Plant Mechanisms Contributing to Acid Impairment of Nodulation of *Medicago murex* and *Medicago sativa* by *Sinorhizobium medicae*

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I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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Yvonne Cheng
Cause is effect concealed, and effect is cause revealed.

The Aghori Vimalananda
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SUMMARY

The widespread sowing of the perennial forage legume *Medicago sativa* to lower groundwater tables in south-western Australia is limited as many soils targeted for its use are too acidic (pH$_{CaCl_2} < 5$) for consistent nodulation with *Sinorhizobium* spp. The annual medic *M. murex* grows and nodulates well in these acidic soils, but it cannot fill the niche of *M. sativa* in lowering groundwater tables. The differential ability of *M. murex* and *M. sativa* to nodulate in acid soils provided the opportunity to compare the nodulation responses between the two species and to identify the mechanisms contributing to the poor nodulation of *M. sativa* in soil of low pH.

An initial glasshouse experiment compared the nodulation of *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius with *S. medicae* strains WSM419 and CC169. Subsequent glasshouse and laboratory experiments used only the more acid-tolerant *S. medicae* strain WSM419. In the glasshouse in soil of pH$_{CaCl_2}$ 4.3, the uppermost nodule on both *M. murex* and *M. sativa* formed at 4-5 cm below the hypocotyl, but the nodules on *M. sativa* formed almost 4 weeks later than those on *M. murex*. The difference in nodulation response between *M. murex* and *M. sativa* was related to numbers of *S. medicae* in the rhizosphere. After 24 d growth in soil of pH$_{CaCl_2}$ 4.3, there were 100-fold higher numbers of *S. medicae* WSM419 associated with the roots of *M. murex* than *M. sativa*. This difference in rhizobial numbers was not due to differences in root growth as there were similar rates of root elongation in *M. murex* and *M. sativa*, or differences in the root products released as root exudates of *M. murex* and *M. sativa* produced at low pH had no significant effect on the growth of *S. medicae*. 
Using a ‘root mat’ approach on soil disks of pH$_{CaCl_2}$ 4.49, *M. sativa* acidified its rhizosphere by approximately 0.2-0.4 pH-units within 4 d, while *M. murex* did not acidify its rhizosphere. Rates of H$^+$ release were higher from *M. sativa* than from *M. murex*. Using videodensitometry with agarose of pH 4.5, mature parts of the tap-root of both species exuded OH$^-$ ions, but this was approximately 2-times higher in *M. murex* than in *M. sativa*. Consequently, young parts of the *M. sativa* rhizosphere were more acidic than that of *M. murex*. The higher rate of acidification by the roots of *M. sativa* made its rhizosphere less favourable for the survival and growth of *S. medicae*.

Root hair development was initially similar for both *M. murex* and *M. sativa*. However by 7 d after sowing in soil of pH$_{CaCl_2}$ 4.3, the density of root hairs on *M. murex* increased to 37 root hairs mm$^{-1}$ root, while the density of root hairs on *M. sativa* decreased to 20 root hairs mm$^{-1}$ root. Due to higher root hair density, the roots of *M. murex* provided a greater surface area for the attachment and colonisation of *S. medicae* compared to the roots of *M. sativa*. Indeed, confocal laser scanning microscopy at 7 d after sowing showed there were larger populations of a green fluorescent protein-marked transconjugant of *S. medicae* WSM419 colonised at 4-5 cm below the hypocotyl on the root of *M. murex* (3.28 pixel intensity units) compared to *M. sativa* (1.78 pixel intensity units). The smaller population of *S. medicae* colonised on the *M. sativa* root resulted in the observed delay in nodule development in *M. sativa* compared to *M. murex*.

Two plant mechanisms contributed to the greater numbers of *S. medicae* in the *M. murex* rhizosphere compared to *M. sativa* rhizosphere when plants were grown in an acidic soil: (1) roots of *M. murex* had a higher density of root hairs, and thus provided a larger root surface area for the growth and
colonisation of *S. medicae* than *M. sativa*, and (2) roots of *M. murex* acidified the rhizosphere less, and thus provided more favourable conditions for the growth and colonisation of *S. medicae* than the rhizosphere of *M. sativa*.

Models explaining the different nodulation responses between *M. murex* and *M. sativa* in soil of pH\(_{\text{CaCl}_2}\) 4.3 and 7.0 are presented.
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And to my family and friends, thank you for everything.
A NOTE ABOUT *Sinorhizobium meliloti* AND *S. medicae* IN THE THESIS

The rhizobial strains used in the experiments described in this thesis are referred to as *Sinorhizobium medicae* based on their 16 rDNA sequence and host nodulation (G Garau, pers. comm., March 2003). However, *S. meliloti* and *Rhizobium meliloti* described in previously published literature are referred to as *S. meliloti*. 
CHAPTER 1. LITERATURE REVIEW

1.1 INTRODUCTION

Legumes are important food and forage crops, and are classified in the family Leguminosae (Royal Botanic Garden, Kew, 2003). This large family, containing 18 000 species (International Legume Database & Information Service, 2003), is further divided into three sub-families, the Caesalpinioideae (mainly trees in the moist tropics), the Mimosoideae (mainly trees and shrubs in the tropic/subtropics, and dry areas) and the Papilinoideae (trees, shrubs and herbs, from tropical to arctic regions, and in dry and flooded areas) (International Legume Database & Information Service, 2003). Some, but not all, legumes form a $N_2$-fixing symbiosis with certain soil bacteria and provide $N$ to the soil (Sprent, 2001). Symbiosis is more common in the Papilinoideae and Mimosoideae sub-families, while very few members of the Caesalpinioideae sub-family form this symbiosis (Sprent, 2001).

In agriculture, the most important benefit of including symbiotic legumes in crop rotations is their ability to fix $N_2$, and consequently enhance the productivity and sustainability of farming systems (Howieson et al., 2000b). Legumes can also provide high protein animal feed, and act as disease and pest breaks. In south-western Australia, legumes are mainly grown for grain or as pastures and forages. The sub-family Papilinoideae contains the tribe Trifolieae, which includes two important pasture genera, *Medicago* and *Trifolium* (Norris, 1965). The perennial species *Medicago sativa* (lucerne or alfalfa) is probably one of the longest used and most important forage crops in agriculture (Michaud et al., 1988).
Once called the “Queen of the Forage Plants”, some of the earliest references to *M. sativa* are from Turkey (1300 B.C.) and Babylonia (700 B.C.) (Michaud et al., 1988). *M. sativa* was probably recognised and domesticated as a valuable agricultural plant for its deep root system which helps its survival through prolonged periods of drought (Michaud et al., 1988). The deep roots of *M. sativa* also use soil water to depths not accessible to annual plants (Ward et al., 2000). Furthermore, deep-set crowns provide protection against frost and cold, and rhizomes enable dormancy during periods of drought or cold. Another possible explanation for the early domestication and cultivation of *M. sativa* was its low dependence on soil N due to its ability to form a symbiotic relationship with N$_2$-fixing soil bacteria. In contemporary times, *M. sativa* has been used to decrease groundwater recharge in areas affected by salinity in North America (Halvorson & Reule, 1980) and south-western Australia (George et al., 1997).

Gram-negative bacteria that form nodules on various leguminous plants and reduce atmospheric N$_2$ to NH$_4^+$ for use by the plant (Phillips, 1999; Sy et al., 2001) are generally called ‘rhizobia’. The most important genera of rhizobia in agriculture are classified into three families of bacteria: Rhizobiaceae, Phyllobacteriaceae and Bradyrhizobiaceae (Sprent, 2001). As defined by 16S rDNA sequence analysis, the family Rhizobiaceae has six genera including *Rhizobium* and *Sinorhizobium*; the family Phyllobacteriaceae has six genera including *Mesorhizobium*; and the family Bradyrhizobiaceae has nine genera including *Bradyrhizobium* (Figure 1.1) (Garrity et al., 2002). New genera and species of rhizobia are being described. Currently, symbionts of less than 50 of the 750 total known legume genera have been identified (Sy et al., 2001).
The genus *Sinorhizobium* currently has seven species, with *S. medicae* and *S. meliloti* being the rhizobial symbionts of the pasture legume genus *Medicago*. Some strains previously classified as *S. meliloti* have been reclassified as *S. medicae* (Rome et al., 1997; Rome et al., 1996). Phenotypically, *S. medicae* nodulates effectively with *M. polymorpha* while *S. meliloti* does not (Rome et al., 1996). Furthermore, the *lpiA* gene which confers acid tolerance and its regulator *fsrR* are both present in *S. medicae*, while only the *lpiA* gene is present in *S. meliloti* (G Garau, pers. comm., March 2003). It is likely that other strains of *S. meliloti* will also be reclassified as *S. medicae*. 
Figure 1.1. Classification of bacteria in the families Rhizobiaceae, Phyllobacteriaceae and Bradyrhizobiaceae (from Garrity et al., 2002). *Indicates microsymbionts of Medicago spp.
Secondary salinity is caused by land management that increases the salt content of soil. It is estimated that $1.8 \times 10^6$ ha (9.4%) of land in Western Australia is affected by salinity (PMSEIC, 1998; George et al., 1997), and $8.8 \times 10^6$ ha (33%) of land is likely to be affected by 2050 (National Land and Water Resources Audit, 2001). In south-western Australia, the major cause of secondary salinity is the inefficient use of soil water in farming systems based primarily on annual crops and pastures. Replacement of native *Eucalyptus pileata* (capped mallee) and *E. eremophila* (tall sand mallee) trees with annual crops and pastures has resulted in decreased transpiration and interception (Nulsen et al., 1986), and increased runoff and recharge, thereby changing the hydrology of the landscape. Salinity greatly affects lower parts of the landscape, degrading rivers and wetlands, and bare salt-affected soils are prone to water and wind erosion. The rehabilitation of salt-affected land requires appropriate water management. On farms, water use can be increased by including more perennial or deep-rooted species in production. For example, plantations of trees such as eucalyptus and *Chamaecytisus proliferus* (tagasaste; tree lucerne) have lowered water tables in south-western Australia (George et al., 1997). However, the relatively high cost of these options (Table 1.1), and the complexity of integrating trees into profitable farming systems has limited their integration at the catchment level (George et al., 1997). Currently, the most economical option is the use of perennial pasture species that can use large volumes of groundwater and be integrated into existing farming systems.
Table 1.1. Costs of management options to control secondary salinity (compiled from George et al., 1997).

<table>
<thead>
<tr>
<th>Salinity management option</th>
<th>Examples</th>
<th>Approximate cost (A$/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drains</td>
<td>• Deep open drains, tube drains</td>
<td>500-4000</td>
</tr>
<tr>
<td>Pumping</td>
<td>• Electric, diesel, air-displacement</td>
<td>750-2000</td>
</tr>
<tr>
<td>Perennial vegetation</td>
<td>• Reforestation with native species</td>
<td>150-&gt;500</td>
</tr>
<tr>
<td></td>
<td>• Trees in agriculture (e.g. E. globulus, Pinus spp.)</td>
<td>250-1200</td>
</tr>
<tr>
<td></td>
<td>• Shrubs (e.g. C. proliferus, Atriplex spp.)</td>
<td>150-800</td>
</tr>
<tr>
<td></td>
<td>• Pastures (e.g. M. sativa)</td>
<td>80-150</td>
</tr>
</tbody>
</table>

Grown in rotation with crops or associated with annual pasture species, *M. sativa* has been identified as the most suited deep-rooted herbaceous perennial for salinity management in south-western Australia (George et al., 1997). In the Great Plains region of the USA, *M. sativa* used soil water to depths of up to 6 m (Halvorson & Reule, 1980) and in Victoria, Australia, incorporating *M. sativa* into a wheat/lupin rotation decreased recharge by 15 mm per year (George et al., 1997). In south-western Australia, *M. sativa* used approximately 50 mm more water per year than a *Trifolium subterraneum* (subterranean clover) pasture by extracting water to depths greater than 1.2 m, compared to 50 cm by the annual pasture roots (Ward et al., 2000). However, the widespread cultivation of *M. sativa* for the control of salinity in south-western Australia is currently limited as the soils targeted for its cultivation are often too acidic (Bettenay & Hingston, 1964) for its nodulation with its rhizobial symbiont *S. meliloti* (Bettenay & Hingston, 1964).

1.2 **SOIL ACIDITY**

It has been estimated that acid soils (pH\textsubscript{H\text{2}O} < 5.5) occupy approximately $3.19\times10^9$ ha (30%) of land in the world (von Uexküll & Mutert, 1995). A large
proportion of these acidic soils occur in the humid tropics, although substantial areas are also found in Australia, North America and Europe. Soil acidification is a natural process favoured by high rainfall, low evaporation, leaching of cations and high oxidative biological activity that produces acids (Jaysundara et al., 1998). However, agriculture and industrialisation have accelerated the natural rate of acidification (Helyar & Porter, 1989). In Australia, approximately $1.5 \times 10^7$ ha of soil is considered strongly acidic ($\text{pH}_{\text{CaCl}_2} < 5.0$) (Cregan et al., 1989).

Soil acidity decreases plant growth due to deficiencies of P, Ca, Mg and K, and toxicities of $\text{H}^+$, Al and Mn (von Uexküll & Mutert, 1995; Ritchie, 1989). In addition to these direct effects on plant growth, soil acidity alters the population and activity of soil microorganisms that transform N, S and P in the soil, thus indirectly affecting the availability of these nutrients to plants (Jackson, 1967). Chemical properties of the rhizosphere and soil microorganisms both affect the availability of nutrients to plants. Furthermore, plant roots can modify the physical, chemical and biological properties of the soil around them and in turn, affect the population of microorganisms in the rhizosphere.

### 1.3 The Rhizosphere

The rhizosphere is defined as the soil adjacent to plant roots, and due to the influence of roots, has a different physical, chemical and biological environment from the bulk soil (Bowen & Rovira, 1999; Hinsinger, 1998). The extent of the ‘rhizosphere effect’ into the soil depends on the roots’ interaction with different nutrients or solutes (Hinsinger, 1998). For example, with water or mobile nutrients, such as nitrates, the rhizosphere extends beyond a few millimetres of soil around the plant roots (Hinsinger, 1998). With poorly mobile nutrients such
as phosphates, the rhizosphere may be limited to less than 1 mm (Hinsinger, 1998). Furthermore, the spatial characteristics for a single nutrient, such as P, vary under different conditions. Physical soil properties that affect the transfer of ionic and molecular compounds, soil particle size and structure, and water content will affect the shape of concentration gradients in the soil. The release of root-derived products will also affect the properties of the rhizosphere (Hinsinger, 1998). During plant growth, the products released by roots may modify the chemical properties of the rhizosphere, in particular pH (Jaillard et al., 2003; Haynes, 1990). As the influence of roots is localised and confined to a relatively small volume of soil, the resulting changes in pH can be one to two pH units different to the bulk soil. This has major effects on the availability of mineral nutrients to roots, and the activity of rhizosphere microorganisms.

