.4±0.1) were lower in the nitrate treated plants than in the controls (18±1.9 and 4.1±0.3 respectively) there appears to be a higher level of N_2 fixation in the larger nitrate treated plants of Sapo. N_2 fixation in the other medic cultivars was markedly inhibited, especially in Parabinga, with the sensitivity being directly related to the decrease in nodulation (Fig. 6.1).

6.3. EXPERIMENT 2: Nodulation and N_2 fixation of *M. rugosa* cvs. Sapo, Paragosa and Paraponto, *M. littoralis* cv. Harbinger, *M. polymorpha* cv. Serena and *M. truncatula* cv. Sephi in the presence of nitrate.

In the first experiment it was confirmed that nodulation in *M. rugosa* cv. Sapo was more tolerant to nitrate than for the *M. truncatula* cultivars tested. In a similar study in this experiment two other *M. rugosa* cultivars were compared with the *M. truncatula* cv. Sephi, *M. polymorpha* cv. Serena and also *M. littoralis* cv. Harbinger which was studied in Chapter 4.

6.3.1. Materials and Methods

All procedures of plant establishment in the growth room and inoculation and data analysis were the same as those in Experiment 1 except that the aeration rate was 0.5 L/min and plants were harvested 14 DAI. The nutrient solution was renewed completely at 5, 8, 10 and 12 DAI and the amount of nitrate taken up by each cultivar determined. Four replicates

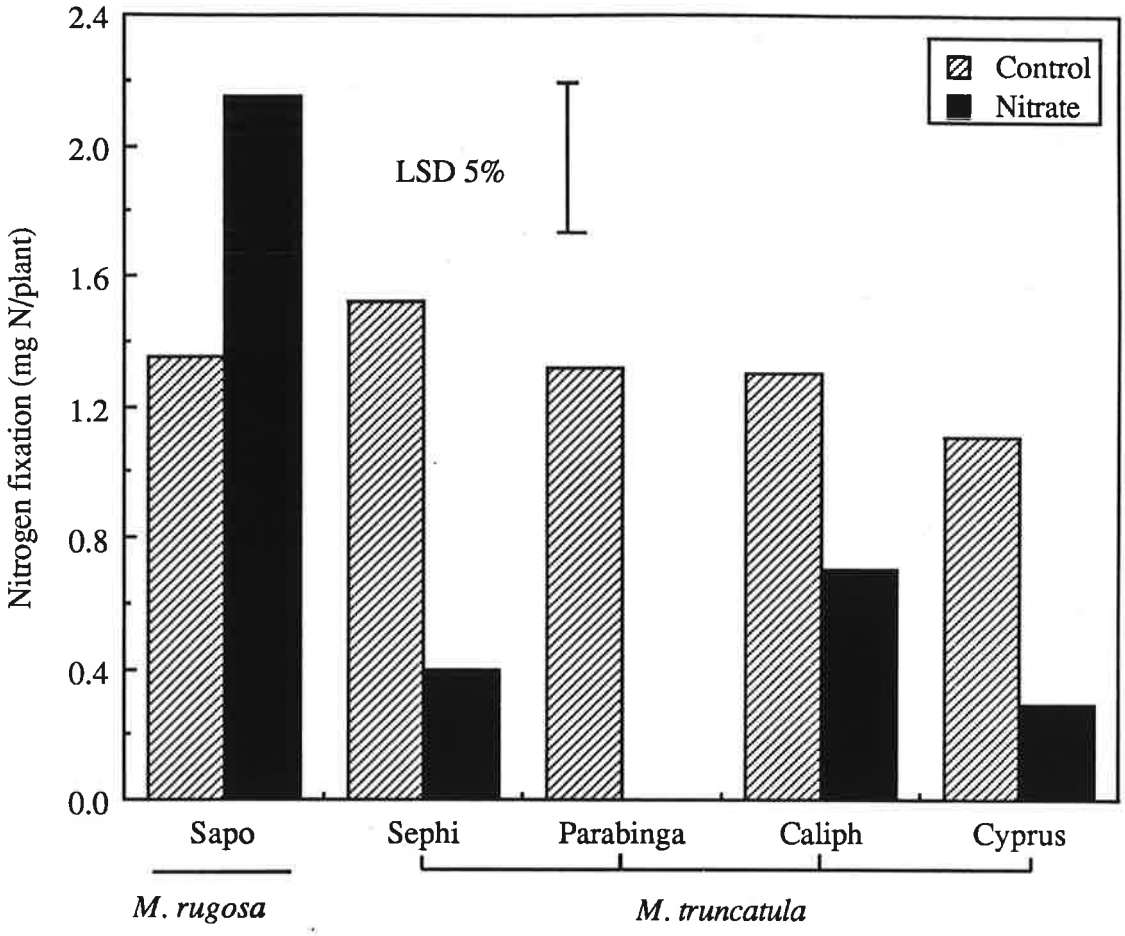


Fig. 6.2. Influence of nitrate on N_2 fixation of five annual medic cultivars.

N_2 fixation estimated between 14 and 20 DAI from data taken from Table 6.2. N_2 fixation in minus nitrate treatment was (c-a). N_2 fixation of nitrate-grown plants was determined by [d-(b+e)]. LSD is for the interaction of plant species and nitrate treatment.

in a completely randomised factorial combination of two levels of nitrate (0 and 1 mM) for the six medic cultivars were used.

6.3.2. Results

The time to nodule appearance in 0 mM nitrate (control plants) was 3 d for Serena, Harbinger and Sephi and 4 d for Sapo, Paragosa and Paraponto (Fig. 6.3). In the presence of 1 mM nitrate, the day on which nodules were first recorded on *M. rugosa* cvs. Sapo, Paragosa, *M. polymorpha* cv. Serena and *M. littoralis* cv. Harbinger was unaffected, but in Paraponto and Sephi it was delayed by 1 and 3 d respectively, compared with the controls.

Nodule number was high in Serena, Paragosa and Sapo, intermediate in Harbinger and Sephi and lowest in Paraponto in the minus nitrate solution. At 14 DAI, nitrate inhibited nodulation in Sapo, Paragosa, Paraponto, Serena, Harbinger and Sephi by 47, 56, 63, 63, 69 and 84% respectively. More than 93% of all nodules on the *M. rugosa* cultivars and 88% on *M. littoralis* cv. Harbinger and *M. polymorpha* cv. Serena grown in the presence of nitrate were pink, while in Sephi this figure was only 60%.

In the absence of nitrate, total plant dry weight was highest for Paraponto and Serena (Table 6.3), while with 1 mM nitrate, dry weights were approximately 2 times those of all the medics without nitrate, the exception being *M. truncatula* cv. Sephi where the increase was 3 times. Sephi had the highest nitrate uptake rate (expressed on the basis of final root dry weight) while *M. rugosa* cultivars (Sapo, Paragosa and Paraponto) had the lowest. Serena and Harbinger had similar rates of nitrate uptake. In the presence and absence of nitrate, the total nitrogen of the plants reflected their total dry weight.

At 0 mM nitrate, N_2 fixation was highest in Serena and similar in the other cultivars (Fig. 6.4). Nitrogen fixation was inhibited by 45, 69, 84 and 89% in Paragosa, Sapo, Paraponto and Harbinger respectively and completely in Serena and Sephi. This is a higher degree of inhibition for Sapo than in Experiment 1 (Fig. 6.2). N_2 fixation in the current experiment

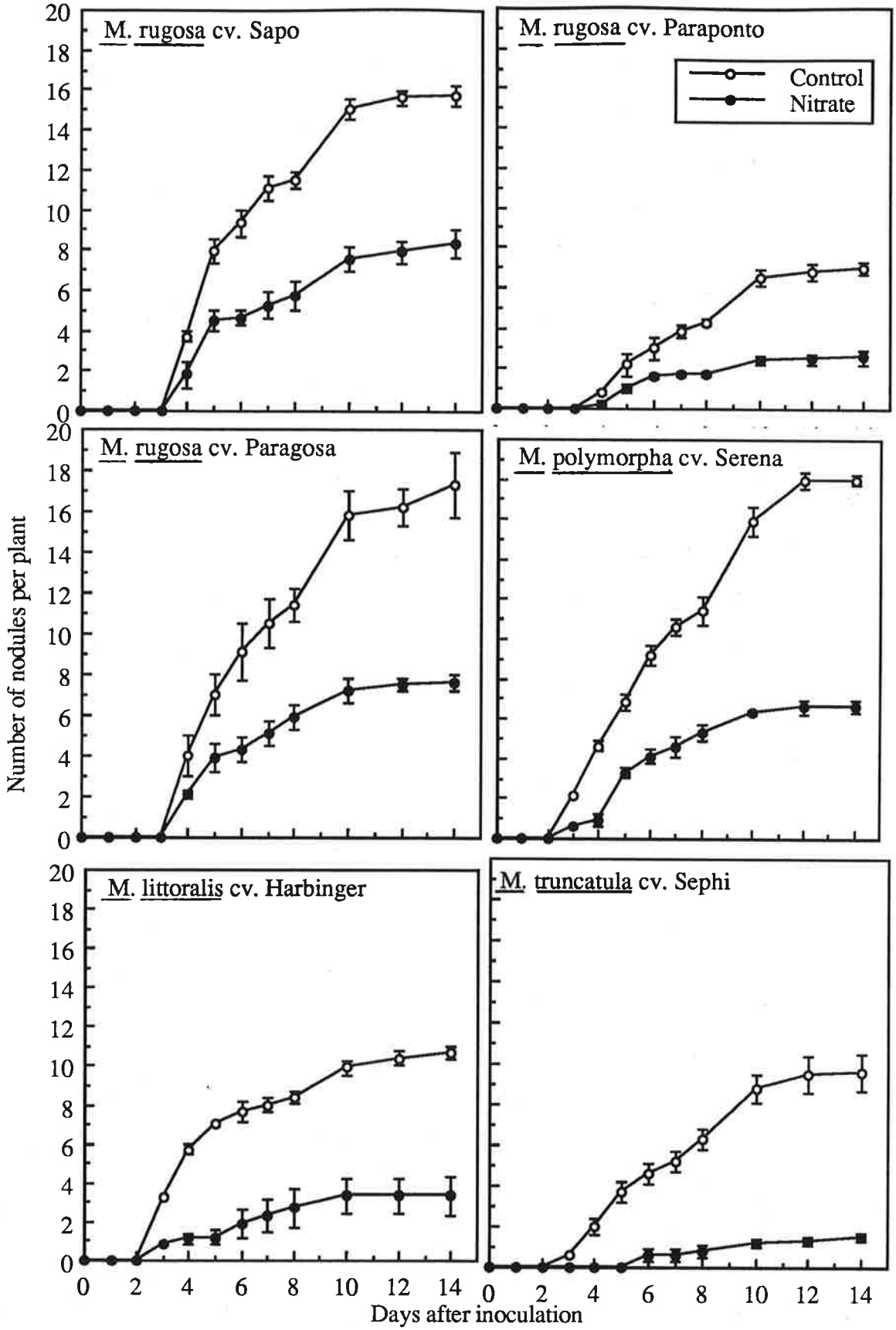


Fig. 6.3. Influence of nitrate on the number of nodules on six annual medic cultivars.

Table 6.3. Influence of nitrate on dry weight and nitrogen accumulation in medics.

The seedlings were grown in minus nitrate nutrient solution or in the presence of 1 mM nitrate and harvested 14 DAI. The dry weight data shown are means of determinations made on 40 plants. Means of the Ln transformed data for total dry weight and total nitrogen are given in parentheses. LSD data are for medic x nitrate interactions.

Nitrate treat. (mM)	Medic cultivar[a]						LSD 5%
	Sapo	Paraponto	Paragosa	Serena	Harbinger	Sephi	
Plant dry weight (mg)							
0	34 (3.5)	44 (3.8)	33 (3.5)	39 (3.7)	26 (3.3)	26 (3.3)	(0.249)
1	58 (4.1)	70 (4.2)	66 (4.2)	89 (4.5)	50 (3.9)	81 (4.4)	
Dry weight ratio (+N/-N)							
	1.7	1.6	2.0	2.3	1.9	3.1	1.02
Total nitrogen (mg/plant)							
0	1.3 (0.27)	1.6 (0.45)	1.4 (0.33)	1.8 (0.58)	1.2 (0.18)	1.1 (0.09)	(0.232)
1	2.5 (0.92)	3.1 (1.13)	2.9 (1.06)	5.0 (1.61)	2.3 (0.81)	4.5 (1.47)	
Nitrate-N uptake (mg/plant)							
1	1.79	2.25	2.02	4.82	1.96	4.16	1.02
Nitrate-N uptake (mmol/g root dry weight/d)							
1	0.66	0.65	0.81	1.05	1.08	1.42	0.095

[a]*M. rugosa* cvs. Sapo, Paragosa and Paraponto, *M. polymorpha* cv. Serena, *M. littoralis* cv. Harbinger and *M. truncatula* cv. Sephi.

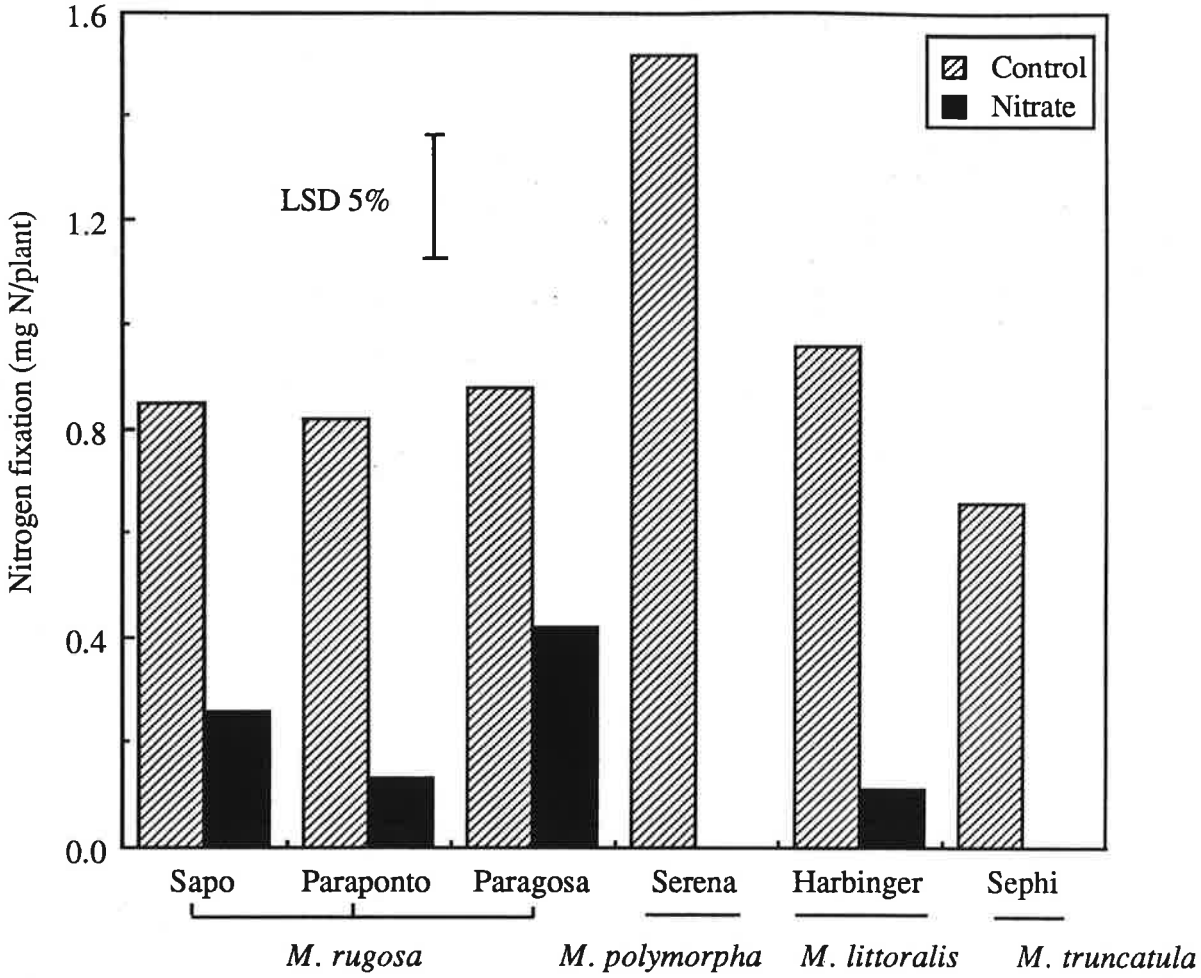


Fig. 6.4. Effect of nitrate on N_2 fixation of annual medics.

Data used were taken from Table 6.3. N_2 fixation was determined by subtracting seed N (Appendix 1) from total plant N in minus-nitrate treatments at the end of the experiment. N_2 fixation of nitrate-grown plants was determined by subtracting cumulative nitrate-N uptake and seed N from total plant N. LSD is for the interaction of plant species and nitrate treatment.

(Fig. 6.4) was assessed between 0 to 14 DAI while in Experiment 1 it was analysed from 14-20 DAI when nodules were more established.

6.4. EXPERIMENT 3: Variation in the sensitivity of nodulation to nitrate in *M. rugosa* cv. Sapo, *M. littoralis* cv. Harbinger AR, *M. polymorpha* cvs. Santiago and Circle Valley, *M. tornata* cv. Tornafield and *M. murex* cv. Zodiac.

Having established that *M. rugosa* cultivars appear more tolerant of nitrate in the nodulation phase than *M. truncatula* cultivars (Experiments 1 and 2), *M. rugosa* cv. Sapo was used as a reference to compare nodulation in cultivars from two other medic species and also an aphid resistant cultivar of *M. littoralis* cv. Harbinger. Sapo was also tested with 5 mM nitrate since Ewing and Robson (1990) reported that the level of inhibition of nodulation in *M. polymorpha* and *M. murex* at 1 mM nitrate was not markedly enhanced by higher levels of nitrate.

6.4.1. Materials and Methods

All procedures of plant establishment in the growth room and experimental design were the same as those described in Experiment 2 except that the aeration rate was 0.8 L/min. Four replicates were used except for Zodiac which had 3 replicates due to poor germination. Total nitrogen and nitrate uptake measurements were as described in Sections 3.4 and 3.5.2.