1.4 FACTORS AFFECTING PRODUCTS RELEASED BY ROOTS IN THE RHIZOSPHERE

Root products are considered as all substances produced by the root and released into the rhizosphere (Uren, 2001). Root products can also be classified on whether they have a perceived role in directly benefiting plant growth (excretions and secretions) or indirectly benefiting plant growth (diffusates and root debris) (Uren, 2001). Excretions are deemed to facilitate internal metabolism, such as respiration, while secretions are deemed to facilitate external processes, such as mineral acquisition (Uren, 2001). Diffusates, such as organic acids, can facilitate plant tolerance to mineral toxicities or deficiencies, while root debris (Uren, 2001) and root exudates, such as the vitamin biotin (Streit et al., 1996), may act as substrates for microbial growth in
the rhizosphere. Many root-released products are organic and are derived from photosynthesis and other metabolic processes. These products may be water-soluble, such as sugars and polysaccharides, amino acids, organic acids, fatty acids, hormones, vitamins, enzymes, flavonones, nucleotides and other miscellaneous compounds such as auxins, glucosides, unidentified proteins and reducing compounds. Root products may also be water-insoluble, such as cell walls, root-cap cells, cell contents, mucilage and root debris (Brimecombe et al., 2001; Uren, 2001). Other root products include ions, water and gases (e.g. O₂, CO₂). Plant species, genotype, age, and nutrition are four important factors determining the relative and absolute amounts of root products released.

1.4.1 Plant species and genotype

The type and amount of products released by roots vary between plant species. Using gas chromatography on the rhizosphere soil solution of two temperate rain forest species, higher concentrations of low molecular weight organic acids were found in the rhizosphere soil of *Yushania niitakeyamensis* (scattered dwarf bamboo) than *Tsuga chinesis* (hemlock) (Chen et al., 2001). Among more closely related species, different amounts of certain sugars (galactose, glucose, rhamnose) were found in root exudates of *Triticum aestivum* (wheat) and *Hordeum vulgare* (barley), while other sugars occurred in similar amounts (Vancura, 1964).

Different genotypes of the same species may also vary in the type and amount of root products released. For example, significant differences were found in the amount of organic acids released from the roots of five genotypes of *T. aestivum* and four genotypes of *Linum usitatissimum* (flax) (Cielinski et al., 1997). The type and quantity of compounds exuded by roots probably relates to
the adaptation of a species to a particular environment. For example, plants adapted to calcareous soils produce more oxalic and citric acids to solubilise P and Fe, respectively, compared to plants adapted to acid soils which exude smaller quantities of these acids (Ström et al., 1994). Research comparing the root products released by perennial and annual species is limited, although Brimecombe et al. (2001) suggested that perennials generally release more fixed C than annuals, which may be related to perennials investing more assimilates in root development in order to survive year round.

1.4.2 Plant age

Root exudation also changes with plant age. A study to compare root products of *Lupinus albus* (white lupin) and *L. luteus* (yellow lupin) showed that organic acids and fatty acids decreased in concentration from flowering to fruiting stages in both species (Lucas García et al., 2001). Among non-legumes, ã-pyrazolylalanine in *Cucumis sativus* (cucumber) root exudates was only detected at the early seedling stage (Vancura & Hovadik, 1965). Total organic C in *Oryza sativa* (rice) root exudates was lowest at seedling stage, increased until flowering but decreased at maturity (Aulakh et al., 2001). It was also found that with increasing plant age, exudation of organic acids substituted exudation of sugars in this species (Aulakh et al., 2001). Changes in the quantity of certain compounds exuded probably correspond to differing requirements for these compounds during different stages of growth.

1.4.3 Plant nutrition

Root exudation is important in plant nutrition, in terms of both nutrient deficiency and toxicity. Deficiencies in N, P and K often increase the rate of exudation
L. albus released organic acids such as succinate, citrate and malate to mobilise P (Kamh et al., 1999). In another study, roots of both L. albus and L. angustifolius dissolved phosphorus rock, although L. albus dissolved twice as much as L. angustifolius (Hinsinger & Gilkes, 1995). It would have been informative if the authors had measured the root biomass of these species to determine whether root biomass could be related to the amount of organic acids released. Under Fe stress, release of nonproteinogenic amino acids (phytosiderophores) increased Fe acquisition in H. vulgare (Takagi et al., 1984). For Al toxicity, extrusion of high concentrations of organic acids by T. aestivum roots chelated Al and thus limited the incorporation of Al into plant tissues (Christiansen-Weniger et al., 1992). The release of substances from roots may contribute to pH change in the rhizosphere. Soil pH is an important chemical soil parameter which influences a wide range of chemical and biological process in the soil.

1.4.4 Microorganisms in the rhizosphere

Microbial biomass and microbial activity are generally much greater in the rhizosphere than in the bulk soil because of the high availability of substrates in the continuous flow of organic compounds from the root (Brimecombe et al., 2001). Under non-sterile conditions, up to 30% of plant photosynthate is lost through the roots, resulting in a continual and readily assimilable source of organic substrates for microbial growth (Bowen, 1991). Populations of microorganisms in the rhizosphere can reach between $10^{10}$ and $10^{12}$ cells g$^{-1}$ rhizosphere soil (Foster, 1988) and are largely supported by C and N released from roots. These microorganisms, in turn, can significantly influence the nutrient supply to the plant and influence the quality and quantity of root
exudates. The presence of microorganisms increased root exudation from *H. vulgare* and *T. aestivum* (Prikryl & Vancura, 1980; Barber & Lynch, 1977). *Azospirillum* and *Azotobacter* spp. and symbiotic microorganisms such as mycorrhizae also increased exudation by plant roots (Brimecombe et al., 2001). Microbial access to substrates in the rhizosphere depends on organisms developing a competitive advantage over other microorganisms, for example, by evolving specificity with a host plant. However, soil physical and chemical properties of the rhizosphere will also affect the availability of these substrates to microbes in the rhizosphere.

## 1.5 Root-Induced pH Changes in the Rhizosphere

Root-induced changes in rhizosphere pH can affect nutrient availability and thereby influence the acquisition of mineral nutrients by plants. A decrease in rhizosphere pH can occur in response to P (Moorby et al., 1988; Grinsted et al., 1982) and Fe deficiencies (Marschner et al., 1986). Decrease in soil pH increased the availability of P, Zn and Fe to the plant by the desorption of P and Zn previously absorbed to soil colloids, and the dissolution of amorphous hydroxy-Fe compounds (Haynes, 1990). Protons excreted by plant roots dissolve phosphate rock and therefore increase its effective use as a source of P (Hinsinger & Gilkes, 1996; Hinsinger & Gilkes, 1995; Haynes, 1990; Bekele et al., 1983). Root-induced pH changes are of particular importance in acidic soils, where elements such as Al are toxic at pH < 5, and a further decrease in pH could lead to significant plant stress. Al-tolerance under acidic conditions in some genotypes of *Zea mays* (maize) (Calba & Jaillard, 1997), *T. aestivum* (Taylor & Foy, 1985a; Taylor & Foy, 1985b) and an Al-tolerant mutant of *Arabidopsis* (Degenhardt et al., 1998) have been related to their ability to
absorb more NO$_3^-$ than NH$_4^+$ compared to sensitive genotypes when grown in mixed solutions of NO$_3^-$ and NH$_4^+$. This pattern of NO$_3^-$-uptake increases rhizosphere pH and decreases the concentration of Al. In a review, Haynes, (1990) suggested that tolerance to Al may be related to tolerance to low pH. In cereal crops and *P. sativum*, the rates of cation uptake and H$^+$ extrusion were less in acid-tolerant genotypes, thereby decreasing Al concentration in the rhizosphere, compared to acid-sensitive genotypes (Haynes, 1990). It would be interesting to determine whether the same mechanism of Al-tolerance would operate if plants were supplied with equal amounts of NO$_3^-$ and NH$_4^+$ at neutral pH, but with high concentrations of Al. Plant-induced pH changes in the rhizosphere can arise from four major sources of H$^+$: (1) root exudation and respiration, (2) cation-anion exchange balance, (3) organic anion release, and (4) redox systems (Hinsinger et al., 2003).

### 1.5.1 Root exudation and respiration

Root respiration as well as catabolism of root released organic carbon by rhizosphere microorganisms produce significant amounts of CO$_2$ in the soil. The consequent dissolution of CO$_2$ in the rhizosphere solution can then contribute to acidification. However, as the first pK value of H$_2$CO$_3$ is 6.36, H$_2$CO$_3$ remains undissociated at acidic pH, and hence does not contribute to acidification in acid soils (Jaillard et al., 2003). Accumulation of CO$_2$ is an important process in the acidification of neutral to alkaline soils (Jaillard et al., 2003).

### 1.5.2 Cation-anion exchange balance

Plants can either acidify their rhizosphere through root uptake of cations (e.g. NH$_4^+$, Ca$^{2+}$, Mg$^{2+}$, K$^+$, Na$^+$), or alkalinise their rhizosphere through root uptake of
anions (e.g. NO$_3^-$, Cl$^-$, SO$_4^{2-}$, H$_2$PO$_4^-$) (Jaillard et al., 2003; Haynes, 1990). To maintain electroneutrality at the root-soil interface and to regulate cellular pH, roots release excess positive charges such as protons when more cations than anions are taken up (Hinsinger, 1998; Haynes, 1990; Hedley et al., 1982; Nye, 1981). Conversely, when more anions than cations are taken up, excess negative charges are released as hydroxyls or bicarbonate ions.

As N is the most demanded mineral nutrient for many plant species and can be utilised by plants as an anion (NO$_3^-$) or a cation (NH$_4^+$), N uptake is important in influencing rhizosphere pH (Jaillard et al., 2003; Hinsinger, 1998; Marschner & Römheld, 1983). Plants supplied with NO$_3^-$ generally release HCO$_3^-$ or OH$^-$ into the rhizosphere and this is associated with rhizosphere alkalisation. In contrast, plants supplied with NH$_4^+$ generally release H$^+$ and this is associated with rhizosphere acidification (Marschner & Römheld, 1996). Plants relying on atmospheric N$_2$, such as N$_2$-fixing legumes, preferentially take up more cations than anions, and hence behave as NH$_4^+$-assimilating plants because symbiotic rhizobia convert atmospheric N$_2$ to NH$_4^+$ utilised by the plant. Therefore, N$_2$-fixing legumes generally acidify their rhizosphere (Tang et al., 1997; Jarvis & Robson, 1983a). However, it is important to note that this process can be oversimplified as P-deficient, NO$_3^-$-fed plants of *Brassica napus* (oilseed rape) (Grinsted et al., 1982) and *Lupinus* spp. (Hinsinger & Gilkes, 1995) have been shown to acidify their rhizosphere.

Furthermore, various parts of a single root or of a whole root system exposed to identical conditions can have localised regions of acidification and alkalisation (Jaillard et al., 1996; Marschner & Römheld, 1983). Often, the region of root just behind the root apex releases H$^+$ ions while other parts of the root release HCO$_3^-$ or OH$^-$ (Plassard et al., 1999; Marschner & Römheld,
15. This pattern of root-induced pH changes was found in 10 d-old *M. sativa* plants grown in soil of pH$_{H_2O}$ 5.5 (Blanchar & Lipton, 1986). The rhizosphere pH at the older taproot of *M. sativa* was more alkaline (pH 6.8), while the rhizosphere pH at the younger lateral roots was more acidic (pH 4.2) (Blanchar & Lipton, 1986). This may be due to the uneven uptake of mineral nutrients along the root and it is possible that older parts of the root system absorb more anions than cations (Blanchar & Lipton, 1986). Uneven nutrient uptake is particularly true for Ca, which is transported through the apoplast to the root cortex (Jaillard et al., 2003). Because of suberisation of the endodermis in older regions of the root, Ca is preferentially taken up at the root apex, causing an excess of cations over anions in this region which subsequently leads to a larger release of H$^+$ in the root apex compared to other parts of the root.

### 1.5.3 Organic anion release

Increased root exudation of organic acids (e.g. citrate, malate, oxalate) in response to P-deficiency also contributes to soil acidification (Neumann & Römheld, 2001; Hoffland et al., 1989). The importance of organic acids to rhizosphere acidification has been regarded as negligible by some researchers (Petersen & Böttger, 1991), while others suggest that due to their low pK values, the contribution of organic acids to rhizosphere acidification is only significant where by H$^+$ release (Jaillard et al., 2003). However, it is difficult to generalise on this issue as different levels of organic acids are released depending on plant species, and the availability or deficiency of certain nutrients.
1.5.4 Redox systems

The major chemical processes that change the oxidation state of Fe, Mn and N involve the production or consumption of H\(^+\), and thereby a change in soil pH (Jaillard et al., 2003). The low solubility of Fe-bearing minerals, such as iron oxyhydroxides and iron oxides, makes the availability of Fe species insufficient for adequate plant growth. The solubility of these minerals is highly dependent on redox conditions (Hinsinger, 1998). The reduction of Fe\(^{3+}\) by oxidation of other compounds, such as organic matter from microbial or root respiration, and by a consumption of H\(^+\) ultimately results in an increase in soil pH. Conversely, the oxidation of Fe\(^{2+}\) by a production of H\(^+\) will be associated with a decrease in soil pH (Jaillard et al., 2003). These redox processes also apply for Mn, N and S.

The interactions between the soil, plants and microorganisms in the rhizosphere is complex. The pH of the rhizosphere does not only affect plants, but also the microbial population of the rhizosphere. Furthermore, compounds released from plant roots also influence microorganisms in the rhizosphere which in turn, affect the growth of plants.

1.6 Acidity and the Legume-Rhizobia Symbiosis

Nodulation of legume roots results from a series of complex and interdependent interactions between the root and rhizobia. The processes and mechanisms of nodulation have mainly been studied with plants and rhizobia grown at neutral pH. However, acid stress on host plants and rhizobia can affect the nodulation process. Low pH decreased nodulation in *Medicago* spp. (Munns, 1970; Robson & Loneragan, 1970a; Robson & Loneragan, 1970c;
Munns, 1968), *Trifolium* spp. (Richardson et al., 1988b; Rice et al., 1977; Small, 1968), *P. sativum* (Lie, 1969), *Glycine max* (soybean) (Sartain & Kamprath, 1975), *Phaseolus vulgaris* (common bean) (Vargas & Graham, 1988) and *Vigna unguiculata* (cowpea) (Hohenberg & Munns, 1984). In most cases, the rhizobia are the more acid-sensitive partner in the rhizobia-legume symbiosis (Hungria & Vargas, 2000; Richardson & Simpson, 1988; Munns, 1986; Robson & Loneragan, 1970a).

Descriptions of stages in the nodulation process have been attempted (Coventry & Evans, 1989; Vincent, 1980), but these tend to be broad and lack detail. The nodulation process between legumes and rhizobia follows a series of eight general steps: (1) rhizobial survival and (2) rhizobial growth in the soil in the absence of the host plant, (3) colonisation of the rhizosphere by rhizobia, (4) molecular signalling between host and rhizobia, (5) chemotaxis of rhizobia to host root hairs, and (6) attachment of rhizobia to host root hairs. For some host legume species such as *Medicago*, this is followed by: (7) root hair curling and development of the infection thread, and (8) cortical cell division and nodule development. The effects of low pH on each step has been studied to varying degrees, but it appears that the impairment by acidity on any of steps 1-7 significantly impacts on the entire nodulation process.

### 1.6.1 Survival of rhizobia

Rhizobial survival and growth are two different phenomena. Survival can be defined as the ability of rhizobia to persist in a particular medium, without increase in numbers over time. In contrast, growth can be defined as the ability of rhizobia to increase in numbers in a particular medium over time. Generally, research on the effect of acidity has emphasised growth rather than survival, as
it is relatively easier to measure growth, while survival may involve measurements to determine the persistence of rhizobia in a particular environment over a longer period of time. In laboratory media where pH values are favourable for both rhizobial survival and growth, the growth rate of a strain (as measured by the mean generation time) will remain relatively constant (‘growth zone’; Figure 1.2) (Watkin et al., 1997). With increasing acidity, rhizobial growth rates decrease until rhizobia no longer grow (‘acid-stress zone’) (Watkin et al., 1997). With further increase in acidity, rhizobia survive although they cannot grow (‘survival zone’) (Watkin et al., 1997), and as pH further decreases, cells begin to die (‘death zone’) (Watkin et al., 1997). The heterogeneous nature of soil may provide less acidic sites for the survival and growth of rhizobia (Coventry & Evans, 1989) compared to laboratory media where conditions are more homogenous. Therefore, even though laboratory media and soil may have the same acidic-pH value, the different conditions between the two types of media has a very different effect on the survival and growth of rhizobia (Howieson et al., 1988).
Figure 1.2. The effect of pH on the mean generation time of rhizobia showing the four zone response of rhizobia to low pH stress. Compiled from Watkin et al. (1997) and Dilworth et al. (2000).

Rhizobial numbers in soil are affected by pH. There were lower numbers of *R. trifolii* in soil of pH 4.5 compared to limed soils where pH ranged from 6.0-7.2 (Rice et al., 1977). A long-term study on the survival of rhizobia in an acidic soil showed that after 25 months incubation in the absence of host plants, the numbers of *Phaseolus*-nodulating rhizobia remained at 90-94% of the initial population in limed soil (pH$_{\text{CaCl}_2}$ 4.60-4.68) (Andrade et al., 2002). In unlimed soil (pH$_{\text{CaCl}_2}$ 3.93-4.12), numbers of *Phaseolus*-nodulating rhizobia decreased to 3-7% of the initial population (Andrade et al., 2002). It is clear that survival of rhizobia in limed soil was not due to effects on the growth of the host (Andrade et al., 2002). The authors suggested that since the decrease in survival was observed over a range of only 0.7 pH-unit, the direct effects of pH on rhizobial survival were not important (Andrade et al., 2002). However, this disregards the important effect of H$^+$ in rhizobial survival (Dilworth et al., 2000; Watkin et al., 1997; Reeve et al., 1993). For *S. meliloti* (Reeve et al., 1993) and
R. leguminosarum (Watkin et al., 1997), a decrease in even 0.1 pH-unit dramatically decreased rhizobial survival. Although there are differences in the conditions which rhizobia encounter in soil and media of low pH, rhizobial survival is highly sensitive to acidity, and this sensitivity should be considered in 0.1 pH-unit increments rather than whole pH-unit increments. Increased Ca concentration significantly improved rhizobial survival at low pH (Dilworth et al., 1999; Watkin et al., 1997; Reeve et al., 1993). An interaction of soil factors, such as Al toxicity with pH, was perhaps also important for the survival of Phaseolus-nodulating rhizobia (Andrade et al., 2002).

Genera of rhizobia differ greatly in their ability to survive under acidic conditions. Bradyrhizobium spp. are relatively tolerant to acidity. Among fast-growing rhizobia, Sinorhizobium spp. are the least tolerant (Dilworth et al., 2000; Graham & Parker, 1964) while R. tropici is the most tolerant (Dilworth et al., 2000). Tolerance to acidity also varies among species of the same genus; for example, R. tropici is more acid-tolerant than R. leguminosarum. Strains within a species also differ in acid-tolerance and this has been demonstrated in B. japonicum (Keyser et al., 1979; Munns et al., 1979), R. phaseoli (Graham et al., 1982), R. leguminosarum bv. trifolii (Lindström & Myllyniemi, 1987; Rice et al., 1977), R. leguminosarum (Watkin et al., 2000; Carter et al., 1995; Hartel & Alexander, 1983; Rai & Prasad, 1983) and S. meliloti (Howieson & Ewing, 1986; Rice et al., 1977). In soil of pH 4.15, numbers of an acid-sensitive strain of R. phaseoli decreased from $10^7$ to $10^2$ cells g soil$^{-1}$ after five d, while numbers of a more acid-tolerant strain decreased only 5-fold after 15 d (Graham et al., 1982).

Variability in rhizobial tolerance to acidity is genetically controlled, and an understanding of these components is being used in the genetic construction of
acid tolerant strains (Dilworth et al., 2000). The Adaptive Acid Tolerance Response (ATR) has been recognised in bacteria, including \textit{E. coli}, \textit{Salmonella typhimurium}, and strains of \textit{Bradyrhizobium}, \textit{R. leguminosarum} and \textit{R. tropici} (O'Hara & Glenn, 1994). This phenomenon occurs where cells that have grown in moderately acidic conditions are more tolerant to subsequent exposure to more extreme acidity than cells that were previously grown at neutral pH (Dilworth et al., 2000). ATR is possibly ecologically important as cells with ATR may contribute to the survival and persistence of bacteria under acidic conditions (Dilworth et al., 2000), and in the case of rhizobia, may affect the establishment of rhizobial populations in acidic soils. ATR involves a variety of structural and regulatory genes essential for cell growth at low pH (Dilworth et al., 2000). Regulatory genes, such as the two-component sensor-regulatory system \textit{actRS} in \textit{S. meliloti}, have a role in tolerance to low pH (Tiwari et al., 1996). These genes are essential for both growth at low pH and the ATR in \textit{S. meliloti}, although it is not known what specific and new proteins are required for the ATR in this species (Dilworth et al., 2000). Other genes, such as the \textit{exoR} gene controlling the production of exopolysaccharides in \textit{R. leguminosarum} bv. \textit{viciae}, and in some strains of \textit{Rhizobium} and \textit{Bradyrhizobium}, may have a role in the tolerance of these strains to acidic conditions (Reeve et al., 1997; Cunningham & Munns, 1984). Dilworth et al. (2000) proposed two ways to construct acid-tolerant strains of rhizobia: (1) by transferring acid-tolerant genes into an acid-sensitive strain (e.g. insertion of acid-tolerance genes from \textit{R. tropici} to other rhizobia (Graham & Vance, 2000)), or (2) by transferring nodulation and \textit{N}_2-fixation genes into an acid-tolerant strain. The genetic complexity of acid tolerance may make the first approach impractical (Dilworth et al., 2000), whereas the concentration of nodulation and
N\textsubscript{2}-fixation genes into megaplasmids may make the second approach more feasible (Dilworth et al., 2000).

1.6.2 Growth of rhizobia

Low soil pH decreased the numbers of \textit{Rhizobium} spp. (Carter et al., 1995; Hartel & Alexander, 1983; Robson & Loneragan, 1970c) and \textit{Sinorhizobium} spp. (Pijnenborg et al., 1990a). In laboratory culture, it has been shown that low pH decreased the rate of growth of \textit{S. meliloti} (Howieson et al., 1992b), \textit{R. trifolii} (Lindström & Myllyniemi, 1987), \textit{R. leguminosarum} bv. \textit{trifolii} (Watkin et al., 1997) and \textit{B. japonicum} (Hartel & Alexander, 1983). Slower growth rates of rhizobia at low pH probably contributed to the low rhizobial numbers observed in acidic soils (Carter et al., 1995; Pijnenborg et al., 1990a; Hartel & Alexander, 1983; Robson & Loneragan, 1970c). Apart from low pH \textit{per se}, Al and Mn toxicities, conditions commonly associated with acid soils, are also the main stresses affecting the growth of free-living rhizobia (Keyser & Munns, 1979b). Unlike for plants, tolerance to acidity in rhizobia does not necessarily relate to Al-tolerance as 40% of the strains \textit{R. leguminosarum} and \textit{B. japonicum} able survive pH 4.5 could not tolerate 50 \textmu M Al, a level that rhizobia could realistically encounter in acidic soils (Keyser & Munns, 1979b). Low concentrations of P (5-10 \textmu M) slowed the growth of some strains of \textit{R. leguminosarum} and \textit{B. japonicum} but with less severity than acidity and Al (Keyser & Munns, 1979b). P concentration was found to have no effect on the critical pH for growth of \textit{R. leguminosarum} (Watkin et al., 1997), although P did interact with pH effects on the growth of \textit{S. meliloti} (Watkin et al., 1997). In both soil and laboratory media, external Ca concentration has a significant effect on the growth of rhizobia at low pH.
Growth of rhizobia at low pH appears highly dependent on the external concentration of Ca. At low pH, increasing Ca concentration increased the rate of growth and survival of *S. meliloti* (Dilworth et al., 1999; Howieson et al., 1992b), *B. japonicum* (Keyser & Munns, 1979a) and *R. leguminosarum* bv. *trifolii* (Watkin et al., 1997). Ca is essential for rhizobial cell wall integrity (Humphrey & Vincent, 1962) and Ca deficiency results in deformities of shape, increased absorption of antibiotics and cell leakiness (Munns, 1978). In acidic conditions where the supply of Ca is limited, cell membrane integrity of rhizobia is affected (Ballen et al., 1998), which may increase H⁺ influx into cells and affect solute uptake. The presence of Ca at low pH may have at least two protective functions: (1) against Al and Mn toxicity by affecting the stability of the cell envelope (Keyser & Munns, 1979a), and (2) against acidity by affecting exopolysaccharide production (e.g. in *S. meliloti*) (Dilworth et al., 1999).

Poor growth of rhizobia in acidic soils is a major factor contributing to decreased nodulation in *M. sativa* (Pijnenborg et al., 1990a); *M. truncatula* (barrel medic) (Robson & Loneragan, 1970c), *V. unguiculata* (Hartel & Alexander, 1983), *P. sativum* (Evans et al., 1993) and *Vicia faba* (faba bean) (Carter et al., 1995). In solution culture, a sufficient population of *S. meliloti* must be attained at the root surface of *M. sativa* before infection will occur (Munns, 1968). Furthermore, growth and survival of *S. meliloti* at low pH is important since the size of the rhizobial population has an effect on the concentration of Nod factor, the signalling molecules from rhizobia required for the nodulation process. Therefore, growth of rhizobia in acidic soils is important because rhizobia must accumulate rapidly at a particular location of the root for successful infection to occur.
Rhizobial survival and growth may be affected by competition with other soil microorganisms (Coventry & Evans, 1989). Rhizobia also compete with other rhizobia for nodulation (Barran & Bromfield, 1997; Ames & Bergman, 1981). Rhizobia reside, interact and compete with numerous and varied microorganisms in both the bulk and rhizosphere soil (Rovira, 1961). In a competitive environment, the survival and growth of rhizobia depends on their ability to locate and colonise suitable sites under pressure from other competing organisms. Suitable sites are generally distributed unevenly in both the soil and on the root surface (Coventry & Evans, 1989). Consequently, populations of rhizobia in acid soils are not only small, but unevenly distributed, with larger populations of rhizobia colonising less acidic sites near the soil surface or senesced nodules (Richardson & Simpson, 1988). In soil where pH is heterogeneously distributed (Coventry & Evans, 1989), populations of rhizobia survive low pH not by tolerating acidity, but by avoiding extreme acidity (Richardson & Simpson, 1988). However, it is likely that both tolerance and avoidance of low pH will affect the rhizobial population size in an acidic soil.

1.6.3 Colonisation of the rhizosphere by rhizobia

Root colonisation can be defined as a process whereby an introduced microorganism on or around roots increases in numbers (Kloepper & Beauchamp, 1992). It is not simply a passive chance encounter of a soil microorganism with a passing root (Kloepper & Beauchamp, 1992). Colonisation of the host plant root by rhizobia is an essential step in the nodulation process (Vincent, 1980). Strong correlations between numbers of rhizobia in the rhizosphere and nodulation have been observed for G. max (Brockwell et al., 1989; Herridge et al., 1984) and annual Medicago spp. (Young
A single rhizobial cell can initiate infection (Purchase & Nutman, 1957). However, in order to infect the host, that single rhizobial cell must multiply as the numbers of rhizobia in the rhizosphere affects the critical concentrations of rhizobial Nod factors required for root hair deformation and cortical cell division (Hirsch, 1992). In acid soils, it is likely that decreased growth rate of rhizobia will delay rhizosphere accumulation and colonisation.