6.4.2. Results

The pattern of nodulation on *M. rugosa* cv. Sapo at 0 and 1 mM nitrate (Fig. 6.5) was consistent with that reported in Experiments 1 and 2 (Fig. 6.1 and 6.3). With 5 mM nitrate, nodule appearance on Sapo was delayed by 1 d, but at 14 DAI, nodule numbers were similar to those on the plants in the 1 mM nitrate treatment. Nodulation on *M. littoralis* cv. Harbinger AR and *M. tornata* cv. Tornafield occurred at the same time both in the presence and absence of 1 mM nitrate but was delayed by 1 d on *M. murex* cv. Zodiac and *M. polymorpha* cv. Circle Valley in the presence of nitrate. At 14 DAI, 1 mM nitrate inhibited

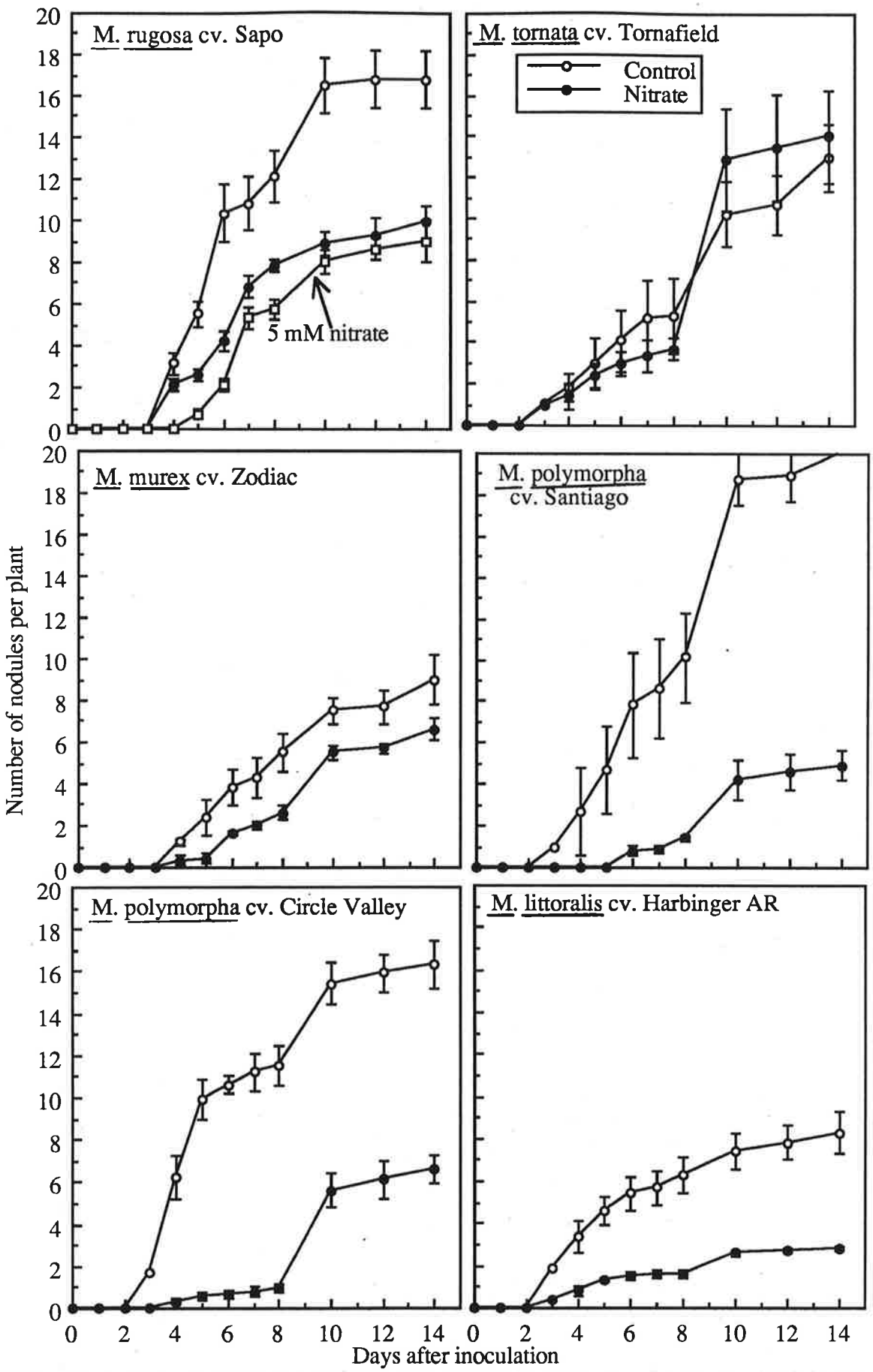


Fig. 6.5. influence of nitrate on nodule numbers of five annual medic species.

nodulation in Sapo, Harbinger AR, Santiago and Circle Valley, by 41, 66, 76 and 60% respectively, but in Zodiac by only 27%. In Tornafield there was a higher number of nodules in the nitrate treated plants. However, only 56% of the nodules in the nitrate treated plants were pink versus 83% of the control, so the nitrate inhibition with respect to pink nodules was 29%. In the other medics, the nitrate treatment had no major influence on the proportion of pink nodules, which was usually about 90% at 14 DAI.

In the absence of nitrate, Sapo had a higher total dry weight than all the other medics in this experiment (Table 6.4). Except for Zodiac however all showed a higher growth response to nitrate than Sapo. The nitrate uptake expressed on the basis of final root weight was higher in *M. polymorpha* cvs. Circle Valley and Santiago, *M. littoralis* cv. Harbinger AR and *M. tornata* cv. Tornafield than in *M. rugosa* cv. Sapo and *M. murex* cv. Zodiac (1.0-1-2 mmol/g root dry weight/day vs. 0.66-0.83). The level of N_2 fixation (Fig. 6.6) was not different between the medic species. The inhibition due to nitrate in Sapo was 69% while in Tornafield, Zodiac, Santiago, Circle Valley and Harbinger it was 76, 78, 78, 98 and 85% respectively.

6.5. EXPERIMENT 4: Nodulation, nitrogenase activity and growth responses to nitrate in *M. rugosa* cvs. Sapo, Paragosa and Paraponto, *M. tornata* cv. Tornafield, *M. truncatula* cv. Parabinga and *M. Murex* cv. Zodiac.

In this experiment the three cultivars of *M. rugosa* plus Tornafield and Zodiac, all of which were shown above to exhibit some tolerance to nitrate during nodulation, are compared further with the nitrate sensitive, *M. truncatula* cv. Parabinga in a study which includes measurements on the nitrogenase activity.

6.5.1. Materials and Methods

All procedures of plant establishment, experimental design and plant analysis were the same as those described in Experiment 1 except that nitrogenase activity (AR) and nodule dry

Table 6.4. Influence of nitrate on dry weight and nitrogen accumulation in medics.

The seedlings were grown in minus nitrate nutrient solution with 1 mM nitrate as indicated and harvested 14 DAI. The dry weight data shown are means of determinations made on 40 plants (except Zodiac 30 plants). Means of the Ln transformed data for total dry weight and total nitrogen are given in parentheses. LSD data are for medic x nitrate interactions.

Nitrate treat. (mM)	Medic cultivar[a]						LSD 5%
	Sapo	Tornafield	Zodiac	Santiago	Circle Valley	Harbinger AR	
Plant dry weight (mg)							
0	37 (3.6)	29 (3.4)	22 (3.1)	25 (3.2)	31 (3.4)	26 (3.3)	(0.19)
1	74 (4.3)	97 (4.6)	33 (3.5)	118 (4.8)	105 (4.7)	85 (4.4)	
5	75 (4.3)						
Dry weight ratio (+N/-N)							
1	2.0	3.4	1.5	4.8	3.4	3.2	0.92
5	2.0						
Total nitrogen (mg/plant)							
0	1.4 (0.36)	1.0 (0.0)	1.0 (0.01)	1.0 (0.01)	1.4 (0.32)	1.0 (0.03)	(0.214)
1	3.4 (1.21)	5.4 (1.7)	1.9 (0.61)	6.6 (1.9)	5.9 (1.8)	4.6 (0.1.5)	
Nitrate-N uptake (mg/plant)							
1	2.62	4.99	1.43	6.16	5.68	4.34	0.659
5	2.58						
Nitrate-N uptake (mmol/g root dry weight/d)							
1	0.66	1.00	0.83	1.18	1.09	1.16	0.050
5	0.61						

[a] *M. rugosa* cv. Sapo, *M. polymorpha* cvs. Santiago and Circle Valley, *M. littoralis* cv. Harbinger AR and *M. murex* cv. Zodiac.

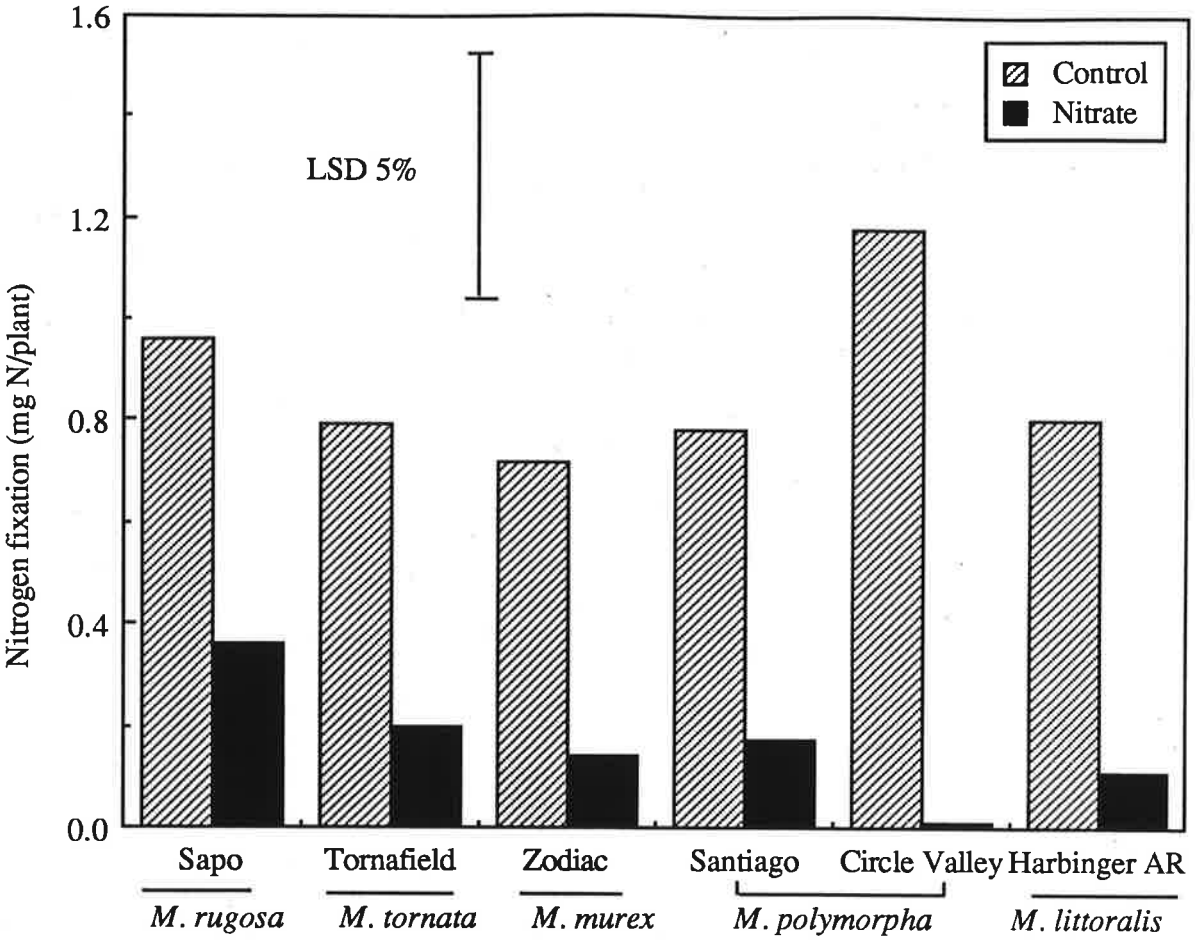


Fig. 6.6. Effect of nitrate on N_2 fixation of annual medics.

Nitrogen fixation was determined by subtracting seed N from total plant N in minus-nitrate treatment at the end of the experiment. N_2 fixation of nitrate-grown plants was determined by subtracting cumulative nitrate-N uptake and seed N from total plant N. LSD is for the interaction of plant species and nitrate treatment.

weight of the plants were measured as described in 3.6 and 3.8. Assay for nitrogenase activity (AR) was made at the end of the experiment. Excess solution was blotted from the root system of plants and whole plants incubated in 10% C_2H_2 in air in 500 mL sealed glass jars. Four replicate pots were used for all the medic cultivars except Zodiac which had 3 replicates due to poor germination.

6.5.2. Results

Nodules appeared 3 DAI on Parabinga and Tornafield (Fig. 6.7) but 1 d later on Sapo, Paragosa, Paraponto and Zodiac. Nitrate delayed nodulation in Parabinga by 3 d but had no effect on the other cultivars. At 14 DAI, inhibition of nodulation due to nitrate was approximately 30% for *M. rugosa* cvs. Sapo, Paragosa, Paraponto and *M. tornata* cv. Tornafield, 48% for *M. murex* cv. Zodiac and 90% for *M. truncatula* cv. Parabinga. Plants of Paragosa and Parabinga from this experiment are shown in Plate 4. Note also that the main root of Paragosa is thicker than that of Parabinga. This feature of Paragosa was a general characteristic of the *M. rugosa* cultivars.

In the absence of nitrate, total dry weight of the medic cultivars was Paraponto > Paragosa = Sapo > Parabinga > Zodiac > Tornafield (Table 6.5). The dry weight in *M. rugosa* cultivars (Sapo, Paragosa and Paraponto) and *M. murex* cv. Zodiac was increased 2 fold with 1 mM nitrate and 3 fold in *M. truncatula* cv. Parabinga and *M. tornata* cv. Tornafield. The shoot : root ratio in nitrate grown plants was significantly higher than those in the nitrate-minus grown plants (data not shown). Nodule dry weights were highest in Paraponto and lowest in Tornafield. In the presence of nitrate, nodule dry weight was reduced on all cultivars, but with *M. rugosa* cultivars the reduction (50%) was less than that found for Zodiac and Tornafield (67%); for Parabinga the few nodules in the presence of nitrate were very small, and a dry weight determination was not possible. Uptake of nitrate (per g final root dry weight) also differed significantly between medic cultivars (Table 6.5), the *M. rugosa* cultivars showed a lower rate than the other cultivars, especially *M. truncatula* cv. Parabinga.

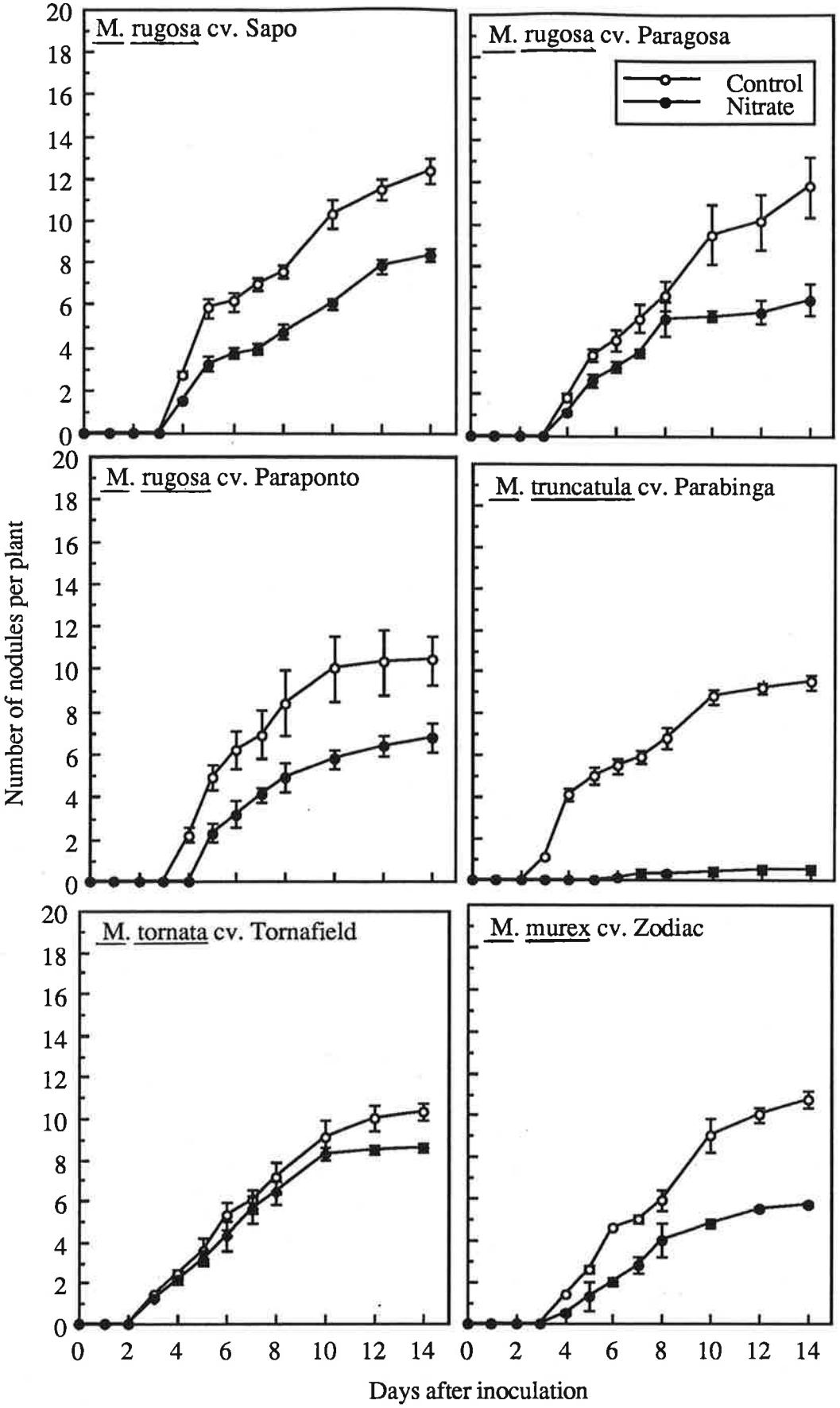


Fig 6.7. Influence of nitrate on the number of nodules in medics.

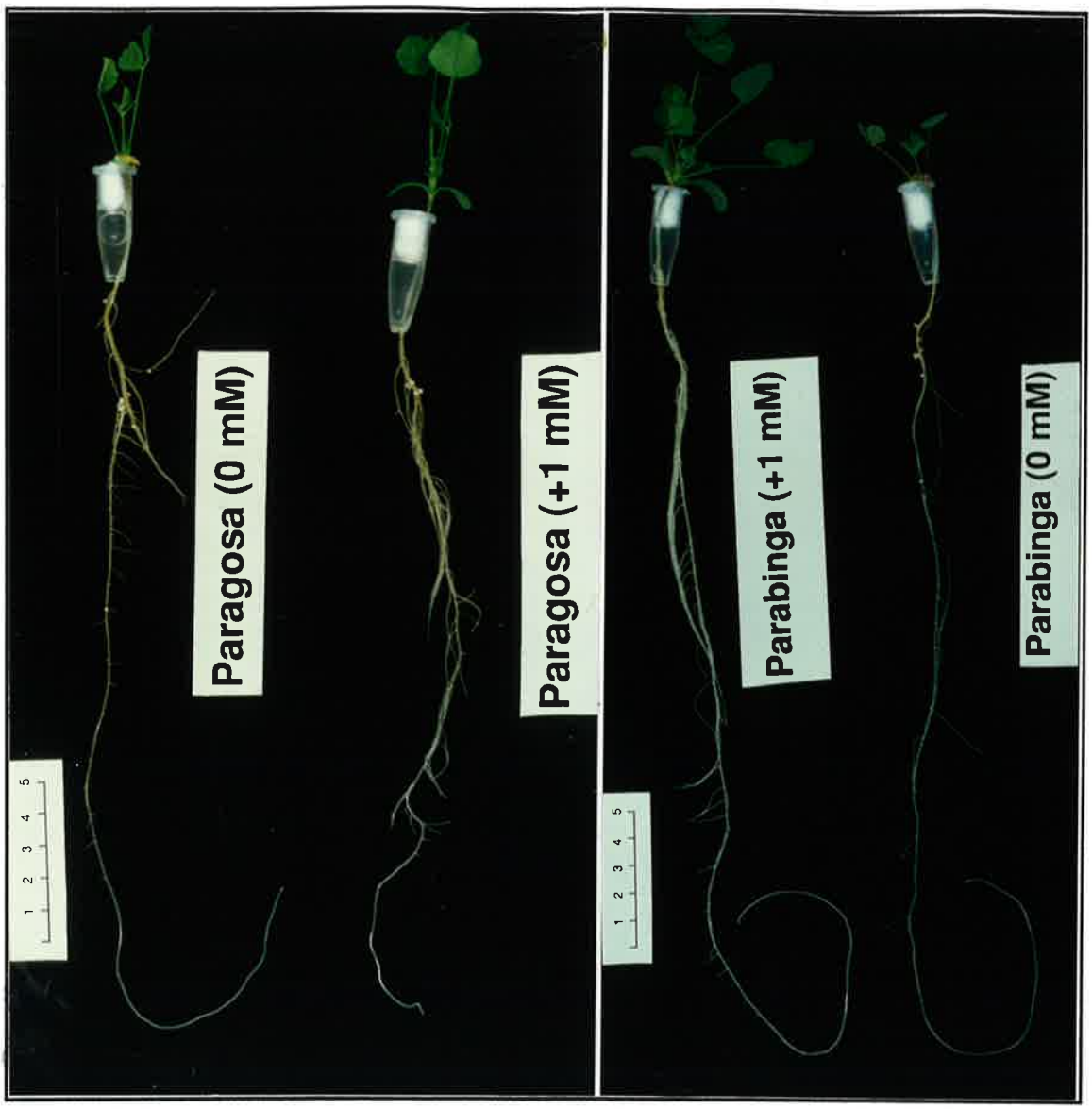


Plate 4. Plants of *M. rugosa* cv. Paragosa and *M. truncatula* cv. Parabinga at 14 DAI and grown in hydroponics with 0 and 1 mM nitrate added (Exp. 4).