Rhizosphere colonisation by rhizobia depends on the interaction between rhizobia and host plant. In acid soils, there was indication that colonisation of the rhizosphere was more successful by acid-tolerant strains of rhizobia on the roots of acid-tolerant plant genotypes than by acid-sensitive strains of rhizobia on the roots of acid-sensitive plant genotypes (Barclay et al., 1994). Although plants were water-stressed and plant growth was adversely affected, there was some indication that in soil of pH\textsubscript{CaCl\textsubscript{2}} 5.26, there were higher numbers of the acid-tolerant strain of \textit{S. meliloti} WSM540 in the rhizosphere of the relatively acid-tolerant species \textit{M. murex} than the more acid-sensitive combination of strain CC169 and \textit{M. truncatula} (Barclay et al., 1994). Plant genotype may affect the extent of colonisation by their differential ability to modify rhizosphere properties (Brimecombe et al., 2001). For example, root-induced rhizosphere pH changes in legumes (Tang et al., 1998; McLay et al., 1997; Tang et al., 1997) may affect the numbers of rhizobia in the rhizosphere, and differential patterns of acidification along a root (Marschner & Römheld, 1983; Marschner et al., 1982) may have an effect on the pattern of rhizobial colonisation. Although there has been extensive research on the pattern of rhizosphere pH (e.g. Marschner et al., 1982) and studies on where microorganisms colonise a root surface (e.g. Prayitno et al., 1999), there is limited work on the pattern of rhizobial colonisation in relation to rhizosphere pH along a root.
1.6.4 Molecular signalling between plants and rhizobia

A key early event in the legume nodulation process is the exchange of specific biochemical compounds between the root and rhizobia (Long, 1996; van Rhijn & Vanderleyden, 1995; Dénarié et al., 1992) in a process called molecular signalling. Different plant parts release different nod gene inducing compounds. For example, young roots of *M. sativa* exuded at least three flavonoid nod gene inducers: 4,4'-dihydroxy-2'-methoxylachalcone, 7,4'-dihydroxyflavone and 7,4'-dihydroxyflavanone (DHF), but none of the flavone luteolin (Maxwell et al., 1989). Luteolin, along with the structurally related 3'-methoxyluteolin (chrysoeriol), and two betaine nod gene inducers, trigonelline and stachydrine, were exuded by germinating seeds of *M. sativa* (Hartwig et al., 1990; Phillips et al., 1992). In the presence of these specific plant compounds, rhizobia exhibit two key responses: (1) increase in growth rate (Hartwig et al., 1991), and (2) nod gene induction (Long, 1996; Downie, 1994; Dénarié et al., 1992). Growth rate of *S. meliloti* was increased by low concentrations (2.5-9.0 µM) of various compounds released from the seed and roots of *M. sativa* (Hartwig et al., 1991).

Exuded plant compounds induce rhizobial nod genes which code for proteins required for the synthesis of host-specific lipochitooligosaccharide nodulation signalling molecules known as Nod factors (Long, 1996; Downie, 1994; Dénarié et al., 1992). Most rhizobial nod genes are transcriptionally silent when bacteria are grown in culture, but their expression can be induced to produce Nod factors by plant compounds (Long, 1996). The concept that compounds exuded from plants could regulate bacterial genes was first demonstrated with luteolin, isolated from germinating *M. sativa* seedlings, which induced the transcription of nod genes in *S. meliloti* (Peters et al., 1986). Similarly, flavones (e.g. 3',5,7-
trihydroxyflavone (apigenin)) and flavanones (e.g. 4',5,7-trihydroxyflavanone (naringenin)) from the non-legume Antirrhinum majus (snapdragon) induced nod genes in R. leguminosarum (Firmin et al., 1986).

Under acidic conditions where rhizobial populations are small, the time taken to produce the concentration of Nod factors required to induce cellular changes in root hairs may be critical, as root hairs are only transiently susceptible to infection (Bhuvaneswari et al., 1981). The dynamics of the production of signal molecules and their exchange between plants and legumes under acidic conditions has not been studied extensively. Depending on pH and the compounds involved, acidity may affect rhizobial nod gene induction in three ways: (1) decrease the activity of nod gene inducers by changing their concentration, (2) decrease the activity of nod gene inducers by changing their structure (Richardson et al., 1989), or (3) adversely affect the response of rhizobia to nod gene inducing compounds (Richardson et al., 1989). Richardson et al. (1988a) collected root exudates from seedlings of T. repens and T. subterraneum between pH 3.0 and 8.0, and assessed the nod gene induction activity of these exudates in R. trifolii. It appeared that pH altered the nod gene induction properties of Trifolium root exudates since nod gene induction in R. trifolii was decreased by: (a) T. repens and T. subterraneum root exudates collected at pH > 7.0, (b) T. repens root exudates collected at pH < 4.0, and (c) T. subterraneum root exudates collected at pH < 5.0 (Richardson et al., 1988a). For the nod gene inducer DHF, its pK value (> 9) would ensure no ionisation of its phenolic hydroxyl groups, and hence no change in its solubility between pH 4.0 and 7.0 (Richardson et al., 1989). Since the structure of DHF was unchanged by acidity, impaired uptake of DHF by R. leguminosarum bv. trifolii may explain the poor nod gene induction by DHF at low pH (Richardson et al.,
Indeed, accumulation of naringenin in the cytoplasmic membrane of *R. leguminosarum* bv. *viciae* was pH dependent: binding was very high at pH 5.7 and not present at pH 9.7 (Recourt et al., 1989). However, the relevance of this finding is questionable since *R. leguminosarum* bv. *viciae* is dead at pH 9.7. It would have been more useful to determine the binding of naringenin to *R. leguminosarum* bv. *viciae* at a pH range (e.g. 4.5-7.0) where rhizobia can at least survive.

In a comparison of *nod* gene induction activity of root exudates from acid-tolerant and acid-sensitive species of *Medicago* in *S. meliloti* at pH 5.8, increasing Ca concentration increased *nod* gene induction activity of *M. truncatula* (acid-sensitive) root exudates, but had no effect on the *nod* gene induction activity of *M. murex* (acid-tolerant) root exudates (Howieson et al., 1992a). It was suggested that the ability of root exudates from acid-tolerant *Medicago* spp. to induce the expression of *nod* genes in *S. meliloti* may be a host contribution to symbiotic acid tolerance (Howieson et al., 1992a). However, another study showed that there was no difference in the ability of root exudates from *M. murex* (acid-tolerant) and *M. sativa* (acid-sensitive) to induce *nod* genes in *S. meliloti*, or a difference in the structural components of root exudates collected from *M. murex* and *M. sativa* (E Watkin, pers. comm., April 2003). Perhaps high concentrations of Ca helped the uptake of *M. truncatula* root exudates in *S. meliloti* in the study by Howieson et al. (1992a). It appears that root exudates alone cannot explain the different nodulation responses between acid-tolerant and acid-sensitive genotypes of *Medicago* (Howieson & Ewing, 1989), and it is likely that other plant-related mechanisms are involved. In the presence of root exudates, rhizobia exhibit chemotaxis and attachment to host plant roots.
1.6.5 Chemotaxis and attachment to host plant roots

Chemotaxis is the free directional movement exhibited by whole organisms in response to a chemical stimulus (Bailey, 1999). For example, rhizobia exhibit positive chemotaxis, moving towards products exuded by roots (Caetano-Anollés et al., 1988a). Chemotaxis in rhizobial cells is important in the symbiosis between rhizobia and host plant, contributing to contact and adherence to host roots (Caetano-Anollés et al., 1988b), rapid infection, efficient nodule initiation (Caetano-Anollés et al., 1988b) and competition for nodule occupancy (Caetano-Anollés et al., 1988b; Gulash et al., 1984; Ames & Bergman, 1981).

Cells of *S. meliloti* exhibit positive chemotaxis towards low concentrations (10^{-8} to 10^{-9} M) of luteolin (Caetano-Anollés et al., 1988a). It appears that rhizobia are selectively attracted to certain compounds, as the closely related compound apigenin was not a chemoattractant while naringenin abolished chemotaxis of *S. meliloti* towards luteolin.

A great deal remains to be understood about the effect of acidity on the motility and chemotaxis of rhizobia. The motility of *R. leguminosarum* in laboratory media was optimum between pH 5.5 and 8.0, and was absent at pH 4.5 (Bowra & Dilworth, 1981). Chemotactic response as affected by pH was not investigated in this study possibly because it was assumed that if motility was impaired by low acidity, chemotaxis would also be impaired. Chemotaxis of *S. meliloti* towards luteolin was dependent on functional *nod* genes (Caetano-Anollés et al., 1988a). However, this study was done at neutral pH. If rhizobial *nod* genes are impaired by low pH, then it may be possible that chemotaxis will be also impaired.
Attachment (also called adsorption) is an early step in the nodulation process. Rhizobia can attach to glass and plastic surfaces, and to the roots of host and non-host plants within seconds or minutes of inoculation in nutrient solution (Bauer, 1981). Rhizobial attachment to host roots may be affected by the growth phase of rhizobia. The attachment of *B. japonicum* to *G. max* roots was maximum in the late-exponential growth phase (Lodeiro & Favelukes, 1999), while the attachment of *R. leguminosarum* bv. phaseoli to *P. vulgaris* roots was maximum in the early-stationary growth phase (Lodeiro et al., 1995). Wisniewski & Delmotte (1996) showed that early to middle exponential phase cultures of *Bradyrhizobium* attached to *L. albus* roots more than older cultures. For *Bradyrhizobium* sp. (Lodeiro & Favelukes, 1999; Wisniewski & Delmotte, 1996) and *R. leguminosarum* bv. phaseoli (Lodeiro et al., 1995), attachment to roots was absent in cultures at the stationary phase. Rhizobial attachment to roots is a 2-step process (Kijne et al., 1988). The first step involves rhizobia loosely attaching to a plant receptor via components on rhizobial surfaces such as extracellular polysaccharides, capsular polysaccharides, lipopolysaccharides (Matthysse & Kijne, 1988) and surface proteins such as rhicadhesin (Matthysse & Kijne, 1988; Smit et al., 1987). The rhizobial polysaccharides may be involved in binding by gelling with plant polysaccharides (Matthysse & Kijne, 1988). Rhicadhesin is a Ca-binding protein that appears to be common among Rhizobiaceae. The second step is a tighter attachment by cellulose fibrils (Smit et al., 1987). Host plants also contribute to attachment by the production of surface proteins (Matthysse & Kijne, 1988) and lectins. A multivalent lectin extracted from *T. repens* seeds is suggested to bind cross-reactive antigen sites on *R. trifolii* and *T. repens* root surfaces (Dazzo et al., 1976). However, the role of lectins in Nod factor reception and recognition remains unclear (Hirsch,
In a review, Hirsch (1992) highlighted that lectins may be involved in invasion rather than attachment of rhizobia. Root exudates may also promote rhizobial attachment as pre-treatment of *S. meliloti* with root exudates of *M. sativa* stimulated the early attachment of cells to roots (Wall & Favelukes, 1988). Attachment to roots of *L. albus* by *Bradyrhizobium* sp. (*Lupinus*) increased in the presence of lupin root isoflavonoids genistein and genistin (Wisniewski & Delmotte, 1996). Rhizobia attach to root hairs all over the root, but hairs that have recently emerged are the most responsive to infection (Bhuvaneswari et al., 1980).

Attachment may vary between strains within a rhizobial species. For example, higher numbers of the more acid-tolerant strain *S. meliloti* WSM540 attached to roots of annual *Medicago* spp. than the more acid-sensitive strain CC169 at low pH (Howieson et al., 1993). It should be emphasised that attachment studies in laboratory media cannot be extrapolated to soils. Plants of *M. sativa* (Pijnenborg & Lie, 1990), *M. polymorpha* and *M. murex* (Howieson & Ewing, 1989) nodulated in soils pH $\leq 6$ and therefore must have had *S. meliloti* cells attached to their roots even though rhizobial attachment to their roots may be impaired in nutrient solution of pH $\leq 6$. Attachment of *Bradyrhizobium* to *L. albus* roots was optimum at pH 6.6, but decreased by 20% at pH 5.6 (Wisniewski & Delmotte, 1996). Although the interaction between Ca and H$^+$ ions has been studied extensively (Keyser & Munns, 1979a; Munns, 1970; Robson & Loneragan, 1970b; Munns, 1968), its role in attachment is still not well understood. The attachment of *S. meliloti* cells to the roots of *M. sativa* (Caetano-Anollés et al., 1989), *M. polymorpha* and *M. murex* (Howieson et al., 1993) was inhibited in laboratory media of pH $\leq 6$, but could be improved by supplying higher concentrations of Ca. In contrast, higher concentrations of Ca
decreased the attachment of *Bradyrhizobium* to *L. albus* roots (Wisniewski & Delmotte, 1996). Other mechanisms such as rhizobial strain, nutrient requirement and growth culture conditions might also influence attachment (Wisniewski & Delmotte, 1996). It may also be possible that Ca has an opposite effect on the attachment of the relatively acid-tolerant *Bradyrhizobium* compared to the more acid-sensitive *Sinorhizobium*. Increased Ca concentration also decreased the attachment of *Azospirillum* strains to *T. aestivum* roots (de Oliveira Pinheiro et al., 2002). Ca concentration may be critical in a Ca$^{2+}$-dependent rhicadhesin in rhizobia that facilitates attachment of bacteria to the tips of developing root hairs (Smit et al., 1989; Smit et al., 1987; Smit et al., 1986). Why root hairs become progressively less infectible by rhizobia with time is unclear, and it may be possible that the Ca$^{2+}$-dependent rhicadhesin is absent in more mature root hairs, thereby decreasing attachment by rhizobia. Like older regions of the root where Ca uptake is lower compared to the root apex (Jaillard et al., 2003), more mature root hair tips may also take up less Ca than younger root hair tips due to increased suberisation of the cell walls.

### 1.6.6 Root hair curling and infection thread formation

Root hairs are projections from single epidermal cells that increase the root surface area for water and nutrient adsorption. In *Vicia, Medicago* and *Tritolium* spp., infection of the root by rhizobia is via a root hair. In other legumes, such as *Arachis hypogaea* (peanut), *Stylosanthes* and *Lupinus*, rhizobia infect via a wound or crack in the root (Sprent, 2001; Sprent, 1989). Root hairs are the first root cells to respond to rhizobial Nod factors (Lhuissier et al., 2001). A small proportion of curled root hairs become colonised with rhizobia and subsequently
form infection threads – tubular structures which elongate in the root hair and carry rhizobia towards the nodule primordium in the root cortex (Gage et al., 1996; Wood & Newcomb, 1988; Bauer, 1981). The susceptibility of root hairs to infection by rhizobia appears to be developmentally regulated and transient in some legumes (Bhuvaneswari et al., 1981; Bhuvaneswari et al., 1980). Therefore, it is critical that root hairs and rhizobia encounter one another at the right time.

The susceptibility of root hairs to rhizobial infection decreases with age. For example, plants of *G. max*, *V. unguiculata* and *M. sativa* formed nodules when inoculated with rhizobia near the root tip, while plants inoculated on the root where more mature root hairs were located formed fewer nodules (Bhuvaneswari et al., 1981; Bhuvaneswari et al., 1980). Variation exists between species, however in general, it appears that the initially susceptible region of the root becomes progressively less susceptible if inoculation is delayed by a few hours. When inoculation of *T. repens* root tips was delayed by 36 h, the number of plants that formed nodules decreased from 93% (no delay) to 18% (Bhuvaneswari et al., 1981). Similarly, root hair cells of *M. sativa* were most susceptible to infection by *S. meliloti* in the first 2 h of root hair initiation (Wood & Newcomb, 1988). When root hairs were inoculated 2-8 h after initiation, root hair cells formed various deformations and the rate of infection was lower (2-17%). It is important to note that although root hairs decrease in susceptibility to infection, it does not mean that all root hairs in more mature regions of the root cannot be infected. Healthy and fully matured root hairs of *T. repens* are still susceptible to infection, although at a lower frequency compared to emerging and younger root hairs (Bhuvaneswari et al., 1981).
Considering the importance of root hair curling in the infection process in some legumes species, the basic mechanism by which the normal synthesis of root hair cell walls are altered through root hair curling and why root hairs are only transiently susceptible to rhizobial infection have not been thoroughly described. It has been suggested that when rhizobia attach close to the root hair tip and release Nod factors, tip growth is stimulated only at the attachment side (Lhuissier et al., 2001). As rhizobia multiply, the area of contact between the root hair and rhizobia increases, and the new root hair tip will touch new rhizobia, which also release Nod factors and again redirect root hair tip growth towards the rhizobia (Lhuissier et al., 2001). As this continues to happen, the root hair tip rotates in one direction to produce a tightly curled tip in which rhizobia are entrapped (Lhuissier et al., 2001). It has also been suggested that the type of root hair deformation (e.g. curling, ballooning, intertwining) was related to the position of the nucleus along the root hair axis (Wood & Newcomb, 1988). Although nucleus position seemed to be related to the age of root hair cell, there was no conclusive evidence to suggest that the nucleus alone was responsible for directing the pattern of root deformation. A speculative model by Bauer (1981) proposed that most successful infections take place at the apical ends of short epidermal cells because these cells have completed most of their axial elongation but their capacity to form cell walls allows the root hair to curl around attached rhizobia (Bauer, 1981). In contrast, if attachment occurred on older root hairs where cell wall formation has been completed, there is decreased capacity for the hair to curl around the attached rhizobia (Bauer, 1981). The hypothesis that cell wall maturity was related to susceptibility of root hairs to infection (Bauer, 1981) was later supported by research on the infection of *M. sativa* root hairs (Mateos et al., 2001). This study
showed that the major site where rhizobial polysaccharide-degrading enzymes were most effective in completely eroding a hole through the root hair wall was highly localised at the amorphous noncrystalline apex of the root hair tip (Mateos et al., 2001), where the cell wall was thinner and less cross-linked than elsewhere along the root hair axis (Hirsch, 1992). A summary of the events leading to root hair curling is shown in Figure 1.3.

Thinner and less cross-linked cell walls of young root hair cells may enable three important processes: (1) the root hair to curl around the attached rhizobial cell, (2) the root hair cell wall to be more easily eroded by rhizobial polysaccharide-degrading enzymes (Mateos et al., 2001), which allow the rhizobial cell to enter the root hair, and (3) Ca\(^{2+}\) to enter root hairs. Oscillations or spikes in Ca\(^{2+}\) concentration in or around the nucleus of root hair cells in *M. sativa* (Ehrhardt et al., 1996), *M. truncatula* (Wais et al., 2000) and *P. sativum* (Walker et al., 2000) begin approximately 10 min after sensing Nod factor at concentrations as low as 10\(^{-13}\) to 10\(^{-12}\) M (Shaw & Long, 2003). At higher concentrations of 10\(^{-10}\) to 10\(^{-9}\) M, Nod factor triggers an influx of Ca\(^{2+}\) into the cytoplasm of the root hair cell within seconds (Cárdenas et al., 1999; Felle et al., 1998; Ehrhardt et al., 1996). In this 2-step response to Nod factor concentration, Ca\(^{2+}\) spiking and Ca\(^{2+}\) influx in root hairs may be mechanisms used by the plant to determine proximity of rhizobia to its roots (Shaw & Long, 2003). In the first step, low concentrations of Nod factor (10\(^{-13}\) to 10\(^{-12}\) M) in soil to induce Ca\(^{2+}\) spiking in the root hair cell serve as a priming signal (Shaw & Long, 2003). In the second step, accumulation of higher concentrations of Nod factor (10\(^{-10}\) to 10\(^{-9}\) M) in the rhizosphere to cause Ca\(^{2+}\) influx into the root hair cell cytoplasm signal to the plant the close proximity of rhizobia (Shaw & Long, 2003). The increase in cytosolic Ca\(^{2+}\) concentration in the root hair cell activates
ion channels leading to an efflux of Cl\textsuperscript{−} ions, causing plasma membrane depolarisation (Donaire et al., 1999; White, 1998; Ehrhardt et al., 1992), which may affect the cell wall formation and a change in a direction of root hair growth leading to curling. Ca\textsuperscript{2+} influx is confined to the apical 20-50 \textmu m of the root hair, depends critically on external Ca\textsuperscript{2+} concentration and pH (White, 1998), and appears to be specifically associated with root hair elongation since it is absent in mature, non-growing root hair cells (White, 1998). Understanding of the effects of low pH on the above process has been substantially limited.
Root hair cell wall structure (Mateos et al., 2001; Hirsch, 1992; Bauer, 1981)

Mature root hair
Cell walls are thicker, cross-linked, crystalline and inflexible

Young root hair
Cell walls are thinner, less cross-linked, noncrystalline and flexible

Erosion of root hair cell wall by rhizobial enzymes
(Mateos et al., 2001)

Ca²⁺ spiking in root hair cells
(Shaw & Long, 2003; Wais et al., 2000; Walker et al., 2000; Ehrhardt et al., 1996)

Ca²⁺ influx in root hair cells
(Shaw & Long, 2003 Cárdenas et al., 1999; Donaire et al., 1999; Felle et al., 1998; White, 1998; Ehrhardt et al., 1996)

No erosion of cell wall by polysaccharide-degrading enzymes from attached rhizobia

Rhizobial Nod factors cause Ca²⁺ spiking

No Ca²⁺ influx into root hair cell

Ca²⁺ influx into root hair cell

Cl⁻ efflux from root hair cell

Plasma membrane depolarisation of root hair cell wall causes curling of root hair

No depolarisation of plasma membrane of root hair cell

No curling of root hair

Curling of root hair

Figure 1.3. Summary of the events leading to root hair curling in young root hair cells. See Section 1.6.6 for full description.
Root hair curling is nonetheless considered an acid-sensitive step in the nodulation of *M. sativa* (Munns, 1970; Munns, 1968), *P. sativum* (Lie, 1969), *P. vulgaris* (Franco & Munns, 1982) and *T. repens* (Wood et al., 1984). In *M. sativa*, low pH delayed the onset of root hair curling (Munns, 1968). However, if root hair curling had already commenced, the numbers of curled root hairs would continue to increase even if the pH was lowered (Munns, 1968). Furthermore, a few hours after curling was completed, the pH could be lowered without hindering infection thread and subsequent steps in nodule development (Munns, 1968). The acid-sensitive step of root hair curling, which occupies less than 12 h of the nodulation process (Munns, 1968), is also the most Ca demanding (Munns, 1970). Low pH and Ca interact on nodulation, so that higher Ca concentrations are needed if the pH is lowered (Munns, 1970; Munns, 1986). The interaction between Ca$^{2+}$ and H$^+$ is not well understood, but the two ions interact closely to affect pre-infection steps in the nodulation process.

**1.6.7 Nodule development**

Concurrent with infection thread formation, cells in the root cortex divide to form the nodule primordium (Dudley et al., 1987). Infection threads grow towards the primordium and once there, rhizobia are released into the plant cytoplasm (Heidstra & Bisseling, 1996). Depending on the host plant, either the inner or outer cortical cells divide to form the nodule primordium (Hirsch, 1992).

There has been limited research on the effect of low pH on the development of the nodule primordium, infection thread growth, and release of rhizobia into the primordium. As these steps take place inside root cells, it is unlikely that external pH has any direct effect on them. Munns (1968) found that
infection thread formation and the subsequent steps in the nodulation process could proceed at low pH provided that infection and root hair curling took place without acid stress, suggesting that the post-infection steps are unaffected by external acidity. Overall, however, research has essentially ‘skipped’ over these steps and continues again when it comes to the effect of low pH on the functioning of established nodules. Low pH (5.2) decreased nitrogenase activity in established *P. sativum* nodules by 63% (Paulino et al., 1987). Furthermore, toxicities associated with soil acidity, such as Al, also affected nodule development and function (Paulino et al., 1987; Hohenberg & Munns, 1984; de Carvalho et al., 1982; Franco & Munns, 1982). Where low pH decreased nodule numbers, compensation in nodule size has been reported in a variety of temperate and tropical legumes (Coventry et al., 1985; Hartel & Alexander, 1983; Keyser et al., 1979; Munns et al., 1977). Although this provides useful information as to how a plant can maximise the effectiveness of a limited number of nodules produced under acid stress, it would be valuable to clearly establish whether acidity has a direct impact on the post-infection steps of nodule primordium development, infection thread growth, or release of rhizobia into the nodule primordium.

1.7 **CONTRIBUTION OF PLANT AND RHIZOBIA TO ACID TOLERANCE IN NODULATION**

Tolerance to acidity in both host plants and rhizobia contributes to a successful symbiosis at low pH. Variation in host plant contribution to nodulation at low pH exists between species of the same genus, and between genotypes of the same species. For example, there was marked variation in the growth and
nodulation in different genotypes of *M. truncatula* (Bounejmate & Robson, 1992), *P. vulgaris* (Vargas & Graham, 1988) and *G. max* (Hungria & Vargas, 2000), and among annual species of *Medicago* (Howieson & Ewing, 1989). There was marked variation in the growth and nodulation of 126 genotypes of *P. vulgaris* by *R. leguminosarum* bv. *phaseoli* in soil of pH 4.5 (Vargas & Graham, 1988), and of *M. truncatula* by *S. meliloti* in soil ranging from pH 4.5-5.4 (Bounejmate & Robson, 1992). Plant growth and nodulation were most affected when both host genotype and rhizobial strain were sensitive to low pH.

Significant contributions have been made in the introduction of selected acid-tolerant rhizobia in the field. In south-western Australia, strains of *S. meliloti* isolated from the moderately acid soils of Sardinia, Italy, showed greater ability to nodulate *Medicago* spp. than commercial inoculant strains (Howieson & Ewing, 1986). Combined with selected acid-tolerant *Medicago* genotypes, this has expanded the area sown to annual pasture legumes in south-western Australia. Another contribution is the introduction of acid-tolerant *R. tropici* as the symbiont for *P. vulgaris* in Brazil (Graham & Vance, 2000; Hungria et al., 2000; Sadowsky & Graham, 1988). The success of this selection has enabled the expansion of *P. vulgaris* cultivation in the predominantly acid soils of Brazil and Africa. Currently, research is being undertaken to insert acid-tolerance genes from *R. tropici* to other rhizobia (Graham & Vance, 2000).

Plants, being less sensitive to acidity than rhizobia, can grow at pH and Ca levels below those necessary for nodulation if supplied with mineral N. *P. sativum* supplied with mineral N grew well in nutrient solution of pH 4.5, but root hair growth was decreased (Lie, 1969). In contrast, low pH did not inhibit the production of root hairs in *M. sativa* (Munns, 1970; Munns, 1968). This difference may be explained by differing patterns of root hair production.
between plant species. Among pasture legumes, *Medicago* spp. are sensitive to soil acidity (Munns, 1970; Robson & Loneragan, 1970a; Robson & Loneragan, 1970b) while *Biserrula pelecinus* and *Ornithopus compressus* (yellow serradella) are relatively tolerant to soil acidity (Howieson et al., 1995).

### 1.8 AIMS OF THE THESIS

Among the acid-sensitive *Medicago* genus (Munns, 1970; Robson & Loneragan, 1970a; Robson & Loneragan, 1970b), the annual species, *M. murex*, nodulated exceptionally well with *S. meliloti* in soils of pH\(_{CaCl_2}\) of 4.9 (Howieson & Ewing, 1989). However, *M. murex* is not commercially attractive and as an annual, would not be suitable for use in lowering groundwater tables in areas affected by secondary salinity in south-western Australia.

As a deep-rooted perennial pasture legume, *M. sativa* is probably the most suitable contemporary species for salinity management in south-western Australia (George et al., 1997). However, the effect of soil acidity on its nodulation with *S. meliloti* is an obstacle to the widespread establishment of *M. sativa*. The nodulation of *M. murex* in soil of low pH (Ewing, 1991; Howieson & Ewing, 1989), and of *M. sativa* in nutrient solution of low pH (Munns, 1970; Munns, 1968) and in soil of low pH (Pijnenborg & Lie, 1990; Pijnenborg et al., 1990a; Pijnenborg et al., 1990b; Pijnenborg et al., 1990c; Munns, 1965) by *S. meliloti* has been previously studied. However, the two species have only been studied separately and there has not been a direct comparison of the nodulation responses of *M. murex* and *M. sativa* under identical conditions of acid stress in soil.

As *M. murex* cannot fill the niche targeted for *M. sativa* in salinity management, it may be possible that in the long term the identification of plant
Chapter 1

genes whose products stimulate *S. meliloti* to nodulate *M. murex* at low pH may be transferred to *M. sativa* using plant transformation technology (Hoffman et al., 1997). If the nodulation of *M. sativa* in acidic soils can be improved, then the projected $8.8 \times 10^6$ ha of land predicted to be affected by salinity by 2050 in south-western Australia may be curtailed.