Table 6.5. Influence of nitrate on dry weight and nitrogen accumulation in medics.

The seedlings were grown in minus nitrate nutrient solution or with 1 mM nitrate as indicated and harvested 14 DAI The dry weight data shown are means of determinations made on 40 plants (except Zodiac, 30 plants). Means of the Ln transformed data for total dry weight and total nitrogen are given in parentheses. LSD data are for medic x nitrate interactions.

Nitrate treat. (mM)	Medic cultivar[a]						LSD 5%
	Sapo	Paragosa	Paraponto	Parabinga	Tornafield	Zodiac	
Plant dry weight (mg)							
0	37 (3.6)	37 (3.6)	50 (3.9)	30 (3.4)	22 (3.1)	27 (3.3)	(0.14)
1	72 (4.3)	70 (4.2)	96 (4.6)	81 (4.4)	64 (4.2)	57 (4.0)	
Dry weight ratio (+N/-N)							
1	2.0	1.9	1.9	2.7	2.9	2.1	0.92
Nodule dry weight (mg/nodule)							
0	0.13	0.16	0.21	0.14	0.09	0.13	0.046
1	0.08	0.10	0.10	0.00	0.03	0.05	
Total nitrogen (mg/plant)							
0	1.2 (0.18)	1.3 (0.26)	1.5 (0.40)	1.1 (0.10)	0.7 (-0.36)	1.2 (0.19)	(0.183)
1	3.2 (1.2)	3.5 (1.2)	4.6 (1.5)	3.9 (1.4)	3.3 (1.2)	3.0 (1.1)	
Nitrate-N uptake (mg/plant)							
1	2.56	2.62	3.26	3.74	3.09	2.60	0.319
Nitrate-N uptake (mmol/g root dry weight/d)							
1	0.62	0.73	0.58	1.21	0.91	0.99	0.062

[a]*M. rugosa* cvs. Sapo, Paragosa and Paraponto, *M. tornata* cv. Tornafield, *M. truncatula* cv. Parabinga and *M. murex* cv. Zodiac.

In the absence of nitrate, there were no differences in N_2 fixation between *M. rugosa* cultivars, *M. truncatula* cv. Parabinga and *M. murex* cv. Zodiac (Fig. 6.8). Nitrate resulted in decreased N_2 fixation in all the plants. However the N_2 fixation of the *M. rugosa* cultivars was less affected than that of *M. truncatula* cv. Parabinga; the latter was completely inhibited by nitrate.

Nitrogenase activity (AR) was significantly different between medic species (Fig. 6.9). Without added nitrate, Paragosa, Paraponto and Zodiac had a higher rate of nitrogenase activity than the other cultivars. Nitrogenase activity of plants grown in the presence of nitrate was markedly decreased, especially in Parabinga and Tornafield. The specific AR activity (μmol/g nodule dry weight) of the *M. rugosa* cultivars was little affected by nitrate whereas there was considerable inhibition of the specific AR activity in the other species examined. The nodule number in *M. tornata* cv. Tornafield was not markedly reduced by the nitrate addition (Fig. 6.7) but nitrogenase activity appeared to be severely inhibited (Fig. 6.9). It was indicated above (see Section 6.4.2) that a small proportion of the nodules on Tornafield were pink compared to the other medics.

6.6. EXPERIMENT 5: Sensitivity of nodulation and nitrogenase activity to nitrate among four annual medic species and a comparison with lucerne (*M. sativa*) and subterranean clover (*Trifolium subterraneum*).

In this final experiment on the sensitivity of nodulation and nitrogenase activity (AR) to nitrate in annual medics, a further comparison was included with lucerne (*M. sativa* L.) and subterranean clover (*Trifolium subterraneum* L.).

6.6.1. Materials and Methods

Subterranean clover (*Trifolium subterraneum* cv. Woogenellup), *Medicago rugosa* cv. Paraponto), *Medicago scutellata* cv. Kelson, *Medicago tornata* cv. Tornafield, *Medicago littoralis* cv Harbinger and *Medicago sativa* cv. Hunter River were germinated in steam sterilised sand and transferred to pots containing 1.5 L of nutrient solution with or without 1

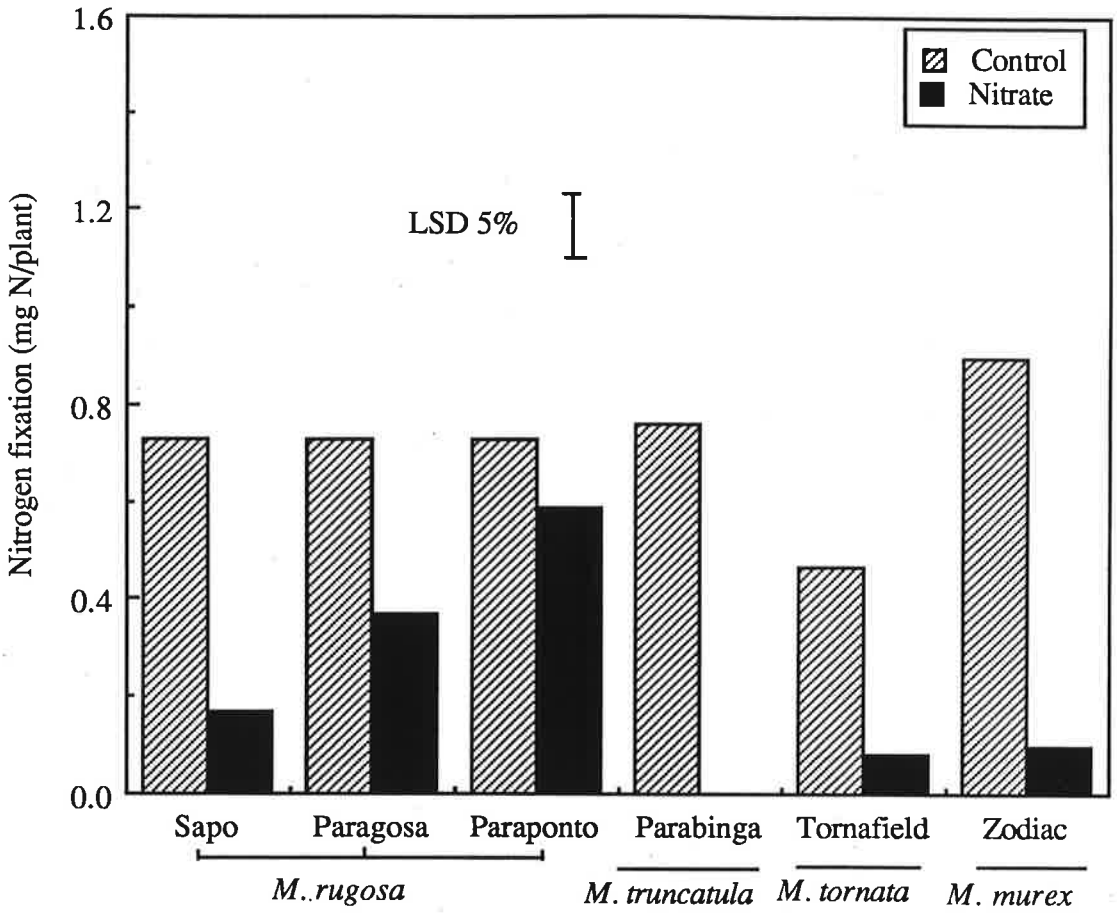


Fig. 6.8. Effect of nitrate on N_2 fixation of annual medics.

Data used were taken from Table 6.5. N_2 fixation was determined by subtracting seed N (Appendix 1) from total N in minus-nitrate treatment at the end of the experiment. N_2 fixation of nitrate grown plants was determined by subtracting cumulative nitrate-N uptake and seed N from total plant N. LSD is for the interaction of plant species and nitrate treatment.

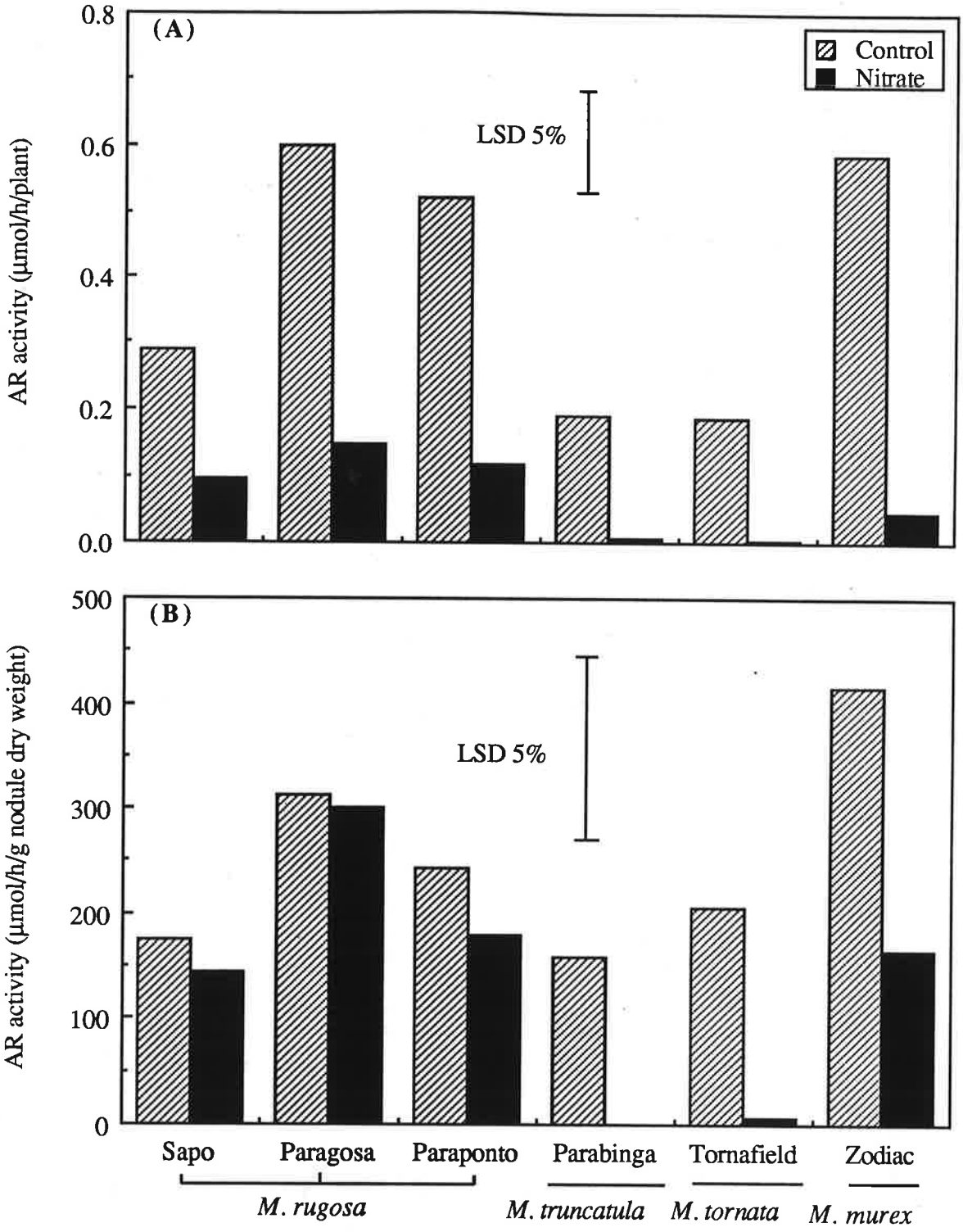


Fig. 6.9. Influence of nitrate on nitrogenase activity (AR) of medics.

The plants were grown as described in Table 6.5. Total nitrogenase activity (A) and its specific activity (B) are shown. LSD are for medic x nitrate interactions.

mM nitrate as described in Section 3.3. The nutrient solution was renewed as described in Section 6.3.1. The subterranean clover was inoculated with *Rhizobium trifolii* strain WU95. In this experiment the medics were inoculated as described in Section 3.1.1 with *Rhizobium meliloti* strain WSM826, the recommended strain for *M. littoralis*, *M. rugosa*, *M. scutellata* and *M. sativa* in southern Australia. Nitrogenase activity (AR) of the plants was estimated by the acetylene reduction technique in a 500 mL glass jar as described in Section 3.6. After the assay, plants were separated into shoot, nodule and root fractions and dried (see Section 3.8). Total nitrogen, nitrate concentration of the shoots and roots and nitrate uptake of the plants were determined as described in Sections 3.4, 3.5 and 3.5.2 respectively. The experiment was analysed as a split-plot using nitrate treatments as the main plot and plant species as the subplot and replicated 4 times.

6.6.2. Results

At 0 mM nitrate, the time from inoculation to first nodule appearance was 3 d or less for all the annual medics and lucerne (Fig. 6.10). Nodule appearance in response to 1 mM nitrate however varied among species: it was delayed by 1 d on *M. rugosa* cv. Paraponto and *M. sativa* cv. Hunter River while *M. scutellata* cv. Kelson showed a 2 d delay. At 14 DAI, nodulation in *M. rugosa* cv. Paraponto, *M. tornata* cv. Tornafield and *M. scutellata* cv. Kelson was inhibited by about 30% by nitrate whereas in *M. littoralis* cv. Harbinger there was a 70% inhibition. The rate of nodulation in subterranean clover with nitrate after a delay of seven d was the same as that in the control and was only inhibited by 30% at 14 DAI. Nodulation in *M. sativa* cv. Hunter River was extremely sensitive to nitrate.

Total dry weight was highest in Kelson and lowest in Hunter River and Harbinger in the nitrate-free treatments (Table 6.6). The relative increase in dry weight due to 1 mM nitrate was less in Kelson, Paraponto and subterranean clover than in the other cultivars. In the absence of nitrate, Kelson and Paraponto had the highest nodule dry weight; subterranean clover was intermediate while Harbinger, Tornafield and Hunter River had the lowest. In the presence of nitrate, nodule dry weight was inhibited by approximately 70% in Kelson,

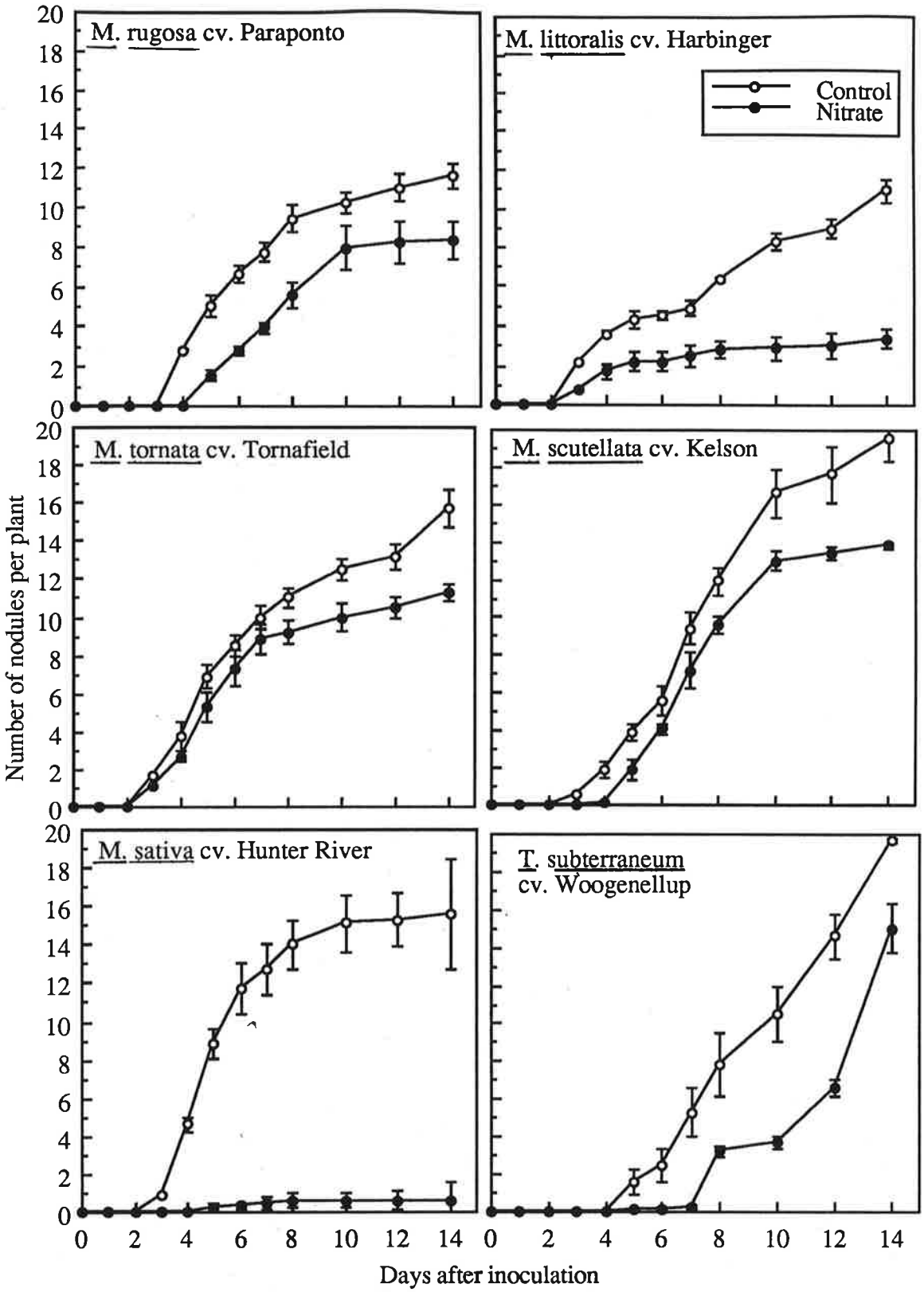


Fig. 6.10. Influence of nitrate on the number of nodules on annual medics, lucerne and subterranean clover.

Table 6.6. Influence of nitrate on dry weight and nitrogen accumulation in medics, lucerne and subterranean clover.

The seedlings were grown hydroponically with or without 1 mM nitrate and harvested 14 DAI. The dry weight data shown are means of determinations made on 40 plants. Means of the Ln transformed data for total dry weight and total nitrogen used for statistical analysis are given in parentheses. LSD data are for plant species x nitrate interactions.

Nitrate treat. (mM)	Plant cultivar[a]						LSD 5%
	Paraponto	Harbinger	Tornafield	Kelson	Hunter River	Sub. clover	
Plant dry weight (mg)							
0	67 (4.2)	16 (2.8)	25 (3.2)	131 (4.9)	17 (2.8)	56 (4.0)	(0.193)
1	110 (4.7)	86 (4.4)	92 (4.5)	194 (5.3)	63 (4.1)	121 (4.8)	
Dry weight ratio (+N/-N)							
1	1.6	5.4	3.7	1.5	3.7	2.2	1.22
Nodule dry weight (mg/nodule)							
0	0.26	0.08	0.09	0.26	0.07	0.16	0.032
1	0.07	0.04	0.03	0.07	0.00	0.08	
Total nitrogen (mg/plant)							
0	1.9 (0.62)	0.7 (-0.31)	0.7 (-0.31)	4.1 (1.39)	0.5 (-0.73)	1.8 (0.59)	(0.188)
1	4.6 (1.52)	4.3 (1.44)	4.3 (1.46)	9.2 (2.21)	2.9 (1.06)	5.2 (1.64)	
Nitrate-N uptake (mg/plant)							
1	3.74	4.07	3.96	7.76	2.78	4.37	0.691
Nitrate-N uptake (mmol/g root dry weight/d)							
1	0.60	1.00	0.85	0.79	1.05	0.73	0.110
Shoot nitrate (µmol/g shoot dry weight)							
1	102	193	122	346	312	195	51.36
Root nitrate (µmol/g root dry weight)							
1	698	656	646	927	807	713	75.89

[a]*M. rugosa* cv. Paraponto, *M. littoralis* cv. Harbinger, *M. tornata* cv. Tornafield, *M. scutellata* cv. Kelson, *M. sativa* cv. Hunter River and *T. subterraneum* cv. Woogenellup

Tornafield and Hunter River, by 77% in Paraponto and by 50% in Harbinger and subterranean clover respectively.

At 14 DAI, total nitrogen contents of the minus-nitrate plants was Kelson > Paraponto > subterranean clover > Harbinger = Tornafield > Hunter River. Total nitrogen of nitrate grown plants was significantly higher than that of minus-nitrate grown plants. It was more than doubled in Paraponto, Kelson and subterranean clover while in Harbinger, Tornafield and Hunter River it was 6-7 times that of control plants. Hunter River and Harbinger took up nitrate at the highest rate, approximately 1.0 mmol/g root dry weight/day. In Paraponto, Tornafield, Kelson and subterranean clover nitrate uptake rates were 0.60, 0.85, 0.79 and 0.73 mmol/g root dry weight/day respectively. The nitrate concentration in root tissues was higher than those in the shoots in all species tested. Kelson and Hunter River had higher nitrate concentrations in their shoots and roots than the other species while Paraponto and Tornafield had the lowest nitrate concentration in their tissues.

At 0 mM nitrate, N_2 fixation varied significantly between the cultivars (Fig. 6.11). *M. scutellata* cv. Kelson fixed 2.8 mg N/plant while *M. rugosa* cv. Paraponto and *T. subterraneum* cv. Woogenellup fixed approximately 1.1 mg N/plant. The remaining cultivars fixed less than 0.5 mg N/plant. N_2 fixation was inhibited in all cultivars in the presence of nitrate. The inhibition was more severe in Kelson and Hunter River (> 95%) than the other cultivars (50-70%).

In the absence of nitrate, *M. scutellata* cv. Kelson had the highest nitrogenase activity (Fig. 6.12). Nitrogenase activity in Paraponto and subterranean clover were similar but lower than Kelson; in the other species it was even lower. In all species the presence of nitrate resulted in a decrease in nitrogenase activity, the inhibition ranging from 55% in subterranean clover to 100 % in *M. sativa.* In the absence of nitrate, specific AR activity (μmol/g nodule dry weight) was the highest in Kelson. There were no significant differences between the other species. 1 mM nitrate inhibited specific AR activity in *M.*

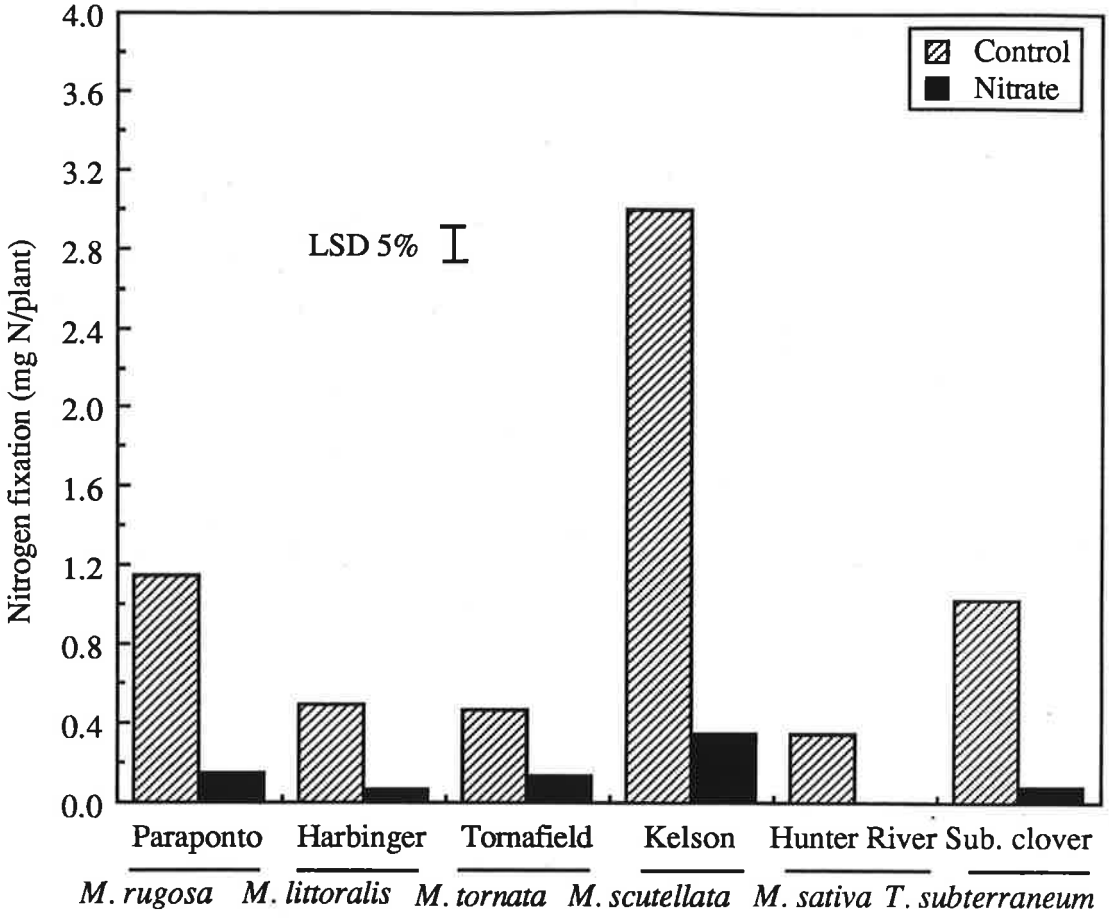


Fig. 6.11. Influence of nitrate on N_2 fixation of four cultivars of annual medics, lucerne and subterranean clover.

Data used were taken from Table 6.6. N_2 fixation was determined by subtracting seed N (Appendix 1) from total N in minus-nitrate treatment at the end of the experiment. N_2 fixation of nitrate grown plants was determined by subtracting cumulative nitrate-N uptake and seed N from total plant N. LSD is for the interaction of plant species and nitrate treatment.

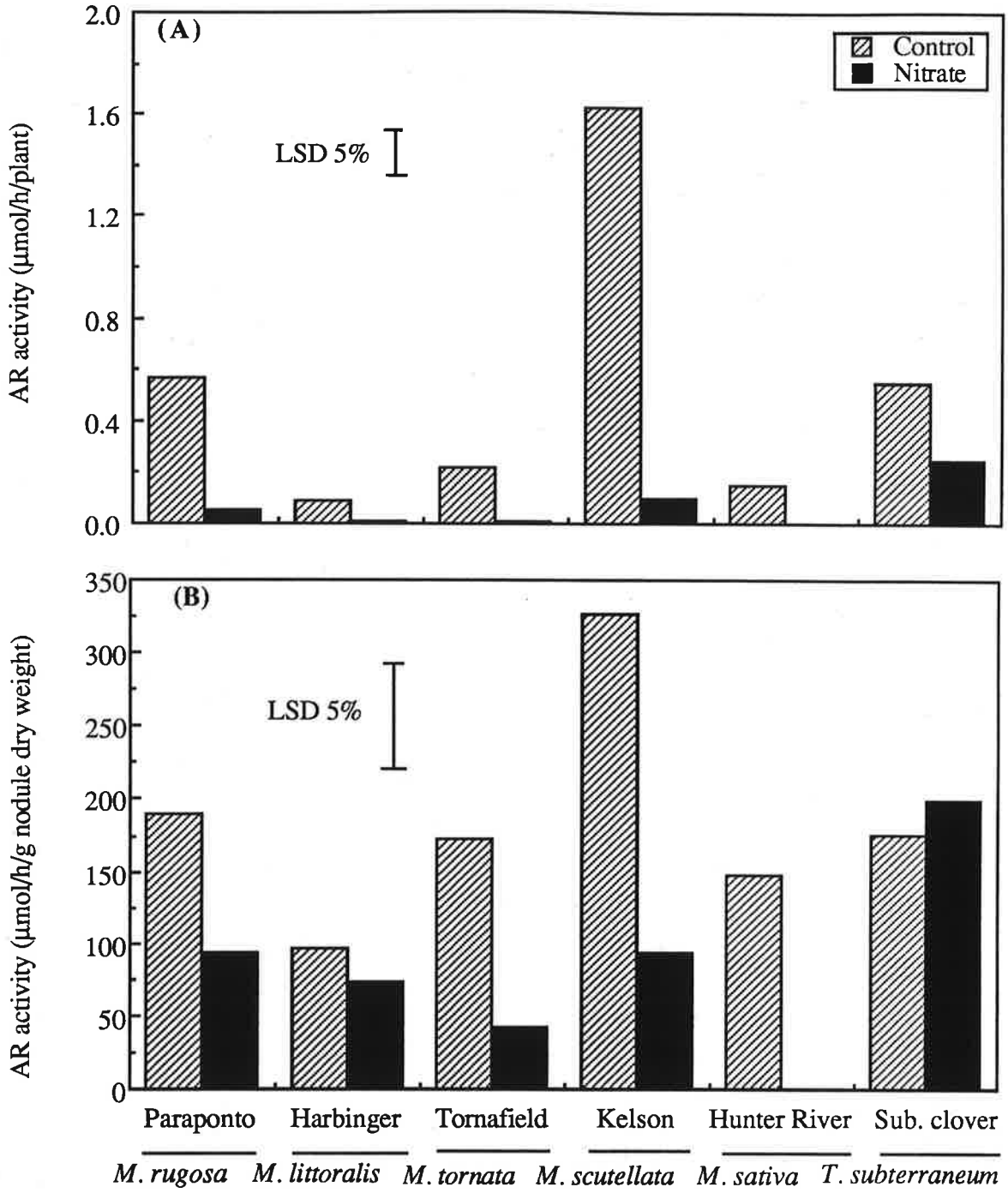


Fig. 6.12. Influence of nitrate on nitrogenase activity (AR) of four cultivars of annual medics, lucerne and subterranean clover.

The plants were grown as described in Table 6.6. Total nitrogenase activity (A) and its specific activity (B) are shown. LSD are for interaction of the plant species and nitrate treatments.

tornata cv. Tornafield by 75%, whereas specific AR activity in subterranean clover was 13% higher than in controls. In Paraponto, Harbinger and Kelson the inhibition of specific AR activity was 50, 14 and 72% of that of control plants respectively.

6.7. EXPERIMENT 6: Nitrate uptake in *M. rugosa* cv. Sapo, *M. polymorpha* cv. Serena and *M. truncatula* cv. Parabinga.

Results from the previous experiments showed that nodulation and N_2 fixation of *M. truncatula* cultivars were more sensitive to nitrate than that of *M. rugosa* cultivars. Differences in nodulation and N_2 fixation between annual medics could be related to a different rate of nitrate uptake. This experiment was done to evaluate the rate of nitrate uptake in three well nodulated medics.

6.7.1. Material and Methods

All procedures of plant establishment in the growth room were the same as those described in Section 3.3. *M. rugosa* cv. Sapo, *M. polymorpha* cv. Serena and *M. truncatula* cv. Parabinga were grown hydroponically (6 plants per pot in a minus-nitrate nutrient solution) and inoculated with *Rhizobium meliloti* strain WSM540. At 26 DAI, when plants were well nodulated, three nitrate treatments (0.25, 0.5 and 1 mM) were introduced. Measurements of nitrate uptake were made at 6, 12, 24, 30 and 36 h at which times the nutrient solutions were renewed. Shoot and root dry weight were determined as described in Section 3.8. Four replicates in a completely randomised factorial combination for the three levels of nitrate and the three medic cultivars were used.

6.7.2. Results

Dry matter accumulation in shoots and roots (data not shown) during the 36 h period of 0.25, 0.50 and 1.0 mM nitrate application was not significantly different between the medic cultivars. The rate of nitrate uptake, as calculated from the depletion of nitrate from solution

(Fig 6.13) was higher in the light period. That in *M. rugosa* cv. Sapo fluctuated less than those of Serena and Parabinga especially at 0.25 and 0.50 mM nitrate treatments.

The effect of external nitrate concentration on nitrate uptake rate was examined in each medic. The mean uptake rate in Sapo was approximately 20% lower than that of Serena and Parabinga (Fig. 6.14). The data were further analysed by a Lineweaver-Burk plot (Fig. 6.15) which allows calculation of V_{max} and the apparent K_m (Stryer 1981). For Sapo, the V_{max} for nitrate uptake was 52 µmol/h/g root dry weight and the apparent K_m 0.11 mM. For Serena and Parabinga the values for V_{max} were 70 and 72 and the K_m was 0.14 .

6.8. EXPERIMENT 7: Nitrate reductase activity in the root and shoot of four medic species.

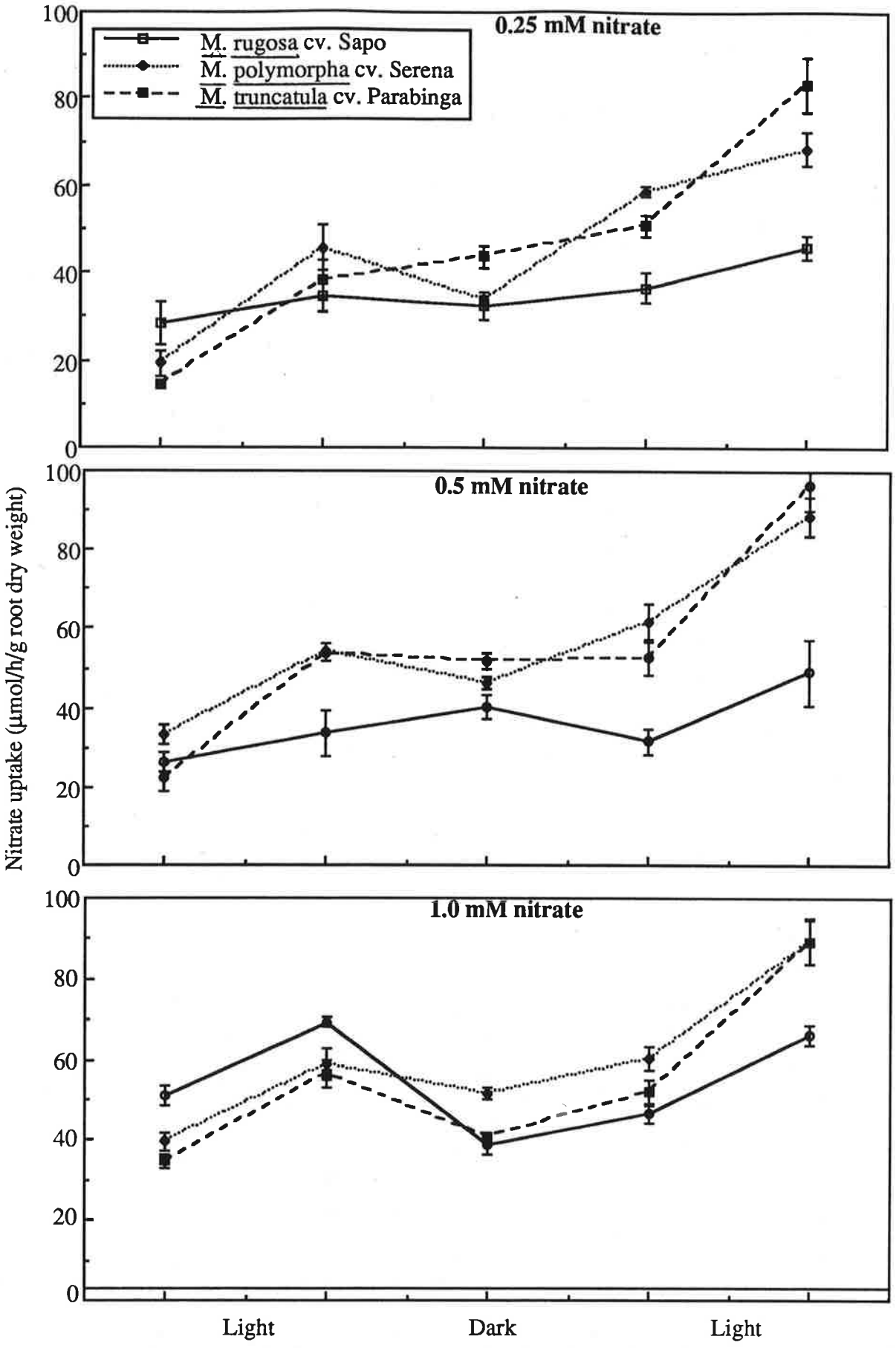
The reduction of nitrate and resultant competition for photosynthate and reductant is one explanation for the inhibitory effect of nitrate on N_2 fixation (see Section 2.7.3). It has been shown that nodulation of *M. rugosa* cv. Sapo, *M. murex* cv. Zodiac and *M. tornata* cv. Tornafield is more tolerant to nitrate than the other medics, e.g. *M. truncatula* cv. Parabinga and this could be related to the apparent lower rate of nitrate uptake in these medics. The level of nitrate reductase could also have an influence and thus in this experiment a comparative study of the influence of nitrate on distribution of nitrate reductase activity (NADH-NR) in the root and shoot of the above four medic cultivars was made.

6.8.1. Materials and Methods

Ten seedlings of *M. rugosa* cv. Sapo, *M. truncatula* cv. Parabinga, *M. murex* cv. Zodiac and *M. tornata* cv. Tornafield were grown in minus-nitrate nutrient solution as described in Section 3.3 with *R. meliloti* strain WSM540 used as inoculum. At 16 DAI, 1 mM nitrate was introduced. NR activity in the shoot and root of the plant was made 2 d after nitrate application as described in Section 3.7. Nitrate accumulation and nitrate uptake were

Fig. 6.13. Time course of nitrate uptake in medics.

At 26 DAI nitrate at the level indicated was added to the nutrient solution (six plants per pot) with other conditions as described in Section 6.8.1. Nitrate uptake was measured at 6, 12, 24, 30 and 36 h as described in Section 3.5.2. and that over the intervals from the period measurement expressed relative to the final root dry weight at 36 h. Data are shown on the graph at the mid point in the interval concerned. The nutrient solution was also renewed at the above times. Each point represents the mean of four replicates while bars indicate ±S.E.


0.25 mM nitrate
M. rugosa cv. Sapo
M. polymorpha cv. Serena
M. truncatula cv. Parabinga
0.5 mM nitrate
1.0 mM nitrate
Nitrate uptake (µmol/h/g root dry weight)
0
20
40
60
80
100
Light
Dark
Light

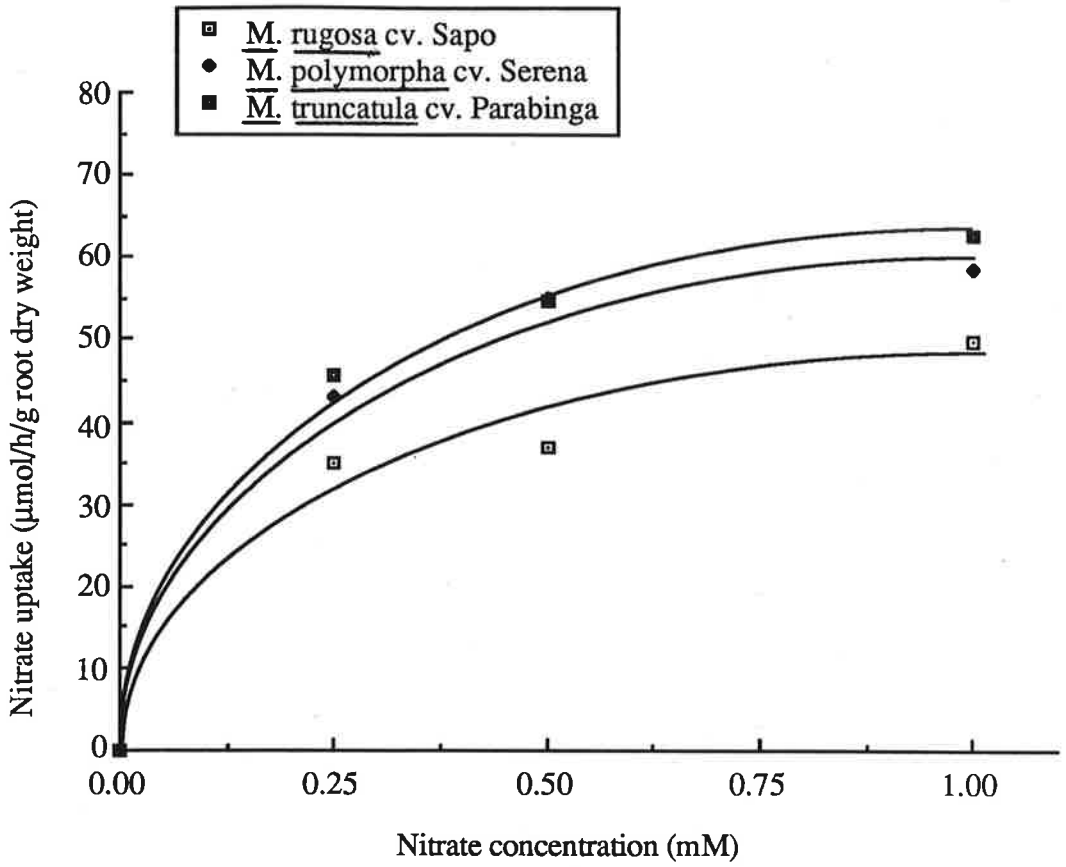


Fig. 6. 14. Nitrate uptake in three annual medic cultivars.