The aims of this research were to:

1. compare the nodulation responses of *M. murex* and *M. sativa* growing in an acidic soil inoculated with *S. medicae*; and

2. determine the plant mechanisms contributing to any differential nodulation response between the two *Medicago* species by comparing:
   a. the rhizosphere pH changes induced by the roots of *M. murex* and *M. sativa* growing in conditions of low pH;
   b. the numbers of root hairs on *M. murex* and *M. sativa* growing in soil of low pH; and
   c. the numbers of *S. medicae* in the rhizospheres of *M. murex* and *M. sativa* growing in an acidic soil.
CHAPTER 2. *M. murex* AND *M. sativa* DIFFER IN THE NODULATION RESPONSE TO SOIL ACIDITY

2.1 INTRODUCTION

The symbiosis between *M. sativa* and *S. medicae* is challenged by acidity. Strains of rhizobia can differ in their ability to survive and grow under acidic conditions (Ballen et al., 1998; Howieson et al., 1988; Lindström & Myllyniemi, 1987), and acid-tolerant strains of *S. meliloti*, such as WSM419 and WSM413, can nodulate well with annual *Medicago* spp. under acidic conditions (Howieson & Ewing, 1989; Howieson & Ewing, 1986). Annual *Medicago* spp. also differ in their ability to nodulate in acidic soils, and *M. murex* has been found to grow and nodulate in soil of pH\( \text{CaCl}_2 \) 4.9 (Howieson & Ewing, 1989). The capacity of *M. murex* to nodulate in acidic soils with strains of *S. medicae* that are also effective on *M. sativa* provides the opportunity to study the differences in symbiotic development between the two plant species at low pH, and to define the acid-sensitive steps in the nodulation of *M. sativa*. This chapter reports an experiment comparing the nodulation of *M. sativa* and *M. murex* in an acidic soil. The experiment tested the hypothesis that soil acidity, combined with low numbers of *S. medicae*, will cause a greater delay in the appearance of nodules in *M. sativa* than in *M. murex*. 
2.2 MATERIALS AND METHODS

2.2.1 Experimental design

The experiment was a split-plot factorial design with six replications, three of which were used for assessment of nodule initials, with the remaining three harvested for determination of nodulation and yield. Two strains of *S. medicae* (WSM419 and CC169) were inoculated to soil of pH$_{\text{CaCl}_2}$ 4.3 at two rates ($10^3$ and $10^6$ rhizobia g$^{-1}$ soil), and into soil of pH$_{\text{CaCl}_2}$ 7.0 at $10^6$ rhizobia g$^{-1}$ soil. Uninoculated control plants were grown in soil of pH$_{\text{CaCl}_2}$ 4.3 with no *S. medicae*. The host *Medicago* species, *M. sativa* cv. Aquarius and *M. murex* cv. Zodiac, were grown in an air-conditioned glasshouse maintained at 22 °C.

2.2.2 Soil

An acidic sandy soil was collected from Western Australian Department of Agriculture’s Merredin Drylands Research Institute (118°17'E 31°29'S). The top 15 cm layer of soil was sampled since toxic concentrations of soluble Al in the subsoil (15-25 cm) can adversely affect plant growth (Carr & Ritchie, 1993). The soil was screened through a 1-cm sieve to remove coarse organic matter, thoroughly mixed with coarse river sand (3:2), finely ground superphosphate (0.15 g kg$^{-1}$ soil) and two rates of lime (0.12 and 1.5 g kg$^{-1}$ soil) in a cement mixer. The amended soils were then placed in separate hessian bags and steamed at 65 °C for 30 minutes. The amended soils used in the experiment had pH 4.3 and 7.0 (0.01 M CaCl$_2$), and low nitrogen status (Table 2.1).
Table 2.1. Physical and chemical properties\(^a\) of unamended soil from Merredin Research Station, and Merredin soil mixed with river sand with 0.12 and 1.5 g kg\(^{-1}\) lime. Soil was sampled for analysis 14 d after mixing.

<table>
<thead>
<tr>
<th></th>
<th>Unamended Merredin soil</th>
<th>Merredin soil: river sand mix (0.12 g kg(^{-1}) lime)</th>
<th>Merredin soil: river sand mix (1.5 g kg(^{-1}) lime)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% sand (2.0 - 0.02 mm)</td>
<td>80.5</td>
<td>88.0</td>
<td>88.0</td>
</tr>
<tr>
<td>% silt (0.02 - 0.002 mm)</td>
<td>3.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>% clay (&lt;0.002 mm)</td>
<td>16.5</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Colour</td>
<td>Brownish yellow</td>
<td>Brownish yellow</td>
<td>Brownish grey</td>
</tr>
<tr>
<td>Nitrate (mg kg(^{-1}))</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium (mg kg(^{-1}))</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Phosphorus (mg kg(^{-1}))</td>
<td>12</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>Potassium (mg kg(^{-1}))</td>
<td>32</td>
<td>21</td>
<td>39</td>
</tr>
<tr>
<td>Sulphur (mg kg(^{-1}))</td>
<td>32.0</td>
<td>31.3</td>
<td>42.2</td>
</tr>
<tr>
<td>Organic carbon (%)</td>
<td>0.51</td>
<td>0.36</td>
<td>0.47</td>
</tr>
<tr>
<td>Iron (mg kg(^{-1}))</td>
<td>598</td>
<td>570</td>
<td>646</td>
</tr>
<tr>
<td>Conductivity (dS m(^{-1}))</td>
<td>0.0430</td>
<td>0.0490</td>
<td>0.0775</td>
</tr>
<tr>
<td>pH (0.01M CaCl(_2))</td>
<td>4.10</td>
<td>4.30</td>
<td>7.00</td>
</tr>
<tr>
<td>pH (H(_2)O)</td>
<td>4.40</td>
<td>4.80</td>
<td>7.40</td>
</tr>
</tbody>
</table>

\(^a\) Soil analysis by CSBP Wesfarmers according to procedures described by Rayment and Higginson (1992).

Free-draining pots (19.5 cm diameter, 19 cm height) were soaked overnight in 4% (v/v) sodium hypochlorite, rinsed with sterile deionised water and lined with a sterile paper towel. In the glasshouse, 3 kg of amended soil was placed in each pot, which were then covered with a plastic sheet. One hundred mL of sterile deionised water was added to each pot 24 h before inoculation.
2.2.3 Rhizobia

*S. medicae* (*S. meliloti*) strain CC169 was originally isolated from a nodule on *M. rugosa* in South Australia (Howieson et al., 2000a). *S. medicae* (*S. meliloti*) strain WSM419 was isolated from *M. murex*, south of Tempio, Sardinia, Italy, growing in soil of pH 6-7 (Howieson & Ewing, 1986). The two strains were obtained from the ‘WSM’ collection at the Centre for *Rhizobium* Studies, Murdoch University, Western Australia.

Cultures were grown at 28 °C in a modified medium (Howieson et al., 1988) which contained: 1.25 g L$^{-1}$ yeast extract, 27 mM D-glucose, 27 mM mannitol, 3 mM glutamate, 3 mM MgSO$_4$, 1.7 mM NaCl, 1.4 mM CaCl$_2$, 98 µM K$_2$HPO$_4$, 98 µM KH$_2$PO$_4$, 18 µM FeSO$_4$, 6.13 µM Na$_2$B$_4$O$_7$, 12 µM MnSO$_4$, 0.76 µM ZnSO$_4$, 0.54 µM Na$_2$MoO$_4$, 0.32 µM CuSO$_4$, and buffered with 20 mM N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES). The pH was adjusted to 7.0 with 1 M NaOH and the media was sterilised at 121 °C for 20 min.

Starter cultures of *S. medicae* were grown for 48 hours in 5 mL of broth and then used to inoculate 50 mL of fresh broth. Flasks were aerated on a platform shaker (Innova 2100, New Brunswick Co. Inc., Edison, New Jersey, USA) at 200 rpm. The rate of cell growth was determined by periodically taking samples to measure absorbance at 600 nm using a spectrophotometer (Hitachi U-1100, Tokyo, Japan) and viable counts using the Miles and Misra drop-plate method (Singleton, 1992) were made in 0.98% (w/v) NaCl. From this, it was established that for both strains, an O.D$_{600}$ of 0.4 represented approximately $10^9$ cells mL$^{-1}$. 


For inoculation of pots in the glasshouse, strains WSM419 and CC169 were grown overnight in 50 mL of broth and adjusted to O.D.<sub>600</sub> of 0.4 with fresh broth the following morning. Suspensions containing approximately $3 \times 10^4$ and $3 \times 10^7$ cells mL<sup>-1</sup> were prepared by diluting the adjusted cultures with sterile deionised water and then 100 mL was poured evenly onto the surface of each pot. One hundred mL of sterile deionised water was added to the surface of pots in the uninoculated treatment. Each pot was then covered individually with plastic wrap secured with an elastic band and allowed to equilibrate for one week.

2.2.4 Most probable number of rhizobia in soil

Seed of <i>M. sativa</i> cv. Aquarius was sorted for uniformity in colour, shape and size. Seeds were washed in absolute ethanol (1 min), soaked in 12% (v/v) hypochlorite solution (20 min), rinsed in six changes of sterile deionised water and left to imbibe overnight in the last change of water. Imbibed seeds were then dipped in 3% (v/v) hydrogen peroxide (30 s) and rinsed in two changes of sterile deionised water. Sterilised seeds were then placed on 1.5% (w/v) water agar (Grade J3, Leiner Davis Gelatin, Australia) and incubated at 20 °C in the dark until radicles were 0.5 cm long. Germinated seeds were transferred aseptically to 200 mL screw-top containers (4 cm diameter, 10.5 cm height) each containing 30 mL of sloped seedling agar (Gibson, 1980), one seedling per container. A small hole was made in the agar with sterile curved forceps; the radicle was inserted in the hole and washed over with the condensation in the tube. Containers were placed in an air-conditioned glasshouse maintained at 22 °C for 5 d before inoculation.
One week after inoculation of rhizobia, 20 samples (1.0 g each) of soil were removed from the top 2-3 cm of each treatment in replicate one with a sterile spatula. The 20 samples were mixed in a sterile container and from this, two duplicate 10 g samples were used in the plant infection test.

The five-fold dilution plant infection test was used (Brockwell, 1982), except that screw-top containers were used instead of glass tubes. Inoculated plants were examined for the presence or absence of nodules after three and five weeks. This assay provides detection ranging from 11 to $3.55 \times 10^5$ rhizobia g$^{-1}$ soil, with 95% confidence limits.

### 2.2.5 Plants for nodulation studies

Seeds of *M. sativa* cv. Aquarius (100 seeds = 0.275 g) and *M. murex* cv. Zodiac (100 seeds = 0.460 g) were sterilised and germinated as described in Section 2.2.4 until their radicles were approximately 1-2 cm long. Ten seedlings of each species were sown into each pot one week after inoculation with *S. medicae*. A sterile plastic, capped watering tube was inserted in the centre of each pot and the soil was then covered with sterilised plastic beads to minimise contamination. Fifty mL of sterile deionised water was added to each pot through the watering tube every day. Nineteen days after sowing, plants were thinned to five plants per species per pot.

### 2.2.6 Assessment of nodule initials

Nodule initials, not detectable macroscopically, were counted after clearing by soaking water-rinsed roots in 10% KOH for 8 h. Roots were then rinsed in running water until the brown colour had disappeared, acidified in 0.25 M HCl.
for 5 min, stained in 0.1% (w/v) Brilliant Green (No. C086, ProSciTech, Queensland, Australia) for 30 min, and destained overnight in water (Tang, 1991). Nodule initials were counted under a dissecting microscope (Olympus SZ40, Olympus Optical Co. Ltd, Tokyo, Japan) at ×20 magnification (Tang, 1991). Nodule initials were distinguished from lateral root initials by their shape – nodule initials are spherical with an origin within the cortex, while lateral root initials are conical with an origin within the pericycle. Plants were carefully removed from pots to minimise damage to the roots, cut at the hypocotyl, roots stained and the numbers of nodule initials and nodules counted. A preliminary assessment was done 7 d after sowing from one plant of each species from each pot. One replicate was harvested at 13 (10 plants per species), 19 and 27 (5 plants per species) d after sowing, respectively.

2.2.7 Assessment of nodulation

The final assessment of nodulation was done at 41 d after sowing on plants from the remaining three replicates. After rinsing roots in water, each plant was cut at the hypocotyl and assessed for the presence or absence of nodules. The distance from the hypocotyl to the first nodule was measured. A score for plant nodulation was developed using a system modified from Howieson and Ewing (1989) where nodulation score is a function of number of nodules, nodule size and position of nodules on the root (Table 2.2).
### Table 2.2. Scoring system for assessment of nodules on *M. murex* and *M. sativa*.

<table>
<thead>
<tr>
<th>Nodule size</th>
<th>Weighting</th>
<th>Nodule position</th>
<th>Weighting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large (&gt; 4 mm)</td>
<td>5</td>
<td>Upper primary root (0-5 cm)</td>
<td>4</td>
</tr>
<tr>
<td>Medium-large (3-4 mm)</td>
<td>4</td>
<td>Lower primary root (below 5 cm)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lateral root within 1 cm of</td>
<td></td>
</tr>
<tr>
<td>Medium (2-3 mm)</td>
<td>3</td>
<td>primary root in upper region</td>
<td>2</td>
</tr>
<tr>
<td>Medium-small (1-2 mm)</td>
<td>2</td>
<td>Nodulation elsewhere</td>
<td>1</td>
</tr>
<tr>
<td>Small (&lt; 1 mm)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 2.2.8 Dry matter production

Shoots and roots from plants from all harvests (except the preliminary assessment) were dried at 70 °C for 48 h and then weighed.

#### 2.2.9 Statistical analysis

A split-plot analysis of variance (ANOVA) using Genstat 5 (Release 4.1, Lawes Agricultural Trust, Rothamsted Experimental Station) was done to test for the effects of *Medicago* spp., soil pH and inoculation level on nodulation at 41 d after sowing. Data of plant nodulation score and distance from the hypocotyl to the uppermost nodule required square root and cube root transformation, respectively, to stabilise variance.

#### 2.3 RESULTS

##### 2.3.1 Most probable number of rhizobia in soil

One week after inoculation, soils inoculated with strain WSM419 had up to 10-fold greater populations than those inoculated with strain CC169 (Table 2.3). The procedure of inoculating soil with two different levels of inoculum resulted in
soils containing realistically low populations of *S. medicae*.

**Table 2.3.** Most probable number (mean of duplicate samples from single replicate pot) of *S. medicae* strains WSM419 and CC169 in Merredin soil of pH\textsubscript{CaCl\textsubscript{2}} 4.3 and 7.0, 7 d after initial inoculation.