Data for nitrate uptake over the 36 h period after its addition were taken from Fig. 6.13 and the average of the uptake rate over each interval of analysis presented.

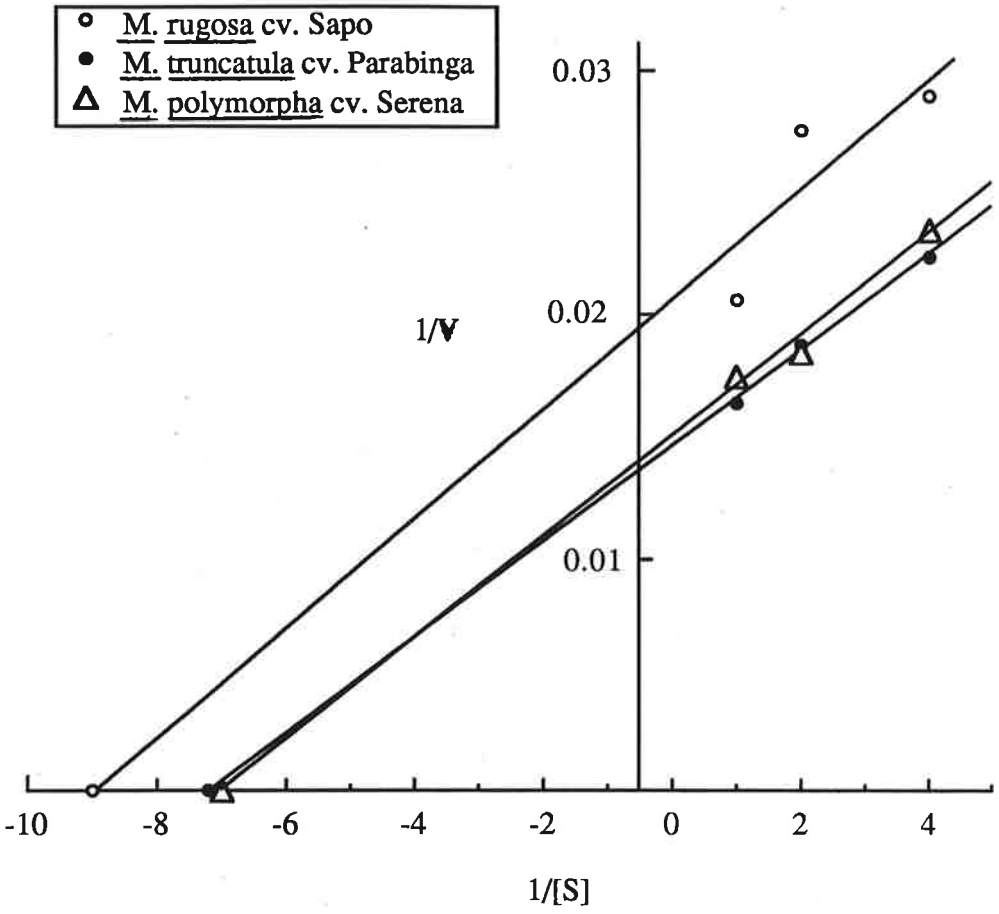


Fig. 6.13. Lineweaver-Burk plot for data on uptake in annual medics.

Data were taken from Fig 6.12.

determined as described in Sections 3.5 and 3.5.2. AR activity in the root and nodule of the plants grown in minus nitrate solution was also determined.

6.8.2. Results

Nitrate reductase activity in the shoot of Sapo and Parabinga was 3.9 and 4.3 µmol nitrite/h/g fresh weight respectively, while for Tornafield and Zodiac it was approximately 2.8 (Table 6.7). There was more variation in the NR activity in the root and Parabinga had the lowest value 15% of the total NR in this plant being in the root. In the other medic cultivars 31-54% of the total NR was in the root. It was again confirmed that Sapo had a lower rate of nitrate uptake than the other medic cultivars. There was also a corresponding lower level of nitrate accumulation in the shoot and root of Sapo. A similar level of nitrate accumulation occurred in the other medics with approximately half being in the root and shoot in all cases.

In control plants grown without added nitrate (nutrient solution contained approximately 0.004 mM nitrate) nitrate reductase was detected in all nodule and root samples (Table 6.8), being highest in Sapo. The nodule cytosol NR per plant (Table 6.8) was 3-5% of that in the equivalent medic root of plants treated with 1 mM nitrate for 2 d (Table 6.7).

6.9. Discussion

This study suggests that variability does exist in the nodulation response of annual medics to nitrate. Nitrate had less effect on the nodulation of *M. rugosa* cultivars than on *M. truncatula* cultivars and some tolerance to nitrate was also observed for *M. tornata* cv. Tornafield, *M. scutellata* cv. Kelson and *M. murex* cv. Zodiac. Other medics tested had intermediate degrees of tolerance.

The addition of nitrate delayed the appearance of the first nodules in some medics and slowed the subsequent rate of appearance of new nodules especially in cultivars of *M. truncatula, M. polymorpha* and *M. littoralis*. This suggests that some of the early stages

Table 6.7. Nitrate uptake and nitrate reductase activity in four medic cultivars.

The plants were grown hydroponically without nitrate for 16 DAI and then exposed to 1 mM nitrate for 2 d. Nitrate uptake was monitored by nitrate depletion from the growth medium. Nitrate reductase (NADH dependent activity) was measured by an *in vitro* procedure and the same extracts were used for nitrate accumulation in plant tissues. Percentage distribution of nitrate and NR in the shoot and root are shown in parenthesis.

Plant part	Fwt[a]. (g)	Nitrate uptake (µmol/g root Fwt/day)	Nitrate accumulation µmol g Fwt.	Nitrate accumulation Total	Nitrate reductase (µmol nitrite/h) g Fwt.	Nitrate reductase Total
Medicago rugosa cv. Sapo						
Shoot	0.28		9.7	2.7 (43)	3.9	1.1 (69)
Root	0.31	33.3	11.6	3.6 (57)	1.6	0.5 (31)
M. truncatula cv. Parabinga						
Shoot	0.25		19.3	4.8 (53)	4.3	1.1 (85)
Root	0.25	53.1	17.2	4.3 (47)	1.0	0.2 (15)
M. tornata cv. Tornafield						
Shoot	0.23		16.0	3.7 (45)	2.7	0.6 (46)
Root	0.25	58.4	18.4	4.6 (55)	3.0	0.7 (54)
M. murex cv. Zodiac						
Shoot	0.23		19.3	4.4 (48)	2.8	0.6 (60)
Root	0.21	51.6	22.2	4.7 (52)	2.1	0.4 (40)
LSD 5% shoot			4.25	1.24	1.34	0.37
LSD 5% root			8.04	1.95	0.54	0.12

[a]Fresh weight/plant

Table 6.8. Nitrate reductase activities in four annual medic cultivars grown without nitrate.

The plants were grown hydroponically for 19 DAI without added nitrate, but the solution did contain 0.004 mM nitrate. Nitrate reductase (µmol nitrite/h) was measured by an *in vitro* procedure using NADH as electron donor. The same extracts were used for nitrate accumulation in plant tissues.

	Plant part	Fwt[a]. (g)	Nitrate accumulation (µmol/g fresh weight)	Nitrate reductase (µmol nitrite/h) g Fwt.	Total
Medicago rugosa cv. Sapo					
	Nodule	0.045	-	0.49	0.022
	Root	0.120	0.08	0.75	0.090
M. truncatula	Nodule	0.016	-	0.39	0.006
cv. Parabinga	Root	0.121	0.09	0.32	0.038
M. tornata	Nodule	0.018	-	0.14	0.003
cv. Tornafield	Root	0.098	0.14	0.43	0.042
M. murex	Nodule	0.024	-	0.97	0.023
cv. Zodiac	Root	0.116	0.06	0.18	0.020

[a]Fresh weight/plant

leading to nodule formation in these cultivars may be more sensitive to nitrate than the early stages are in the other annual medics. Several investigators demonstrated that the greatest inhibitory effects of nitrate are on infection events during the first days after inoculation. For example, Dazzo and Brill (1978) and Dazzo *et al.* (1981) found that nitrate inhibited nodulation in white clover by altering the composition of the plant recognition component thus preventing attachment and invasion of the root hair by *Rhizobium.* Munns (1968a, b) also reported that root hair curling and formation of infection threads were more sensitive to nitrate than later stages of nodulation.

In the majority of the medics studied there was a decrease in the overall rate of nodule production 8-10 DAI. The formation of the first nodules has been suggested to have an inhibitory effect on further infection and nodule development (Dart and Pate 1959, Roughley *et al.* 1970). Mature nodules were reported to elicit feedback regulation of nodule number in lucerne (Caetno-Anolles and Gresshoff 1991) and soybean (Caetno-Anolles *et al.* 1991).

Nodulation of *M. rugosa* cvs. Sapo, Paragosa and Paraponto was more tolerant to nitrate than that of *M. truncatula* cvs. Parabinga, Sephi, Caliph and Cyprus. Nodule number was decreased by more than 80% in the *M. truncatula* cultivars but only by 30% in *M. rugosa* cultivars. Nodulation of *M. tornata* cv. Tornafield, *M. murex* cv. Zodiac and *M. scutellata* cv. Kelson was relatively tolerant to nitrate and was inhibited by 30-40%, whereas nodulation of *M. polymorpha* cvs. Serena, Circle Valley and Santiago and *M. littoralis* cv. Harbinger was decreased by 40-70%. Ewing and Robson (1990) also reported that nodulation of *M. truncatula* cv. Cyprus was more sensitive to nitrate than that of *M. murex* cv. N3172 and *M. polymorpha* cv. Santiago. A comparison of nodulation in annual medics with *Trifolium subterraneum* and *M. sativa* showed that *M. scutellata* cv. Kelson and *M. rugosa* cv. Paraponto had similar tolerance to nitrate as subterranean clover, while *M. littoralis* cv. Harbinger and *M. sativa* were more sensitive to nitrate.

Harper and Gibson (1984) suggested that variation in the sensitivity may be due to differences in nitrate uptake and assimilation by the host plant. In this study, there was a

positive relationship between the inhibition of nodulation and the rate of nitrate taken up (Fig. 6.16). The greater tolerance to nitrate in *M. rugosa* cultivars (Sapo, Paragosa and Paraponto) was found to be associated with a low uptake rate of nitrate, with nitrate uptake per g root dry weight by *M. rugosa* cultivars being approximately half of that in *M. truncatula* cultivars. Nitrate had a relatively small effect on the initiation of nodules in *M. tornata* cv. Tornafield although its rate of uptake was relatively high. However, the nodules formed in Tornafield in the presence of nitrate were much smaller (Table 6.5) and a lower proportion of the nodules were pink and apparently active.

Plants of *M. rugosa* cv. Sapo grown in the presence of 5 mM or 1 mM nitrate had similar number of nodules. This suggests that higher concentrations of nitrate caused no additional suppression of nodulation and supports the observations of Ewing and Robson (1990) that there was no further inhibition of the nodulation of medics by nitrate at concentrations over 1 mM. The uptake rate of nitrate was almost saturated at a concentration of 1 mM (Fig. 6.14). Thus the mechanism of tolerance could lie in reduced uptake of nitrate. That of faba beans has also been related to the uptake of low amounts of nitrate (Chalifour and Nelson 1988a). Since less uptake of nitrate appears to be linked to its enhanced tolerance during the nodulation process, it suggests that the inhibitory effect of nitrate is internal rather than external. Internal factors that could influence the nodulation process and which could be sensitive to nitrate are the induction of nodulation genes, hormone balance, and export of flavonoids (see Section 2.4.3). Malik *et al.* (1987) showed that the degree of inhibition of nodulation did not correlate well with the nitrate concentration in the zone of the root undergoing infection and concluded that it was not nitrate itself which was inhibitory.

Cho and Harper (1991) reported that nitrate decreased the production of flavonoids and isoflavonoids within a root. Flavonoids regulate the expression of rhizobial *nod* genes and the subsequent production of bacterial *nod* factors which are responsible for nodule initiation (Verma 1992). The greater uptake and accumulation of nitrate in *M. truncatula* cultivars could result in a greater impairment of flavonoids.

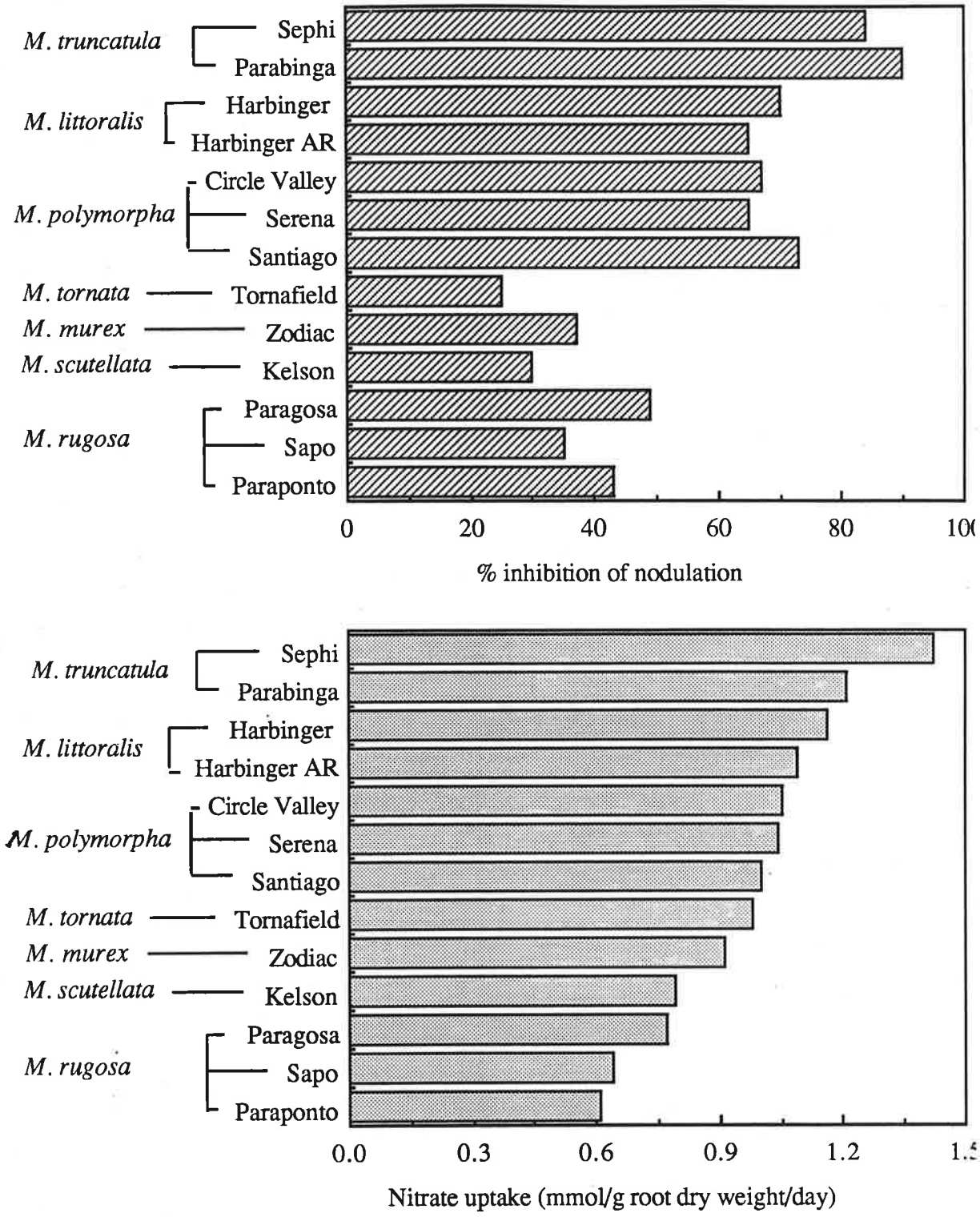


Fig. 6.16. Relationship between nitrate uptake and inhibition of nodulation in annual medics.

The data were taken from Experiment 2-5.

Ethylene is an inhibitor of nodulation (Ligero *et al.* 1987a) and the inhibitory effect of nitrate on nodulation is alleviated by an inhibitor of ethylene production (Ligero *et al.* 1991). Variation in tolerance to nitrate in the medic could be related to differences in ethylene production.

In the young plants used in the main part of this study (14 DAI), nodule dry weight was generally affected to a slightly greater extent (0-40%) by nitrate than nodule number. Wiersma and Orf (1992) and Harper and Cooper (1971) also reported that the effects of nitrate on nodule growth were greater than the effects of nitrate on nodule number. However in older plants (20 DAI, Table 6.1) and in the studies in Chapter 5 (22 DAI) nodules formed in the presence of nitrate appeared to have been less inhibited in growth.

In nodules formed in the presence of nitrate (Fig 6.9) there was less effect of the nitrate on their specific activity for nitrogenase than when nitrate was applied to nodulated plants (Chapter 4). In subterranean clover the presence of nitrate resulted a small increase in specific AR activity (Fig. 6.12). Ralston and Imsande (1983) reported that in soybean specific AR activity for nodules grown in the presence of nitrate is greater than that of nodules formed in the absence of nitrate.

Although it was clearly established in this study that nitrate uptake and accumulation were lower in *M. rugosa* cv. Sapo than in other annual medic species, the level of nitrate reductase in Sapo was not lower than the other species. No relationship between the level of nitrate reductase and nodulation was established. Harper and Gibson (1984) also reported that the site of nitrate reductase was not related to nodulation tolerance in a range of temperate and tropical legumes. When nodulated plants are exposed to nitrate, induction of nitrate reductase is inevitable and this is accompanied by inhibition of nitrogenase activity (Silsbury *et al.* 1986, Carroll and Gresshoff 1983).

It has been reported that the uptake of nitrate is dependent on nitrogen demand for growth (see Section 2.5). A greater plant growth response to nitrate was observed for cultivars of *M. truncatula*, *M. polymorpha* and *M. tornata* than for the cultivars of *M. rugosa* and *M. murex*. Nitrate resulted in a 3-4 fold increase in total dry matter of *M. truncatula* cultivars but only a 1.5-2.0 fold increase in *M. rugosa* cultivars. The greater growth response of *M. truncatula* to nitrate was associated with a higher nitrate uptake (Table 6.1).

Under hydroponic culture it was possible to measure nitrate uptake and the fraction of this that was assimilated by the plants. Using this and the total nitrogen data, the amount of N_2 fixed in the presence of nitrate could be calculated. N_2 fixation calculated by this method showed that N_2 fixation in *M. rugosa* cv. Sapo was more tolerant to nitrate than that in the cultivars of *M. truncatula* (Fig. 6.4, 6.8). The differences between medics in N_2 fixation to nitrate was almost identical to that of nodulation. An inverse relationship between N_2 fixation and nitrate uptake per g root dry weight held true for all the medic cultivars studied except Tornafield. The inhibition of N_2 fixation measured by nitrogen balance study did not always show a close agreement with nitrogenase activity of the plant as measured by the AR procedure. Several possible explanations could account for this discrepancy between these two methods. The AR assay is a point measurement made at the end of the experiment on the rate of nitrogenase activity at that time while the nitrogen balance study provides a complete record of the N_2 fixation throughout the experiment.

Although none of the medic cultivars examined were completely tolerant to nitrate, some cultivars continued to form relatively high numbers of nodules and fixed N_2 at moderately high levels under conditions where nitrate was non-limiting. Such cultivars can be classified as tolerant to nitrate while those which failed to nodulate or nodulated poorly can be classified as sensitive. The medic species studied can be divided into three groups:

- tolerant - *M. rugosa*, *M. murex*, *M. scutellata* and *M. tornata*
- moderately tolerant - *M. polymorpha*
- sensitive - *M. truncatula* and *M. littoralis*

The low uptake of nitrate under non-limiting conditions appears to be the basis of nitrate tolerance in medics with the effect perhaps mediated on the nodulation processes via some influence on the hormonal balance in the root or export of nodule initiation factors.

CHAPTER 7

INHIBITION OF NITROGENASE ACTIVITY (AR) BY NITRATE IN ANNUAL MEDIC SPECIES AND RECOVERY AFTER ITS WITHDRAWAL

7.1. Introduction

It was established in Chapter 6 that variation exists in the sensitivity of different species of medic to the effect of nitrate on nodulation and nodule development. Several *M. truncatula* cultivars tested were more sensitive to nitrate than *M. rugosa* cultivars. In this chapter the influence of nitrate on nitrogenase activity is examined further with particular attention to *M. truncatula* and *M. rugosa* cultivars. Data for nitrogenase activity assessed by AR activity are compared with the level of N_2 fixation deduced from nitrogen balance studies. In one experiment the recovery of nitrogenase activity is monitored after withdrawal of the nitrate supply.

7.2. EXPERIMENT 1: Sensitivity of nitrogenase activity (AR) to nitrate in *Medicago rugosa* cv. Sapo, *M. polymorpha* cv. Serena, *M. truncatula* cvs. Parabinga and Sephi.

7.2.1. Materials and Methods

Seeds of *M. rugosa* cv. Sapo, *M. polymorpha* cv. Serena , *M. truncatula* cv. Parabinga and Sephi were sown in sand. Four d after sowing, ten seedlings of each cultivar were transferred to 2.5 L pots containing minus-nitrate solution. The plants were inoculated with *Rhizobium meliloti* strain WSM540 as described in Section 3.3.1. At 27 DAI, plants from 16 pots were harvested (H1), the remaining 32 pots were divided into two groups and supplied with either 0 (H2) or 1 mM (H2N) nitrate solution for a further 7 d. At both harvests, nitrogenase activity (AR), dry weight and total nitrogen of the plants were measured. The nutrient solutions were renewed daily and the nitrate level in the discarded solution measured in order to determine nitrate uptake. The aeration rate was 0.8 L/min.

AR activity and relative growth rates were measured as described in Sections 3.6 and 3.8 respectively. Nitrate assimilation was determined by subtracting the total amount of nitrate in plant tissues from total uptake of nitrate. A balance sheet was constructed to show the relationship between total nitrogen, nitrate uptake and N_2 fixation for each cultivar. Total nitrogen and nitrate content of shoot and root were determined as described in sections 3.4 and 3.5. The experimental design was a split-plot with two concentrations of nitrate (0 and 1 mM) as main plots and four cultivars of medic as subplots, with four replicates of each.

7.2.2. Results

At 27 DAI (H1), there were no significant differences between the medic cultivars in total dry weight (Table 7.1). After a further 7 d, plants of *M. truncatula* cvs. Parabinga and Sephi transferred to 1 mM nitrate (H2N) had approximately 30% greater dry weight than those maintained on minus nitrate solution (H2). The corresponding increase for *M. polymorpha* cv. Serena and *M. rugosa* cv. Sapo were 3 and 10% respectively Nitrate severely retarded further nodule development relative to the controls; 80% for Sapo, 93% for Serena and 100% for Parabinga and Sephi. The increase in relative growth rate for the nitrate-treated plants over the controls was significant ($P<0.05$) for Parabinga and Sephi, but for Sapo and Serena the increase was marginal.

In *M. truncatula* cvs. Parabinga and Sephi total N approximately doubled with the addition of nitrate (Table 7.2) whereas in Sapo and Serena the increase was 26 and 20% above that of control plants respectively. Percentage nitrogen in Serena was however higher than that of the other medics in this study (Table 7.2). Total nitrate uptake (as determined by cumulative nitrate depletion from the nutrient solution) was similar for Serena, Parabinga and Sephi. The *M. rugosa* cv. Sapo however took up less nitrate than the other species. Nitrate content in shoot tissues was approximately twice that of the roots in all the species (Table 7.2) and the proportion of the nitrate taken up, over the 7 d period that was assimilated was approximately 83% for all species.

Table 7.1. Influence of nitrate on the growth and nodule development in four medic cultivars.

Plants were grown without nitrate for 27 DAI as described in Section 7.2.1. At 27 DAI, one third of the plants were harvested (H1). The remainder were either transferred to 1 mM nitrate (H2N) or left in minus nitrate solution (H2). These were harvested at 34 DAI. Relative growth rate was calculated for the 7 d period from d 27 to d 34. Data in the Table are means of determinations made on 40 plants.

		Medic cultivar[a]				
	Harvest time	Sapo	Parabinga	Sephi	Serena	LSD 5%
Total dry weight (mg/plant)	H1	116.7	113.7	128.8	123.7	23.82
	H2	266.7	237.6	266.6	318.8	47.92
	H2N	292.3	307.8	349.8	329.0	
Nodule dry weight (mg/plant)	H1	4.65	4.00	4.65	4.40	0.85
	H2	9.78	8.95	10.50	16.50	2.01
	H2N	5.68	4.00	4.37	5.25	
Relative growth rate (mg/mg/day)	(H2-H1)	0.12	0.11	0.10	0.14	0.03
	(H2N-H1)	0.13	0.14	0.14	0.14	

[a]*M. rugosa* cv. Sapo, *M. truncatula* cvs. Parabinga and Sephi, *M. polymorpha* cv. Serena

Table 7.2. Total nitrogen, nitrate uptake, nitrate content of shoots and roots and nitrate assimilation by the medics.

The plants were grown as described in Section 7.2.1. Nitrate uptake (27-34 d) was determined by its disappearance from the nutrient solution. Nitrate content of the shoot and root were determined as described in Section 3.5. Percentage nitrogen in the plants are shown in parentheses.

	Medic cultivar[a]				
Harvest time	Sapo	Parabinga	Sephi	Serena	LSD 5%
Total nitrogen (mg/plant)					
H1(a)	3.93 (3.4)	3.85 (3.4)	4.24 (3.3)	5.43 (4.4)	0.74
H2 (b)	9.23 (3.5)	7.67 (3.2)	8.62 (3.2)	13.68 (4.3)	1.86
H2N (c)	11.68 (4.0)	14.38 (4.7)	15.60 (4.5)	16.46 (5.0)	
Nitrate-N uptake (d) (mg/plant)	5.55	9.76	10.46	9.03	1.33
Shoot nitrate-N (mg/plant)	0.54	1.16	1.23	1.06	0.21
Root nitrate-N (mg/plant)	0.31	0.53	0.59	0.49	0.11
Nitrate-N assimilation (mg/plant)	4.70	8.07	8.64	7.48	1.14
% Nitrate assimilation	84.6	82.6	82.7	82.9	1.92

[a] as in Table 7.1.

The amount of N_2 fixed by the medic species (Fig. 7.1) ranged from 3.81 in *M. truncatula* cv. Parabinga to 8.24 mg nitrogen/plant in *M. polymorpha* cv. Serena for plants supplied with 0 mM nitrate. The corresponding value for nitrate-treated plants was between 0.77 in *M. truncatula* cv. Parabinga and 2.20 mg nitrogen/plant in *M. rugosa* cv. Sapo. Based on the nitrogen balance study N_2 fixation was inhibited by 59% in Sapo and approximately by 80% in the other cultivars.

At 27 DAI (H1), there were no significant differences in nitrogenase activity (μmol C_2H_4/h/plant) between Sapo, Parabinga and Sephi (Fig. 7.2A). The *M. polymorpha* cv. Serena however had a higher nitrogenase activity than all the other species. At 34 DAI, nitrogenase activity in Sapo, Parabinga and Sephi was two fold the activity at 27 DAI but in Serena the increase was 5 fold. Nitrogenase activity of the nitrate treated (H2N) plants was inhibited by 88% for Sapo and approximately 96% for the other species when compared to that of the control plants (H2). The specific activity of the nitrogenase (μmol C_2H_4/h/g nodule dry weight) for Sapo and Serena (Fig. 7.2B) showed a similar trend as for the total activity per plant, though the increase from 27 to 34 DAI in the control plants was smaller. A slight decrease occurred in the control plants of Parabinga and Sephi in the same period. Nitrate treatment resulted in a major decrease in the specific nodule activity, especially in the two *M. truncatula* cultivars.

7.3. EXPERIMENT 2: Recovery of nitrogenase activity (AR) after withdrawal of nitrate

7.3.1. Materials and Methods

M. rugosa cv. Sapo, *M. polymorpha* cv. Serena, *M. scutellata* cv. Kelson, *M. littoralis* cv. Harbinger, *M. tornata* cv. Tornafield and *M. truncatula* cv. Parabinga were grown hydroponically (16 plants per pot in a nitrate-free culture) as described in Section 3.3. The aeration rate was 0.8 L/min per pot. Sapo and Serena were inoculated with *Rhizobium meliloti* strain WSM540 which was shown in Chapter 5 to be a good inoculant for these two medics. WSM826, the recommended strain, was used for Harbinger, Tornafield and

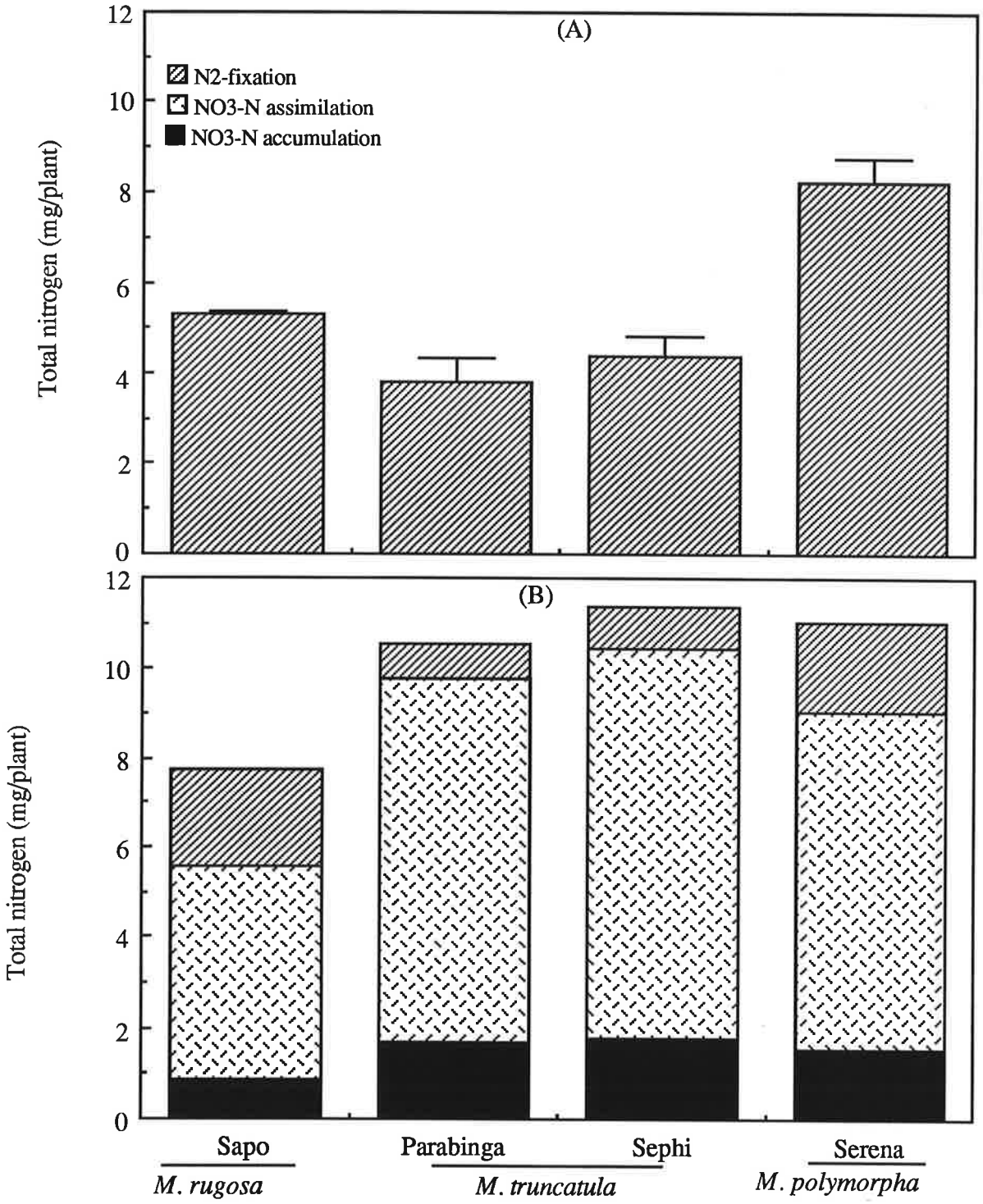


Fig. 7.1. Nitrate uptake and assimilation and its influence on N_2 fixation in medics.

The four medic cultivars were grown in the absence (A) and presence of 1 mM nitrate (B) from 27 to 34 DAI. The data is derived from Table 7.2. The estimate of N_2 fixed made by (b-a) in minus nitrate treatment and c-(a+d) in 1 mM nitrate treatments.

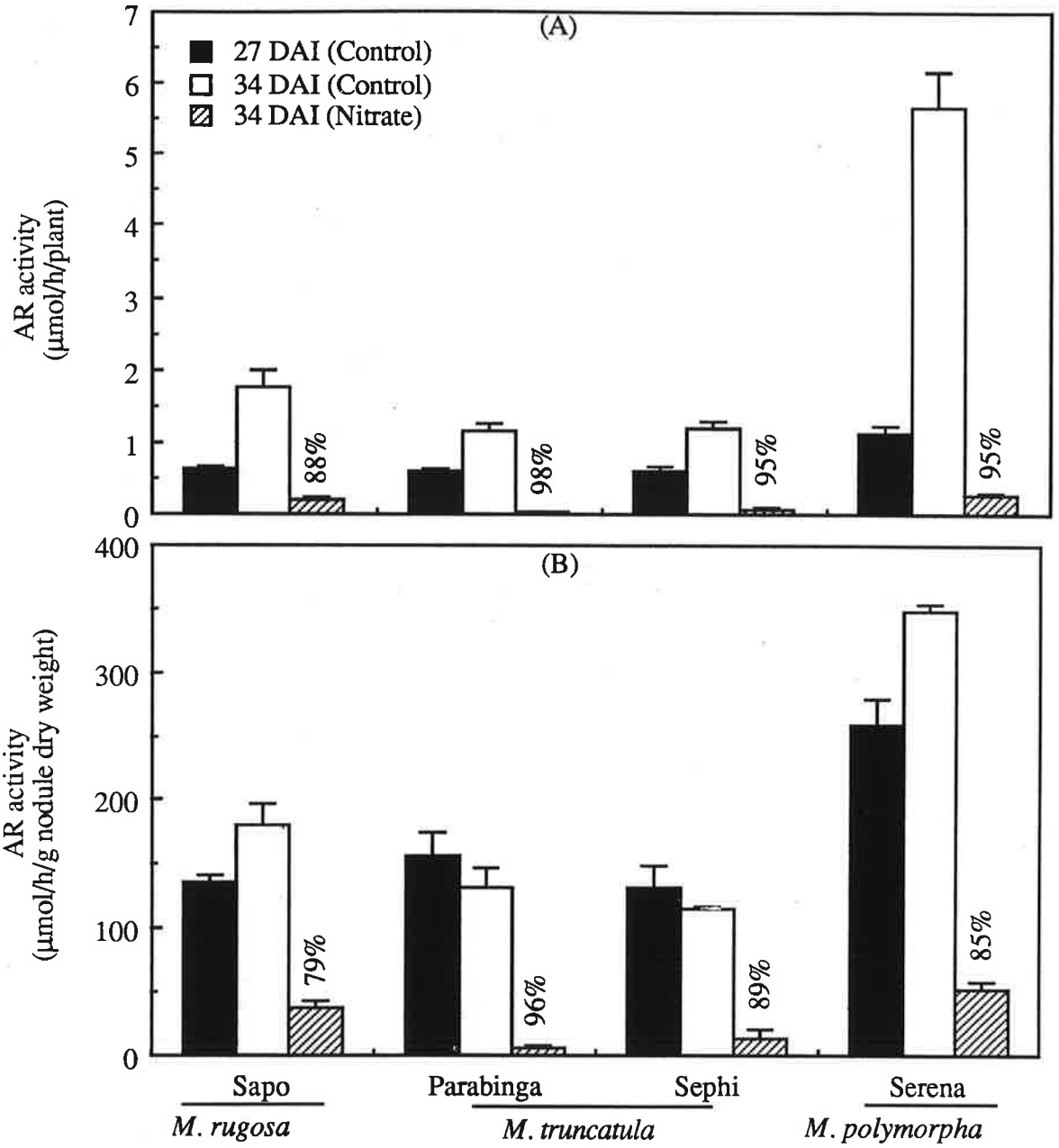


Fig 7.2. Influence of nitrate on nitrogenase activity (AR) of four medic cultivars.

Plants were grown as described in Table 7.1. Nitrogenase activity per plant (A) and per g nodule dry weight (B) are shown for each cultivar and the percentage inhibition by the 1 mM nitrate treatment in each case indicated. Values represent the mean of four replicates while the bars indicate ±S.E.

Kelson. For Parabinga, WSM540 and WSM826 were tested to see if there was an effect of the rhizobial strain in this experiment. At 16 DAI, when the plants were nodulated they were assigned either to treatment (A) in which plants were maintained in minus-nitrate solution for 14 d or (B) in which plants were transferred to a nutrient solution containing 1 mM nitrate for 7 d, then grown for a further 7 d in minus-nitrate solution. Five plants in each pot were harvested at 16 and 23 DAI, while 3 plants per pot were harvested at 26 and 30 DAI. Measurements of nitrate uptake were made between 16-23 DAI (see Section 3.5.2). At each harvest, total nitrogen, nitrogenase activity (AR), shoot, root and nodule dry weights were measured as described in Sections 3.4, 3.6 and 3.8 respectively. Nitrate concentration of the shoots and roots was measured at 23, 26 and 30 DAI as described in Section 3.5. Due to the small amount of plant material available for the determination of total nitrogen and nitrate concentration of the shoots and roots, the two replicates of each treatment were pooled and analysed as a single unit. The plants were arranged in a completely randomised block design.

7.3.2. Results

Except for Kelson, the nodule number between 16-23 DAI either did not increase (Parabinga) or increased only slightly (Fig. 7.3) in the absence of nitrate. In the presence of 1 mM nitrate this increase in nodulation was slightly reduced (Sapo and Serena) or eliminated (Kelson, Tornafield and Harbinger). There was no difference in the nodulation pattern when Parabinga was inoculated with either *Rhizobium* strain WSM540 or WSM826. When the nitrate was removed, new nodules were formed in all the species except Parabinga. The rate of nodulation in plants exposed to nitrate was higher than that of the controls in the same period for Kelson, whereas in Serena, Tornafield and Harbinger it was similar in both nitrate treated and control plants.

At 16 DAI, total nodule dry weight (Table 7.3) was highest in Kelson > Sapo = Serena > Tornafield = Parabinga > Harbinger. Changing from minus-nitrate to 1 mM nitrate at 16

Fig. 7.3. Influence of nitrate supply and its withdrawal on nodule numbers in six medic species.

Plants were grown in a minus nitrate nutrient solution. At 16 DAI, half the plants were exposed to 1 mM nitrate (solid line) and the other half maintained in nitrate free solution (broken line) for further 7 d. All plants were then grown in minus nitrate solution until the end of the experiment. Each point represents the mean of four replicates while the bars indicate ±S.E.