<table>
<thead>
<tr>
<th><em>S. medicae</em> strain</th>
<th>Soil pH ((0.01 \text{ M CaCl}_2))</th>
<th>Approximate inoculum level ((\text{rhizobia g}^{-1} \text{ soil}))</th>
<th>Most probable number of rhizobia (\text{g}^{-1} \text{ soil})</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSM419</td>
<td>7.0</td>
<td>(1\times10^6)</td>
<td>(1.85\times10^5)</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>(1\times10^6)</td>
<td>(9.42\times10^2)</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>(1\times10^3)</td>
<td>(5.30\times10)</td>
</tr>
<tr>
<td>CC169</td>
<td>7.0</td>
<td>(1\times10^6)</td>
<td>(2.93\times10^4)</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>(1\times10^6)</td>
<td>(3.32\times10^2)</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>(1\times10^3)</td>
<td>(5.02)</td>
</tr>
<tr>
<td>Uninoculated control</td>
<td>4.3</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

### 2.3.2 Nodule initials

In soil of pH\textsubscript{CaCl\textsubscript{2}} 7.0, nodule initials were seen at 7 d after sowing in roots of *M. sativa* and *M. murex* grown in soil inoculated with either strain of *S. medicae* (Figure 2.1, Table 2.4). With strain WSM419, nodule initials were observed 7 d after sowing in *M. murex* grown in soil of pH\textsubscript{CaCl\textsubscript{2}} 4.3 containing either \(9.42\times10^2\) or \(5.3\times10\) rhizobia g\(^{-1}\) soil. However, when *M. sativa* was grown in soil of pH\textsubscript{CaCl\textsubscript{2}} 4.3 with \(9.42\times10^2\) rhizobia g\(^{-1}\) soil, nodule initials were not seen until 19 d after sowing. In soil of low pH with \(5.3\times10\) rhizobia g\(^{-1}\) soil, nodule initials were not detected in *M. sativa* at any harvest.
Figure 2.1. Root of *M. murex* (stained with Brilliant Green) grown in soil of pH\textsubscript{CaCl$_2$} 7.0 inoculated with *S. medicae* strain WSM419 showing nodules (asterices), nodule initial (black arrow) and lateral root initial (red arrow) at 13 d after sowing. Magnification ×20.

With CC169 in soil of pH\textsubscript{CaCl$_2$} 7.0, nodule initials were seen in both *M. murex* and *M. sativa* 7 d after sowing (Table 2.4). However when grown in soil of pH\textsubscript{CaCl$_2$} 4.3 containing $3.32 \times 10^2$ rhizobia g$^{-1}$ soil, nodule initials were seen earlier in *M. murex* (13 d after sowing) than *M. sativa* (27 d after sowing). In conditions of low soil pH and 5.02 rhizobia g$^{-1}$ soil, nodule initials were not seen in either *Medicago* spp.

No nodule initials were observed in uninoculated treatments.
Table 2.4. Average number of nodule initials in plants of *M. sativa* and *M. murex* grown in soil of pH\textsubscript{CaCl\textsubscript{2}} 7.0 and 4.3, inoculated with *S. medicae* strains WSM419 and CC169.

<table>
<thead>
<tr>
<th><em>S. medicae</em> strain</th>
<th>Soil pH, MPN\textsuperscript{a}</th>
<th><em>Medicago</em> species</th>
<th>Average no. of nodule initials/plant</th>
<th>Average no. of nodules/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Days after sowing</td>
<td>MPN = most probable number of rhizobia g\textsuperscript{-1} soil, 7 d after inoculation (i.e. at time of planting).</td>
</tr>
<tr>
<td>WSM419</td>
<td>7.0, 1.85×10\textsuperscript{5}</td>
<td><em>M. sativa</em></td>
<td>7\textsuperscript{b}</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4</td>
<td>0.4</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>4.3, 9.42×10\textsuperscript{2}</td>
<td><em>M. sativa</em></td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>0.2</td>
<td>1.3</td>
</tr>
<tr>
<td>CC169</td>
<td>7.0, 2.93×10\textsuperscript{4}</td>
<td><em>M. sativa</em></td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.6</td>
<td>0.6</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>4.3, 3.32×10\textsuperscript{2}</td>
<td><em>M. sativa</em></td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
</tr>
</tbody>
</table>

*All uninoculated treatments remained nodule free.*

\textsuperscript{a}MPN = most probable number of rhizobia g\textsuperscript{-1} soil, 7 d after inoculation (i.e. at time of planting).

\textsuperscript{b}Preliminary assessment, counts from one plant/species/pot.
2.3.3 Nodulation and nodule location

Nodules were present on roots of *M. murex* at 13 d after sowing when grown in soil of pH$_{\text{CaCl}_2}$ 7.0 and 4.3, inoculated with strain WSM419 (Table 2.4). In the same soils, the appearance of nodules on *M. sativa* occurred at a slower rate. In soil of pH$_{\text{CaCl}_2}$ 7.0, *M. sativa* had nodules 19 d after sowing, while *M. sativa* grown in soil of pH$_{\text{CaCl}_2}$ 4.3 containing 9.42×10$^2$ rhizobia g$^{-1}$ soil were nodulated at 27 d after sowing, and plants grown in soil of pH$_{\text{CaCl}_2}$ 4.3 containing 5.30×10$^2$ rhizobia g$^{-1}$ soil were not nodulated until the final harvest (41 d after sowing).

A different pattern of nodulation was observed for the acid-sensitive strain CC169 (Table 2.4). At pH$_{\text{CaCl}_2}$ 7.0, *M. sativa* nodulated earlier (7 d after sowing) than *M. murex* (13 d after sowing). By contrast in the acid soil containing higher numbers of rhizobia (3.32×10$^2$ cells g$^{-1}$ soil), *M. murex* was nodulated 13 d after sowing, whereas *M. sativa* was not nodulated until 41 d after sowing. In the acid soil containing lower numbers of rhizobia (5.02 cells g$^{-1}$ soil), nodulation of both species was delayed until 41 d after sowing.

All uninoculated treatments remained nodule free.

There was a significant effect of *Medicago* spp., and soil pH and inoculum level on the location of the uppermost nodule in plants (Table 2.5). Except in soil of pH$_{\text{CaCl}_2}$ 7.0, there was no significant difference in the distance from the hypocotyl to the uppermost nodule between the two *Medicago* spp. in any soil pH and inoculation level. In soil of pH$_{\text{CaCl}_2}$ 7.0 with either strain of *S. medicae*, *M. murex* usually formed the uppermost nodule approximately 0.3 cm below the hypocotyl, with 100% plants forming crown nodules. In the same soil, *M. sativa* formed the uppermost nodule significantly further down the
primary root than *M. murex*, with approximately 40-50% of plants forming crown nodules. With strain WSM419, low soil pH and low rhizobial numbers did not affect the location of the uppermost nodule in *M. sativa*, as this was formed at approximately the same location on the primary root as those of plants in soil of pH$_{\text{CaCl}_2}$ 7.0. However, low soil pH and $5.30 \times 10^4$ rhizobia g$^{-1}$ soil resulted in the uppermost nodule forming significantly lower down on primary root of *M. murex*. The uppermost nodule in both *M. sativa* and *M. murex* formed significantly lower down the primary root on plants grown in the acid soil containing a low population of CC169.
Table 2.5. Distance from hypocotyl to uppermost nodule in plants of *M. sativa* and *M. murex* grown in soil of pH$_{CaCl_2}$ 7.0 and 4.3, inoculated with *S. medicae* strains WSM419 and CC169.

<table>
<thead>
<tr>
<th><em>S. medicae</em> strain</th>
<th>Soil pH, MPN$^b$</th>
<th>Distance from hypocotyl to uppermost nodule (cm)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSM419</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0, 1.85×10$^5$</td>
<td></td>
<td>4.73 (1.68)</td>
</tr>
<tr>
<td>4.3, 9.42×10$^2$</td>
<td></td>
<td>8.05 (2.00)</td>
</tr>
<tr>
<td>4.3, 5.30×10$^3$</td>
<td></td>
<td>4.95 (1.70)</td>
</tr>
<tr>
<td>CC169</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0, 2.93×10$^4$</td>
<td></td>
<td>3.44 (1.51)</td>
</tr>
<tr>
<td>4.3, 3.32×10$^2$</td>
<td></td>
<td>4.90 (1.70)</td>
</tr>
<tr>
<td>4.3, 5.02</td>
<td></td>
<td>11.07 (2.23)</td>
</tr>
</tbody>
</table>

All uninoculated treatments remained nodule free.

$^a$ Values in parentheses show cube root transformation upon which statistical analyses are based.

$^b$ MPN = most probable number of rhizobia g$^{-1}$ soil, 7 d after inoculation (i.e. at time of planting).

$P$ values for *Medicago* spp. = 0.004; *S. medicae* strain = 0.606; soil pH-inoculum level < 0.001; *Medicago* spp.$\times$soil pH-inoculum level < 0.001.

LSD ($P = 0.05$) is 0.4474, for comparison of transformed values between *M. sativa* and *M. murex* in the same *S. medicae*/soil pH/inoculum level combination.

LSD ($P = 0.05$) is 0.4568, for comparison of transformed values not from same *S. medicae*/soil pH/inoculum level combination.

There was a significant effect of *Medicago* spp., *S. medicae* strain, soil pH and inoculum level on the nodulation score of plants (Table 2.6). *M. murex* grown in soil of pH$_{CaCl_2}$ 7.0 achieved the highest nodulation score regardless of the strain of *S. medicae*. When grown at pH$_{CaCl_2}$ 4.3 with 5.30×10$^3$ of WSM419 g$^{-1}$ soil, *M. murex* produced a greater nodulation score than *M. sativa*. However, a significant decrease in nodulation score was seen in *M. murex*, but not *M. sativa*, when grown in acidic soil with higher or lower numbers of either strain of *S. medicae*.
Table 2.6. Nodulation score and average number of nodules at 41 d after sowing in plants of *M. sativa* and *M. murex* grown in soil of pH\textsubscript{CaCl\textsubscript{2}} 7.0 and 4.3, inoculated with *S. medicae* strains WSM419 and CC169.

<table>
<thead>
<tr>
<th><em>S. medicae</em> strain</th>
<th>Soil pH, MPN$^b$</th>
<th>Nodulation score$^a$</th>
<th>Average number of nodules/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>M. sativa</em></td>
<td><em>M. murex</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSM419</td>
<td>7.0, 1.85×10$^5$</td>
<td>25.0 (5.0)</td>
<td>83.7 (9.1)</td>
</tr>
<tr>
<td></td>
<td>4.3, 9.42×10$^2$</td>
<td>22.0 (4.7)</td>
<td>16.2 (4.0)</td>
</tr>
<tr>
<td></td>
<td>4.3, 5.30×10</td>
<td>5.2 (2.3)</td>
<td>24.4 (4.9)</td>
</tr>
<tr>
<td>CC169</td>
<td>7.0, 2.93×10$^4$</td>
<td>19.8 (4.4)</td>
<td>55.0 (7.4)</td>
</tr>
<tr>
<td></td>
<td>4.3, 3.32×10$^2$</td>
<td>4.5 (2.1)</td>
<td>5.1 (2.2)</td>
</tr>
<tr>
<td></td>
<td>4.3, 5.02</td>
<td>7.0 (2.6)</td>
<td>7.7 (2.8)</td>
</tr>
</tbody>
</table>

All uninoculated controls remained nodule free.

$^a$ Values in parentheses show square root transformation upon which statistical analyses are based.

$^b$ MPN = most probable number of rhizobia g$^{-1}$ soil, 7 d after inoculation (i.e. at time of planting).

$P$ values for *Medicago* spp. = 0.002; *S. medicae* strain = 0.024; soil pH-inoculum level < 0.001; *Medicago* spp.×soil pH-inoculum level = 0.007.

LSD ($P = 0.05$) is 2.119, for comparison of transformed values between *M. sativa* and *M. murex* in the same *S. medicae*/soil pH/inoculum level combination.

LSD ($P = 0.05$) is 2.400, for comparison of transformed values not from same *S. medicae*/soil pH/inoculum level combination.
2.3.4  Dry matter production

At the final harvest, *M. murex* produced significantly more dry matter per plant than *M. sativa* when grown with strain WSM419 at both pH\(_{\text{CaCl}_2} 4.3\) and 7.0, (Figure 2.2). *M. murex* grown in acidic soil with low numbers of rhizobia (5.30×10\(^{\text{cells g}^{-1} \text{ soil}}\)) produced approximately 51% more dry matter than *M. sativa*. With strain CC169, there was no significant difference in the amount of dry matter produced between the two *Medicago* spp. except in soil of pH\(_{\text{CaCl}_2} 4.3\) containing 3.32×10\(^2\) rhizobia g\(^{-1}\) soil where *M. murex* produced almost twice as much dry matter than *M. sativa*. Uninoculated plants of both species produced less dry matter than plants grown in soils inoculated with rhizobia, but the difference between *M. murex* and *M. sativa* in this treatment was not significant.
Figure 2.2. Dry matter production in plants of *M. sativa* and *M. murex* grown in soil of pH$_{CaCl_2}$ 4.3 and 7.0 containing *S. medicae* strains WSM419 or CC169, 41 d after sowing. Bars represent standard error.

### 2.4 DISCUSSION

*M. murex* and *M. sativa* grown in the same soil enabled a comparison of the effect of soil acidity on the two *Medicago* spp. Soil acidity had different effects on the nodulation and growth of the *Medicago* spp. in this experiment. When grown in an acidic soil, *M. murex* produced fewer nodules than plants grown in soil of pH$_{CaCl_2}$ 7.0, but nodules formed at a similar rate to those on plants grown in soil of neutral pH. However, not only were fewer nodules produced by *M. sativa* when grown in the acidic soil, but the nodules appeared later compared to plants grown in soil of pH$_{CaCl_2}$ 7.0. The similar location of the uppermost nodule in *M. sativa* when inoculated with *S. medicae* strain WSM419 in soils of pH$_{CaCl_2}$ 4.3 and 7.0, but the delay in the appearance of nodules in
plants grown in soil of pH$_{\text{CaCl}_2}$ 4.3 may be explained by two possibilities: (1) low pH delayed either the initiation of infection, or (2) it delayed development of the subsequent nodule.

In the first possibility, infection would be initiated early in a root hair near the root tip, but nodule development would be delayed by acidity. In the second possibility, infection would be initiated later in a root hair in the more mature region of the root, away from the root tip where root hairs are considered most susceptible to infection (Bhuvaneswari et al., 1981). Development of the subsequent nodule would then proceed without delay. It is suggested that the second possibility, where delayed infection took place in the more mature region of the root, is more likely. This is based on previous studies on rhizobial survival and growth at low pH (Ballen et al., 1998; Howieson et al., 1988; Lindström & Myllyniemi, 1987), the susceptibility of M. sativa root hairs to infection (Wood & Newcomb, 1988; Bhuvaneswari et al., 1981), root hair curling and infection thread formation in M. sativa under acidic conditions (Munns, 1970; Munns, 1968).

Decreased saprophytic growth and colonisation of an acid sandy soil by S. meliloti decreased the nodulation of M. truncatula (Robson & Loneragan, 1970c). The dynamics of rhizobial growth and colonisation is important because M. sativa root hairs become progressively less susceptible to infection by rhizobia with time (Wood & Newcomb, 1988; Bhuvaneswari et al., 1981). In acidic soils where rhizobial populations are small and growth of rhizobia is slow (Rice et al., 1977), rhizobia must multiply fast enough to reach a threshold population at a particular location of the root where root hairs are most
susceptible to infection. Therefore, initiation of infection is a function of rhizobial numbers and time (Coventry & Evans, 1989).