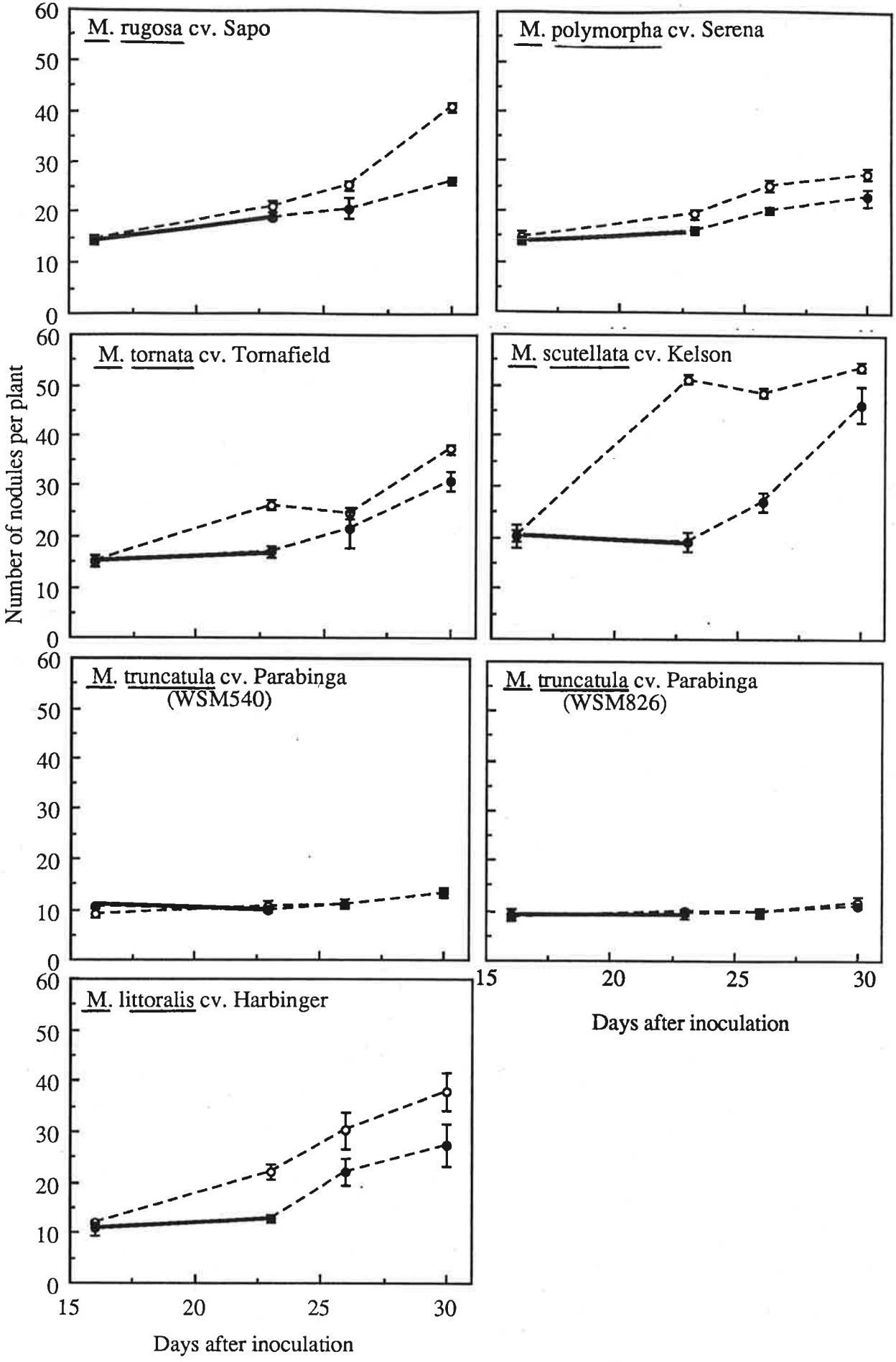

M. rugosa cv. Sapo
M. polymorpha cv. Serena
M. tornata cv. Tornafield
M. scutellata cv. Kelson
M. truncatula cv. Parabinga (WSM540)
M. truncatula cv. Parabinga (WSM826)
M. littoralis cv. Harbinger
Number of nodules per plant
0
10
20
30
40
50
60
15
20
25
30
Days after inoculation

Table 7.3. Influence of nitrate on nodule growth in six medic species.

Seedlings were grown in a minus nitrate nutrient solution in hydroponic culture. At 16 DAI, half the plants were transferred to 1 mM nitrate (B) and the other half maintained in minus nitrate nutrient solution for a further 7 d (A). All plants were then grown in minus nitrate solution until the end of the experiment (30 DAI). Data shown are means ±S.E. of the four replicates of 5 plants (16 and 23 DAI) or 3 plants (26 and 30 DAI).

	Medic cultivars and *Rhizobium* strains[a]						
DAI and nitrate treatment	Sapo 540	Serena 540	Tornafield 826	Kelson 826	Parabinga 540	Parabinga 826	Harbinger 826
Nodule dry weight (mg/plant)							
16 (0)	1.5±0.08	1.5±0.09	1.1±0.09	4.0±0.44	1.1±0.09	1.1±0.14	0.7±0.06
23 (0) A	3.0±0.29	4.2±0.39	2.4±0.13	8.7±0.33	2.9±0.13	2.8±0.36	1.5±0.21
23 (1) B	2.4±0.14	2.3±0.10	1.4±0.10	3.9±0.22	1.6±0.08	1.2±0.05	0.9±0.06
26 (0) A	3.9±0.16	5.0±1.10	3.1±0.28	10.7±0.71	3.8±0.17	3.6±0.77	1.8±0.21
26 (0) B	3.9±0.49	5.3±0.64	2.0±0.41	5.9±0.25	1.8±0.32	2.0±0.36	1.1±0.21
30 (0) A	6.4±0.66	8.7±2.53	5.7±0.58	15.9±0.55	5.2±0.17	4.7±0.93	2.3±0.29
30 (0) B	7.9±0.63	12.5±2.21	3.3±0.59	12.4±1.14	2.4±0.48	2.7±0.36	1.9±0.21

[a]*M. rugosa* cv. Sapo, *M. polymorpha* cv. Serena, *M. tornata* cv. Tornafield, *M. Scutellata* cv. Kelson, *M. truncatula* cv. Parabinga, *M. littoralis* cv. Harbinger
R. meliloti strain WSM540,
R. meliloti strain WSM826.

DAI resulted in a decreased nodule dry weight to approximately 40-60% of the control for all medic species except *M. rugosa* cv. Sapo (20%). At 30 DAI, nodule dry weight in Sapo and Serena given 1 mM nitrate (from 16-23 DAI), was 1.2-1.4 times greater than control plants grown in minus nitrate solution throughout. In all other cases there was a considerable reduction in nodule weight in plants which had an exposure to nitrate. This inhibition was either the same as that at 23 and 26 DAI (Tornafield, Parabinga) or showed some increase (Kelson and Harbinger).

Analyses of the total nitrogen of the plants in this experiment are shown in Table 7.4. At 16 DAI, the percentage nitrogen of all the medics except Harbinger was at least 2.9. That in Serena was 3.8. This cultivar of *M. polymorpha* also had the highest percentage nitrogen in experiment 1 (Table 7.2). The percentage nitrogen of the Harbinger plants did increase to 2.8 % by 30 DAI. The total nitrogen of all the medics increased 1.5 to 2-fold after 7 d treatment (16-23 DAI) with 1 mM nitrate. The percentage nitrogen values showed a pronounced increase as the result of this nitrate treatment but a further seven d after the nitrate had been removed the percentage nitrogen of the treated plants was less than that of the control plants maintained in minus nitrate solution. Nitrate uptake by *M. rugosa* cv. Sapo was lower than the other medics and that in *M. scutellata* cv. Kelson was intermediate (Table 7.5). In this medic there was a higher level of nitrate accumulation in the shoot at 23 DAI than in the other plants. In all medics, the nitrate concentration of the root was 2-4 times that of the shoot. Removing nitrate from the nutrient solution resulted in a marked decrease in nitrate concentration of the plant tissues.

Nitrogenase activity per plant increased in all cultivars between 16 to 23 DAI (Fig. 7.4), but nitrate applied at day 16 was inhibitory. With Sapo and Serena the level at 16 DAI was maintained but in the other species it was inhibited to about 60-70% of controls. Seven d after removing the nitrate, the nitrogenase activity of all cultivars, except Harbinger and Tornafield had increased to approximately that of the controls.

Table 7.4. Total nitrogen and percentage nitrogen in six annual medic species.

Seedlings were grown as described in Table 7.4 in which the nitrate treatment between 16-23 DAI is B. Total nitrogen was determined as described in Sections 3.4. Data shown are means ±S.E. of the four replicates.

	Medic cultivars and *Rhizobium* strains[a]						
DAI and nitrate treatment	Sapo 540	Serena 540	Tornafield 826	Kelson 826	Parabinga 540	Parabinga 826	Harbinger 826
Total nitrogen (mg/plant)							
16 (0)	1.1±0.03	1.1±0.06	0.7±0.05	2.7±0.18	0.9±0.07	1.0±0.11	0.3 ±0.02
23 (0) A	1.8±0.16	2.8±0.23	1.0±0.06	5.2±0.18	2.0 ±0.10	1.9 ±0.24	0.5 ±0.03
23 (1) B	3.4±0.17	3.9±0.35	1.8±0.16	8.8±0.35	2.9 ±0.19	2.9 ±0.26	0.8 ±0.10
30 (0) A	4.0±0.34	7.4±1.42	2.9±0.31	11.3±0.34	3.5 ±0.27	3.5 ±0.57	0.7 ±0.04
30 (0) B	5.7±0.45	7.7±1.09	2.7±0.44	13.3±0.45	3.9 ±0.52	4.2 ±0.77	1.0 ±0.17
Percentage nitrogen (N%)							
16 (0)	3.27±0.01	3.83±0.01	2.90±0.02	2.93±0.11	3.11±0.07	3.23±0.06	2.18 ±0.01
23 (0) A	3.03±0.08	3.97±0.06	3.07±0.01	2.91±0.07	2.83±0.03	2.84±0.01	2.57±0.07
23 (1) B	4.18±0.03	4.71±0.08	4.17±0.02	4.29±0.10	3.97±0.09	4.13±0.10	3.74±0.03
30 (0) A	3.59±0.14	4.29±0.05	3.67±0.07	3.05±0.05	3.21±0.01	3.08±0.08	2.79±0.03
30 (0) B	2.74±0.01	2.98±0.04	3.14±0.05	2.89±0.01	3.20±0.01	3.17±0.01	2.87±0.04

[a]As in Table 7.3.

Table 7.5. Nitrate uptake and nitrate concentration of the shoots and roots in six annual medic species.

Seedlings were grown as described in Table 7.4 in which the nitrate treatment between 16-23 DAI is B. Nitrate uptake were determined as described 3.5.2. Nitrate concentration of the shoots and roots was determined as described in Section 3.5. Data shown are means ±S.E. of the four replicates.

DAI and nitrate treatment	Medic cultivars and *Rhizobium* strains[a]						
	Sapo 540	Serena 540	Tornafield 826	Kelson 826	Parabinga 540	Parabinga 826	Harbinger 826
Nitrate-N uptake (mmol/g root dry weight/day)							
	0.57±0.01	1.17±0.04	1.00±0.03	0.86±0.04	1.00±0.04	0.99±0.05	1.40±0.1
Shoot nitrate (µmol/g dry weight)							
23 (1) B	80±2.7	108±8.1	85±5.4	249±8.1	207±5.4	195±5.7	113±5.4
26 (0) B	14±0.0	14±0.0	23±0.0	18±3.0	41±5.3	23 ±0.0	8.20±0.6
30 (0) B	8.6±0.0	8.6±0.0	12.6±2.1	8.6±0.0	12.6±3.0	17.6±3.0	6.60±0.0
Root nitrate (µmol/g dry weight)							
23 (1) B	347±16	235±27	235±5.4	723±16	413±22	488±32	472±25
26 (0) B	41 0±0.0	46±2.6	64±2.6	178±10	178±5.3	158±7.9	55±7.9
30 (0) B	26.4±0.0	26±0.0	30.9±3.1	66.3±4.2	48.7±8.6	48.9±6.4	22±3.2

[a]As in Table 7.3.

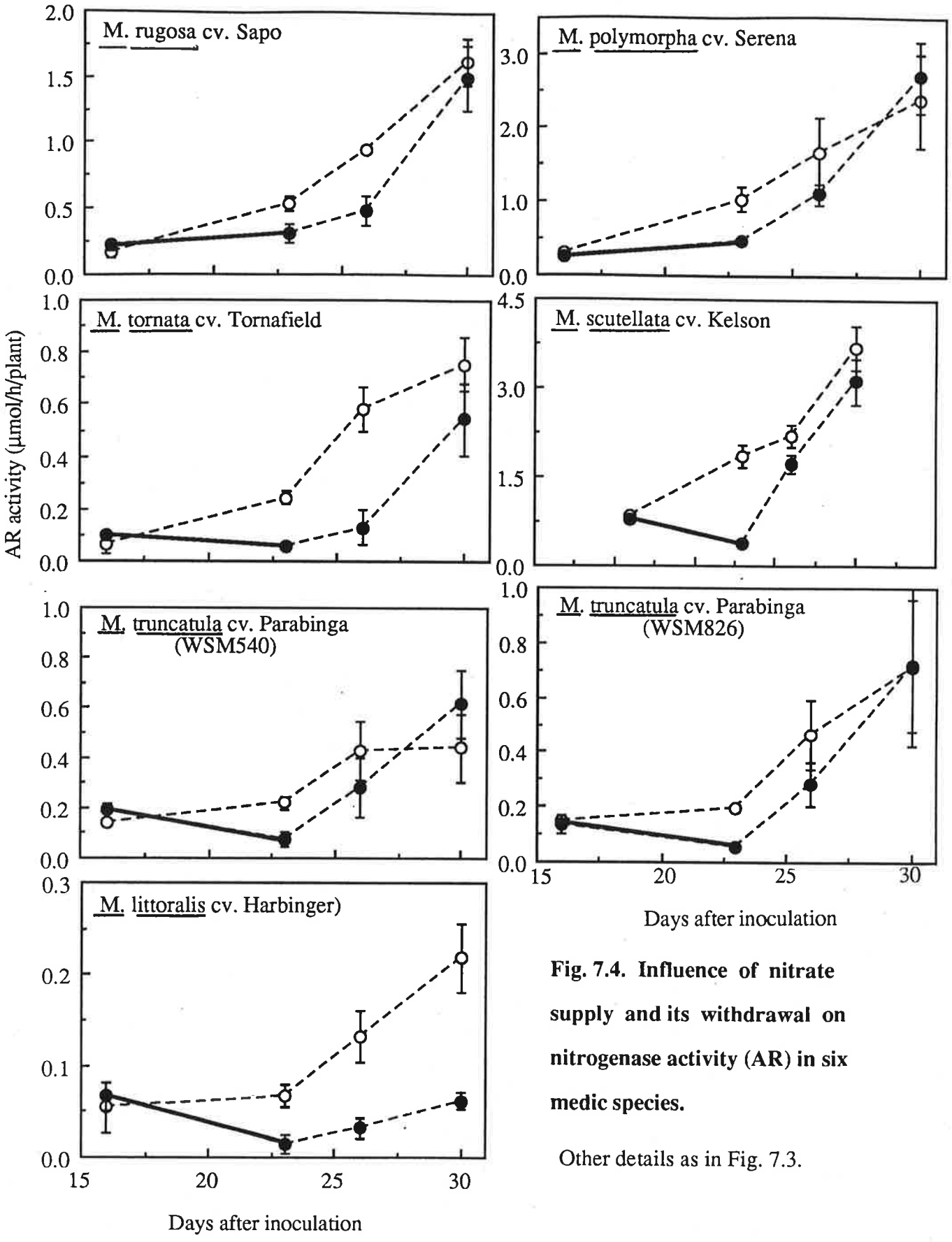


Fig. 7.4. Influence of nitrate supply and its withdrawal on nitrogenase activity (AR) in six medic species.

Other details as in Fig. 7.3.

Specific AR activity (μmol/g nodule dry weight) in Sapo, Serena and Tornafield grown in minus nitrate solution increased up to 26 DAI, while in other species it remained approximately constant (Fig. 7.5). Specific AR activity in all the cultivars except Serena decreased following nitrate treatment. When the nitrate was removed, there was a pronounced rapid recovery of specific AR activity in Kelson and both Parabinga treatments, a delayed and smaller recovery in Sapo and Tornafield, but no significant increase in Serena and Harbinger.

7.4. Discussion

Measurement of total nitrogen and nitrate uptake enabled the direct estimation of the amount and proportion of nitrogen derived from the atmosphere and from nitrate uptake. *M. rugosa* cv. Sapo had the highest N_2 fixation and derived 2.20 mgN/plant or 41% of its nitrogen from the atmosphere in the presence of nitrate; *M. truncatula* cvs. Parabinga and Sephi, on the other hand, fixed only 0.77 and 0.90 mgN/plant or 20% of the total nitrogen assimilated under identical conditions (Fig. 7.2). When the nitrogenase activity was measured at the end of the 7 d nitrate treatment, by AR assay, a higher level of inhibition due to nitrate was apparent and Sapo was only slightly less inhibited (88%) than Parabinga or Sephi (98% and 95% respectively).

It was again confirmed that *M. rugosa* cv. Sapo took up less nitrate than the *M. truncatula* cvs. Parabinga and Sephi and also *M. polymorpha* cv. Serena. In all four medics 83-84% of the nitrate taken up was assimilated. Variation between the medics in nitrate content was related to differences in their capacity for nitrate uptake and dry matter accumulation.

When nitrate was applied at 16 DAI, the appearance of nodules of a range of medics examined was stopped (Fig. 7.3). When the nitrate was removed after 7 d, nodulation resumed at a rate similar to that in the controls at the time of its addition. In Parabinga no further nodules appeared after 16 DAI. During the 7 d nitrate treatment the total AR activity of the plants was maintained or decreased slightly but increased rapidly in all medics after

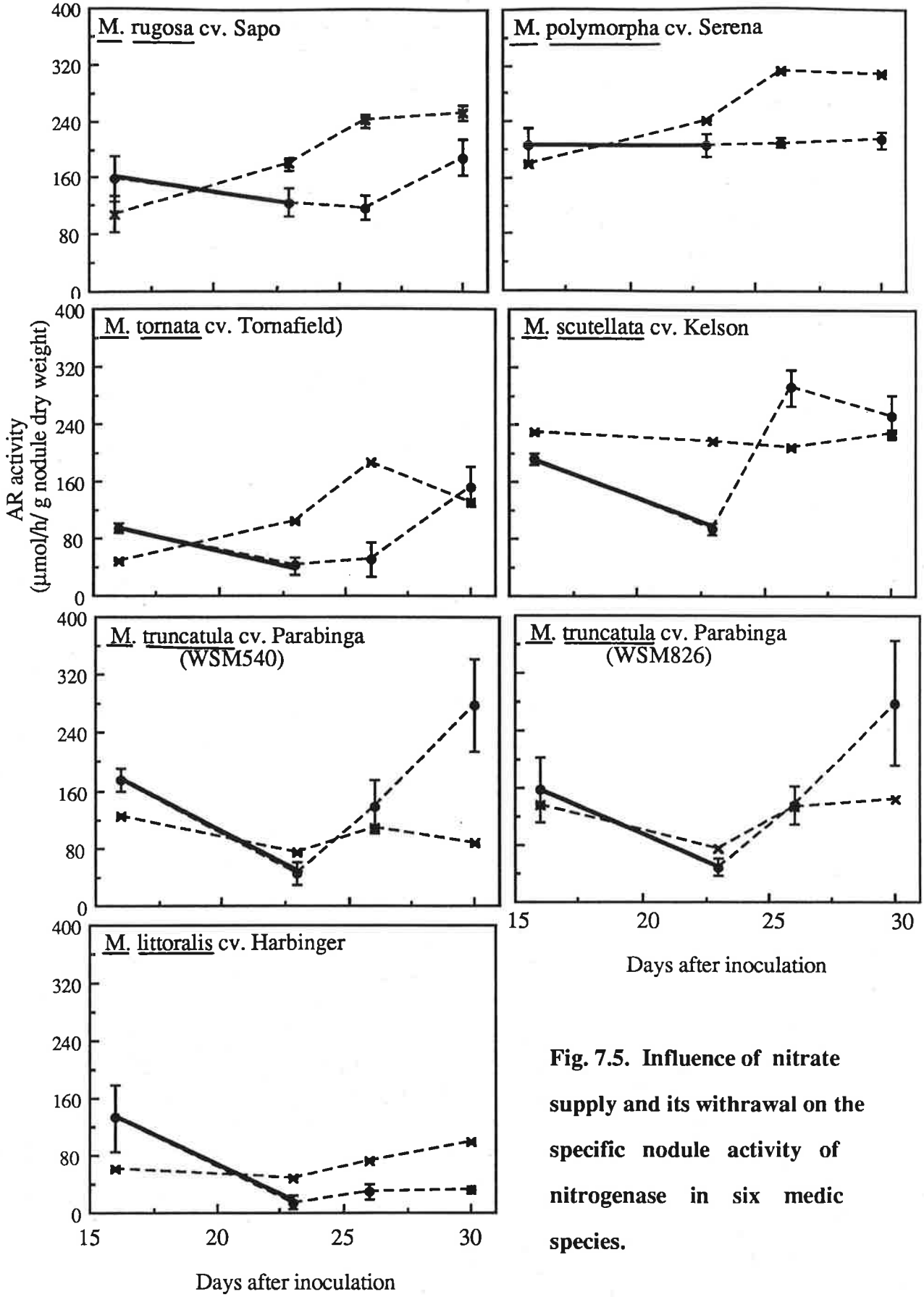


Fig. 7.5. Influence of nitrate supply and its withrawal on the specific nodule activity of nitrogenase in six medic species.

the nitrate was removed. The rapid increase in nitrogenase activity after nitrate was removed indicates that negligible nodule senescence was occurred during the nitrate treatment. Schuller *et al.* (1986) and Kamberger (1977) reported that nitrogenase activity of the bacteroids isolated from the soybean nodules was not inhibited by exposure to nitrate until at least 7 d after commencement of the treatment. Bisseling *et al.* 1978 and Noel *et al.* (1982) also showed that there is no destruction of nitrogenase after a short term exposure of nodulated plants to nitrate. The inhibition of nitrogenase could be due to decreased carbohydrate supply due to competition with the assimilation of nitrate (Section 2.7.3), an interference in the oxygen supply to the bacteroid (Section 2.7.2), or a feedback effect due to the resultant accumulation of organic nitrogen in the nodules (Section 2.7.4).

There have been several reports of recovery of nitrogenase activity after nitrate removal from rooting medium. For example, Faurie and Soussana (1993) showed that 3 to 5 d after nitrate removed, nitrate inhibition was fully alleviated in white clover. Silsbury *et al.* (1986) also showed that when subterranean clover plants previously grown with 15 mM nitrate were exposed to nitrate-free solution, nitrogenase activity recovered after 7 d. Gibson (1976) found a recovery of nitrogenase activity in soybean plants 4 d after removing nitrate and Streeter (1981) reported the complete recovery of nitrogenase activity 7 d after nitrate removal. The results of the current study, in which a rapid recovery of nitrogenase activity was found with nitrate removal, are in agreement with the above findings. They also support the observations in Chapter 4, in which an inverse relationship between nitrogenase activity (AR) and nitrate content of the plants.

The inhibition of nitrogenase activity due to nitrate application was higher in Experiment 1 (nitrate treatment 27-34 DAI) than in Experiment 2 (nitrate treatment 16-23) (Fig 7.2 and Fig 7.4). This discrepancy in the response of AR activity to nitrate could be due to nodule age, the older nodules being more sensitive to nitrate. Rigaud (1981) reported that the nitrogenase activity in younger nodules was less inhibited than that of mature nodules.

The earlier observation in Chapter 4 that there was little variation in the effect of nitrate on nitrogenase activity in medics has been confirmed in this chapter. The nitrate effect on the specific AR activity of the medics (Fig 7.5) was similar and the smaller effect on inhibition of the level of N_2 fixation per plant in *M. rugosa* cv. Sapo (Fig. 7.1) was due to the corresponding smaller inhibition of nodulation in this species described in Chapter 6.

CHAPTER 8

GENERAL DISCUSSION

One of the successes of Australian agriculture over the past 50 years has been the use of self-regenerating pasture legumes with the potential to fix high levels of N_2 and maintain soil N levels in farming systems. Prior to this study, investigation of the effect of nitrate on nodulation and N_2 fixation by medics had attracted only limited attention. In a comparative study on several legume species it was found that the medic tested, (*M. truncatula* cv. Akbar), was the most sensitive (Harper and Gibson 1984). However it has been found in this study that natural variation exists between medics in the sensitivity of their nodulation to nitrate.

Nitrate had less of an inhibitory effect on nodulation of the *M. rugosa* cultivars than on that of the *M. truncatula* cultivars. Studies on other medic species provided evidence for a range of tolerance in different species of medics between that of *M. rugosa* and *M. truncatula* with *M. murex*, *M. tornata*, *M. scutellata* being equally tolerant to nitrate as *M. rugosa*. Ewing and Robson (1990) showed that nodulation in *M. murex* cv. N3172 and *M. polymorpha* cv. Santiago was more tolerant to nitrate than *M. truncatula* cv. Cyprus.

Those medics which exhibited tolerance to nitrate during nodulation had a lower rate of nitrate uptake and accumulation (Fig. 6.16, Table 6.2) compared with *M. truncatula* cultivars. An inverse relationship between nitrate uptake during nodulation and tolerance of nitrate was also observed by Harper and Gibson (1984) and Chalifour and Nelson (1988a). In a kinetic analysis of nitrate uptake in *M. rugosa* cv. Sapo and *M. truncatula* cv. Parabinga, the K_m values (0.11 and 0.14 mM respectively) were similar but the differences in V_{max} (52 and 72 μmol/h/g root dry weight respectively) correlated with the differences in

uptake consistently observed between these two species throughout this study. The actual mechanism of nitrate uptake is not completely understood, e.g. the magnitude of active and passive components (Ullrich 1992), and the differences described in this study could reflect inherent differences in these processes in the roots of different medic species. The efflux component of nitrate may also vary (Section 2.5).

The *M. rugosa* cultivars and also the other relatively tolerant species e.g. *M. scutellata* cv. Kelson had thicker roots than *M. truncatula* cultivars, but how this might be relate to a lower uptake of nitrate by them is not understood. No correlation was observed between water uptake and nitrate uptake in the medics studied. Laine *et al.* (1993) also found no obvious relationship between maximum nitrate uptake per unit weight of roots and transpiration per unit weight in a range of plant species. Oscarson and Larsson (1986) showed that the capacity of pea plants to absorb nitrate from the medium was several-fold higher than the N requirement for sustaining maximum growth rate. Yet in the medics examined in this study about 85-90% of the nitrate taken up between 14-20 DAI was assimilated.

In the studies described in Chapter 6, the differences in tolerance to nitrate during nodulation were apparent as early as 4 DAI. Even at 2 DAI it could be seen with a microscope that a distinction between *M. rugosa* cv. Sapo and *M. truncatula* cv. Parabinga, with respect to nitrate tolerance, was evident. This agrees with the results of Munns (1968a, b) who found that the early stages of nodulation, involving root hair curling, infection of root hairs and development of infection threads, were most sensitive to nitrate in *M. sativa.* Truchet and Dazzo (1982) in a study on nodulation in the same species concluded that the major symbiotic steps restricted by nitrate were the release of the rhizobia from the infection threads, and formation of the bacteroids. In soybean, Malik *et al.* (1987) found that the nitrate-sensitive events of infection were completed within 18 h after application of the rhizobia. Thus, in further studies to define the basis of the tolerance to nitrate, more detailed

microscopy and accompanying biochemical analysis will be required on these medics and the examination should commence immediately after inoculation.

The inhibitory effect of nitrate on medic nodulation appears to be controlled by internal factors, since it is related to uptake and accumulation rates in the plant (Table 6.2). However, Malik *et al.* (1987) in their careful studies on application of nitrate to different zones of soybean root demonstrated that the extent of the inhibition of nodulation in these regions of the root did not correlate with the internal nitrate concentration at that site. Thus nitrate itself does not appear to be involved in the infection-inhibiting responses in the host. In contrast to the direct relationship between the level of nitrate accumulation in the root and shoot of the medics and the inhibition of nitrogenase activity in the nodules (Chapter 4), it was demonstrated in *M. rugosa* cv. Sapo (Fig. 6.5), that the inhibition of nodulation by 1 mM nitrate (40%) was not increased by 5 mM nitrate. Ewing and Robson (1990) found that the degree of inhibition of nodulation by 1 mM nitrate in *M. murex* and *M. polymorpha* was not enhanced by applying up to 10 mM nitrate. It would appear that physiological processes influenced by nitrate concentration (e.g. oxygen diffusion and carbohydrate availability, Sections 2.7.2 and 2.7.3) are not directly involved in mediating the inhibitory effect of nitrate on the nodulation process.

In medic plants studied 14-20 DAI the presence of 1 mM nitrate inhibited nodule growth to the same extent as nodule initiation (Table 6.1, Fig. 6.1). Competition for photosynthate with nitrate assimilation (Vessey *et al.* 1988a) is likely to be the main factor causing the retardation of nodule development. Parsons *et al.* (1993), have recently proposed that reduced nitrogen compounds e.g. from nitrate assimilation in the shoot and supplied to nodules by the phloem, inhibit nodule growth. However in older plants (22 DAI) it was found (Table 5.1 and 5.3) that nodules that had formed in the presence of 1 mM nitrate had similar dry weight as those on the plants in the minus nitrate medium. Either there is less

competition for photosynthate in the older plants or less feedback by reduced nitrogen occurs.

The level of nitrate chosen for the main part of the current study is relevant to the accumulation of nitrate found in soils in which medics grow in southern Australia. Alston and Graham (1982) measured 25-45 mg nitrate/kg soil in the soil in South Australia during autumn, when germination and nodulation of medics occurs. Assuming the moisture content of soil at field capacity is 20% (v/v), the above quantities of nitrate are equivalent to a concentration of 9-16 mM. However, the available nitrate in the soil will depend on cropping history and environmental conditions at the start of the growing season, and the concentration at the root surface will be also limited by diffusion from the surrounding soil. By contrast a constant level of nitrate is maintained in the hydroponic situation.

No major variation was found in the effect of nitrate on nitrogenase activity in different medic species. In each case there was a direct relationship between the concentration of nitrate in the root and shoot and the inhibition of nitrogenase activity (Fig. 4.2). Although the basis of the inhibitory effect of nitrate on nitrogenase activity is still open to debate (Section 2.6.2), explanations involving carbohydrate deprivation, interference in nodule physiology or biochemistry and feed back inhibition are subject to common biochemical and physiological processes which are unlikely to vary significantly between plant species.

The recovery of nitrogenase activity after the removal of nitrate suggests a complementary relationship between nitrogenase activity and nitrate assimilation. Seven days after withdrawal of nitrate from the rooting medium, total nitrogen accumulation of the plants did not differ from those which had relied on N_2 assimilation throughout the growth period (Table 7.5) indicating that the plants were able to satisfy their nitrogen requirement from both nitrate assimilation and N_2 fixation. Increases in nodule dry weight (Table 7.4) and

specific AR activity (Fig 7.5) after nitrate removal confirmed that the symbiotic system was still able to respond after 7 d exposure to nitrate. This shows that the N nutrition of the legume plant is dynamic, the predominate source of N (i.e. atmospheric N_2 or nitrate) varying according to the availability of nitrate in the root medium. The feed-back mechanism proposed by Silsbury *et al.* (1986, Section 2.7.4 and also Parsons *et al.* 1993) appears best to explain the integration of nitrogen fixation and nitrate assimilation.

Ewing and Robson (1990) considered that the tolerance of nodulation to nitrate in medics might be related to the fertility of the soils in which they evolved. *M. truncatula* is found in the more fertile soils of the Mediterranean region, and therefore the greater availability of mineral N throughout the growing season may mean that the dependence on N_2 fixation is less and they are responsive to nitrate levels in the soil. The present study has characterised the nitrate sensitivity among a wider rage of cultivars and species, and when the sensitivity to nitrate is compared with the areas and soils of adaptation, the link between soil fertility and sensitivity to nitrate suggested by Ewing and Robson does not seem consistent. For example, nodulation in *M. rugosa* cultivars was found to be less sensitive to nitrate than *M. truncatula*, but cultivars of *M. rugosa* are widely grown on heavy textured, grey alkaline soils in South Australia (Oram 1990), which are some of the most fertile soils in the state. On the other hand, *M. littoralis* cv. Harbinger was sensitive to nitrate, but they are adapted to sandy soils which are likely to be low in available nitrate. Therefore, there does not appear to be a strong association between soil fertility and tolerance to nitrate.

In has been clearly demonstrated in this study that *M. rugosa* is more tolerant to nitrate than *M. truncatula* during the nodulation phases of these medics. Other medics such as *M. scutellata, M. tornata* and *M. murex*, capable of nodulating in the presence of nitrate were identified (Chapter 6). However, only one cultivar of each species was used (*M. scutellata* cv. Kelson, *M. tornata* cv. Tornafield and *M. murex* cv. Zodiac), so it would be worthwhile

to examine other cultivars of these species. Further physiological and biochemical studies are required on the inverse relationship between nitrate uptake and the inhibition of nodulation. If low nitrate uptake is confirmed as a good predictor of tolerance of nodulation to nitrate it would be an easy parameter to screen for.

Appendix 1. The origin, seed weight and seed nitrogen content of the medics and subterranean clover used in this study.

Plant species	Cultivar	Origin	Seed dry weight (mg/seed)	Seed N content (mg/seed)
Medicago sativa	Hunter River	France	2.31	0.15
M. truncatula	Borung	Tunisia	3.50	0.26
" "	Caliph	Naturalised S.A[b]	4.33	0.32
" "	Cyprus	Cyprus	3.51	0.24
" "	Jemalong	Naturalised S.A.	4.19	0.32
" "	Mogul	Naturalised S.A.	4.05	0.30
" "	Parabinga	Naturalised S.A.	4.45	0.34
" "	Paraggio	Naturalised S.A.	5.19	0.38
" "	Sephi	Palestine	5.36	0.41
M. rugosa	Paraponto	Italy	11.3	0.75
" "	Paragosa	Portugal	7.85	0.51
" "	Sapo	Portugal	7.91	0.47
M. littoralis	Harbinger	Iran	3.06	0.20
" "	Harbinger AR[a]	Naturalised S.A.	2.68	0.19
M. polymorpha	Circle Valley	Naturalised W.A[c]	4.00	0.21
" "	Santiago	Chile	3.84	0.25
" "	Serena	Naturalised x Chilean	4.51	0.26
M. murex	Zodiac	Tunisia	5.03	0.30
M. scutellata	Kelson	Hungary	18.9	1.10
M. tornata	Tornafield	U.S.A.	5.11	0.23
Trifolium subterraneum	Woogenellup	Australia	10.7	0.79

[a]Aphid Resistance. [b]South Australia. [c]Western Australia

Appendix 2. Composition of nutrient solution used in sand culture.

Stock solution

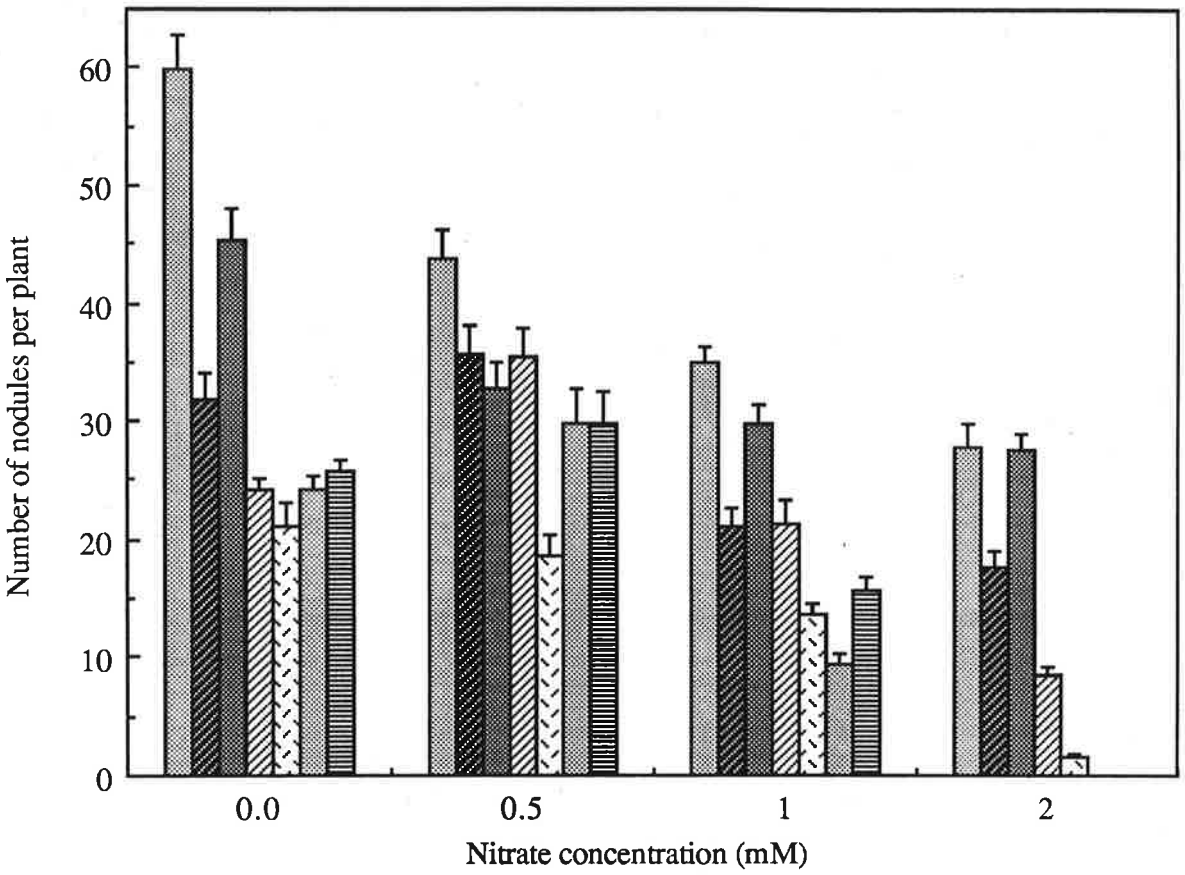
Name	Chemical	g/L
A	KNO_3	50.55
B	$Ca(NO_3)2.4H_2O$	118.07
C	$MgSO_4.7H_2O$	49.27
D	KH_2PO_4	27.20
E (Trace elements)	$Na_2MoO_4.2H_2O$	0.12
"	$CuSO_4.2H_2O$	0.80
"	$ZnSO_4.7\ H_2O$	0.22
"	$MnCl_2.4H_2O$	1.81
"	H_3BO_3	2.86
F	EDTA	5.99
"	$FeSO_4.7H_2O$	4.98
G	K_2SO_4	43.55

Composition of nutrient solution (mL stock solution or g chemical/L)

	Nitrate				
Reagent stock	0.0 mM	1.0 mM	2.5 mM	5.0 mM	10 mM
Solution A (mL)	-	0.67	1.66	3.32	6.65
Solution B (mL)	-	0.67	1.66	3.32	6.65
Solution G (mL)	5.00	4.35	3.33	1.67	0.83
$CaSO_4.2H_2O$ (g)	0.43	0.37	0.29	0.14	0.00

All solutions contained 5 mL C, 1.25 mL D, 0.5 mL E and 4 mL F.

Appendix 3. Data from preliminary experiment to examine the response in nodule number to different rates of nitrate (Chapter 5).



Effect of increasing levels of nitrate on the nodulation of M. rugosa cv. Paraponto, M. rugosa cv. Sapo, M. rugosa cv. Paragosa, M. littoralis cv. Harbinger, M. truncatula cv. Borung, M. truncatula cv. Parabinga, M. truncatula cv. Sephi

Nodulation of seven medic cultivars in the presence of nitrate.

The plants were inoculated with *Rhizobium meliloti* strain CC169 and grown in the presence of 0.0, 0.5, 1 and 2 mM nitrate. The plants were harvested at 42 d (0.0 mM), 36 d (0.5 mM), 30 d (1 mM) and 24 d (2 mM). Each value represents the mean of 35 to 40 plants while bars indicates S.E.

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