In the acidic soil in this experiment, it is suggested that low populations of *S. medicae* required several days to accumulate to sufficient numbers to initiate infection in *M. sativa*. Some of these infections occurred in the older part of the root where root hairs are susceptible to infection, although at a much lower frequency than ones in the younger region of the root (Wood & Newcomb, 1988; Bhuvaneswari et al., 1981). Root hair curling is considered an acid-sensitive step (Munns, 1968), but shortly after root hair curling is complete, infection thread and nodule development in *M. sativa* progresses without being hindered by acidity (Munns, 1968). These findings support the suggestion that acidity, by decreasing the rhizobial population, delayed the initiation of infection and hence appearance of the subsequent nodule, rather than a delay in nodule development after infection was initiated. In contrast to this experiment, *M. sativa* grown in solution culture of pH 5.2 formed nodules lower on the primary root (Munns, 1970). This difference may be explained by the different rhizosphere conditions in plants grown in soil and hydroponic systems.

Numbers of rhizobia in the rhizosphere can also explain the different nodulation response of *M. murex* grown in an acidic soil. In soil of pH*CaCl*₂ 4.3, the population of rhizobia may have accumulated more rapidly in the rhizosphere of *M. murex* than *M. sativa*, reaching the threshold population required for infection of a root hair near the root tip. Soil acidity did not delay the appearance of nodule initials and nodules in *M. murex* since nodule initials formed within 7 d after sowing in plants grown in soil of both pH*CaCl*₂ 4.3 and 7.0. However, the uppermost nodule in plants grown in the acidic soil developed
lower down the primary root than those grown in soil of pH\(_{\text{CaCl}_2}\) 7.0 indicating that initiation of infection occurred later within the initial 7 day period. An assessment for nodule initials earlier than 7 d after sowing could reveal any difference in time of initiation of infection in \(M. murex\) grown in acidic and neutral soils.

Growth and survival of \(S. medicae\) is an important step in the nodulation of \(Medicago\) spp. at low pH since the size of the rhizobial population has an effect on the concentration of Nod factor produced by rhizobia. For infection to be initiated, multiplication and accumulation of rhizobia in the rhizosphere is essential for the release of critical concentrations of Nod factor required for root hair deformation \((10^{-11} \text{ M})\), cortical cell division \((10^{-7} \text{ M})\) (Hirsch, 1992) and the attachment of rhizobial cells to root hairs (Bauer, 1981).

Differences in survival and growth between the two strains of \(S. medicae\) were observed in this study as measured by numbers of rhizobia in the soil one week after inoculation. The strain with the greater numbers (WSM419) formed nodules higher on the primary root of both \(Medicago\) spp. than CC169. However, the reliability of using distance from the hypocotyl to the uppermost nodule as an indication of the ‘speed’ of initiation of infection is open to question as some root hairs in the older part of the root remain susceptible to infection (Bhuvaneswari et al., 1981). Nonetheless, this method still provides a comparative estimate of where successful infection occurred. Even in soil of high pH, nodules formed lower down the primary root in \(M. sativa\) than in \(M. murex\), suggesting a difference in plant response in nodulation between the two species.
Low pH decreased the adsorption of rhizobial cells to roots (Howieson et al., 1993; Caetano-Anollés et al., 1989) which probably resulted from decreased survival and growth of rhizobia in acidic conditions. As infection by rhizobia require the adsorption of rhizobia to plant roots, it is likely that decreased rhizobial adsorption contributed to decreased nodulation in soil of low pH.

It is possible that fewer numbers of rhizobia were associated with the rhizosphere of *M. sativa* than *M. murex* as the root exudates of *M. murex* may be more favourable for rhizobial growth. In addition, the ability of different legumes to modify their rhizosphere pH varies within and between species (Tang et al., 1997). *M. murex* may be more efficient in increasing the pH of its rhizosphere than *M. sativa* when grown at low pH, thereby developing conditions more favourable for rhizobial survival and growth. This may be related to different patterns of cation/anion uptake between the two species (Haynes, 1990). It would be instructive to compare the numbers of rhizobia associated with the roots of *M. sativa* and *M. murex* seedlings growing in low pH soil, and relate any differences to compounds exuded by the roots and pH changes in the rhizosphere of both species.
Chapter 3. Numbers of S. medicae Associated with Roots and Root Exudates of M. murex and
M. sativa at Low pH

3.1 Introduction

Poor survival and growth of rhizobia in acidic soils is a major factor contributing to decreased nodulation of M. sativa (Pijnenborg et al., 1990a), M. truncatula (Robson & Loneragan, 1970c), V. unguiculata (Hartel & Alexander, 1983), P. vulgaris (Vargas & Graham, 1988), P. sativum (Evans et al., 1993) and V. faba (Carter et al., 1995). Survival and growth of rhizobia is important because a sufficient population of rhizobia must be attained in the host rhizosphere for root hair curling to occur (Munns, 1968). Rhizobial Nod factors induce Ca\(^{2+}\) spiking in root hair cells (Cárdenas et al., 1999; Felle et al., 1998), root hair curling, and cortical cell division in the host plant (Hirsch, 1992), and the concentration of Nod factors to induce these events is dependent on rhizobial numbers. Furthermore, root hairs are only transiently susceptible to infection by rhizobia (Bhuvaneswari et al., 1980). Interactions between rhizobia and host plants can affect the population size of rhizobia as compounds released by plant roots can affect rhizobial growth rates in the rhizosphere.

Compared to the bulk soil, where supply of C can be limited (Cheng et al., 1996; Bazin et al., 1990), rhizosphere soil, containing root-derived organic compounds such as sugars, amino acids and polysaccharides (Brimecombe et al., 2001; Bringhurst et al., 2001; Uren, 2001), support the growth of large bacterial populations. The relative and absolute amounts of compounds
released by plant roots vary with plant species (Vancura, 1964), plant age (Aulakh et al., 2001; Lucas García et al., 2001; Vancura, 1964), and the availability of nutrients (Kamh et al., 1999).

When grown in an acidic soil of pH$_{CaCl_2}$ 4.3, *M. murex* nodulated earlier and in some cases, produced more nodules than *M. sativa* (Chapter 2). This difference in nodulation between the two species may be related to the size of the *S. medicae* populations accumulated in the rhizospheres. It may be possible that higher numbers of *S. medicae* associate with the roots of *M. murex* than *M. sativa* because the rhizosphere of *M. murex* is more favourable for the survival and growth of the rhizobia. This chapter describes two experiments which test the hypotheses that: (1) at low pH, there are higher numbers of *S. medicae* associated with the roots of *M. murex* than *M. sativa*, because (2) the compounds released by the roots of *M. murex* at low pH are more favourable for the growth of *S. medicae* than the compounds released by roots of *M. sativa*.

**3.2 Materials and Methods**

**3.2.1 Experiment 3.1. Numbers of *S. medicae* associated with the roots of *M. murex* and *M. sativa* in an acidic soil**

**3.2.1.1 Experimental design**

Experiment 3.1 was a split-plot design with seven replications. Seedlings of
Chapter 3

*M. sativa* cv. Aquarius and *M. murex* cv. Zodiac were grown in soil of pH$_\text{CaCl}_2$ 4.3 and 7.0 into which *S. medicae* strain WSM419 was inoculated ($10^6$ rhizobia g$^{-1}$ soil). An uninoculated control treatment was also used. Between 3 and 24 d after sowing, numbers of rhizobia associated with the roots of *M. murex* and *M. sativa* were determined by the Miles and Misra drop-plate method (Section 2.2.3), and the spread-plate method.

### 3.2.1.2 Soil and plants

The soil was the acidic sand described in Section 2.2.2 and Table 2.1. A total of 200 g of the air-dried soil mix was placed in plastic, screw-top containers (6.5 cm diameter, 8 cm height). Each container was moistened to field capacity (10% w/v) with deionised water before sterilisation at 121 °C for 20 min.

Seeds of *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius were sterilised and germinated as described in Section 2.2.4, except seeds were treated in 4.5% (v/v) H$_2$O$_2$ for 2 min after imbibing overnight in sterile deionised water.

### 3.2.1.3 Rhizobia

*S. medicae* strain WSM419 was grown as described in Section 2.2.3. As it is more acid-tolerant than strain CC169, WSM419 was used to minimise the possibility that very poor rhizobial survival and growth would limit the detection of rhizobial numbers on the roots of *M. murex* and *M. sativa* in soil of pH$_\text{CaCl}_2$ 4.3. Immediately before sowing, suspensions containing approximately $4 \times 10^7$ rhizobia mL$^{-1}$ were prepared by diluting the cultures with sterile deionised water and then 5 mL was poured evenly onto the surface of the soil in each container. A total of 5 mL of sterile deionised water was added to the soil in
each container of the uninoculated control treatments. Five seedlings of each species were sown in each container, with lids loosely screwed to allow for air exchange. All containers were then placed in an air-conditioned glasshouse maintained at 22 °C.

3.2.1.4 Root length

Four replicates of each inoculated treatment were harvested at each sampling to determine root length.

3.2.1.5 Enumeration of rhizobia on plant roots

Rhizobia were counted on the media described in Section 2.2.3, which also contained 1.5% (w/v) agar (Grade J3, Leiner Davis Gelatin, Australia). Plates were incubated at 28 °C until single colonies appeared. A sterile phosphate buffered saline (PBS) solution was used as the diluent for rhizobial counts. This contained 180 mM polyvinyl-pyrrolidone, 136 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl and 1.5 mM KH₂PO₄. The pH was adjusted to 7.4 with 0.1 mM KOH before sterilising at 121 °C for 20 min. With soil of pH$_{\text{CaCl}_2}$ 4.3, all plants from three replicates of the inoculated treatment were sampled between 3 and 24 d after sowing, and all plants from three replicates of the uninoculated control treatments were sampled only at 4 and 14 d after sowing. With soil of pH$_{\text{CaCl}_2}$ 7.0, all plants from three replicates of both uninoculated and inoculated treatments were sampled at 3 and 7 d after sowing. At each sampling, plants were carefully removed from the container under aseptic conditions and the roots dipped for 2 s in sterile PBS to remove excess soil. Remaining soil adhering to the roots was considered soil associated with the roots. Roots of
each species were grouped and homogenised in 1 mL of sterile PBS using a sterile mortar and pestle. As lower numbers of rhizobia were expected to be associated with roots grown in soil of pH \(4.3 \text{CaCl}_2\) than pH \(7.0\), slightly different methods were used to count rhizobia. For plants grown in soil of \(4.3 \text{CaCl}_2\), rhizobia were counted by dividing the root homogenate into three fractions: (1) an aliquot of 100 µL was removed and viable counts made by the Miles and Misra drop-plate method as described in Section 2.2.3; (2) the remaining homogenate was then centrifuged at 1 000 rpm for 30 s (IEX MicroMax, International Equipment Company, Needham Heights, MA, USA) and viable counts made by spreading 20, 40, 60, 80 and 100 µL of the undiluted supernatant onto five plates. The excess supernatant was then discarded; and (3) the soil pellet was resuspended in 0.5 mL of sterile PBS, vigorously vortexed and viable counts made by the Miles and Misra drop-plate method (Section 2.2.3). Numbers of rhizobia from each fraction was then summed. For plants grown in soil of \(7.0 \text{CaCl}_2\), rhizobia were counted by taking 100 µL of the root homogenate and viable counts made by the Miles and Misra method described in Section 2.2.3.

3.2.2 Experiment 3.2. Effect of \(M. murex\) and \(M. sativa\) root exudates collected at low pH on numbers of \(S. medicae\)

3.2.2.1 Experimental design

Experiment 3.2 was a randomised design with four replications. Root exudates from seedlings of \(M. sativa\) cv. Aquarius and \(M. murex\) cv. Zodiac were
collected in nutrient solution of pH 5.6. Nutrient solution of pH 5.6 without root exudates was also used. After 72 h of collection, the nutrient solution was adjusted to either pH 5.6, 5.8 or 7.0 and *S. medicae* strain WSM419 was inoculated to the solutions. Between 22 and 118 h after inoculation, numbers of *S. medicae* in the nutrient solution were determined by the Miles and Misra drop-plate method (Section 2.2.3), and the spread-plate method.

### 3.2.2.2 Plants

Seeds of *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius were sterilised and germinated as described in Section 2.2.4. After germination, 40 seedlings were placed on a sterile wire mesh, immersed in 3% (v/v) H$_2$O$_2$ for 30 s and rinsed in two changes of sterile deionised water. The wire mesh with the seedlings was then placed inside a sterile screw-top plastic container (5.5 cm diameter, 7.0 cm height) containing 10 mL of sterile plant nutrient solution which contained: 500 µM K$_2$SO$_4$, 200 µM MgSO$_4$, 100 µM FeEDTA, 100 µM Ca(NO$_3$)$_2$, 10 µM KH$_2$PO$_4$, 3 µM H$_3$BO$_3$, 1 µM MnCl$_2$, 0.75 µM ZnSO$_4$, 0.2 µM CuSO$_4$ and 0.03 µM Na$_2$MoO$_4$, and buffered with 10 mM 2-[N-Morpholino]ethane-sulphonic acid (MES). The pH was adjusted to 5.6 with 0.1 M KOH before sterilisation at 121 °C for 20 min. A plastic ring at the bottom of each container ensured that only the radicles were immersed in the nutrient solution. To prevent photosynthesis, each tub was then wrapped with aluminium foil, and incubated at 20 °C on a platform shaker (Bio-Line Incubator Shaker, Edwards Instrument Company, NSW, Australia) at 65 rpm. After 72 h, the wire mesh containing seedlings was removed, a 5 mL aliquot of the nutrient solution taken from each container and the pH measured (Orion 410A pH meter, Orion Research Inc.,
Boston, USA). From titration curves (data not shown), the remaining 5 mL of nutrient solution in each container was adjusted to either pH 5.6, 5.8 or 7.0 with sterile solutions of 0.1 M H$_2$SO$_4$ or 1 M KOH. Aliquots of 2 mL were then aseptically removed from each container and placed into two sterile screw-top tubes (2.5 cm diameter, 8 cm height, 35 mL volume), into which rhizobia were inoculated.

3.2.2.3 Rhizobia

Twenty-four to 48 h before inoculation, cultures of S. medicae strain WSM419 were grown to approximately 10$^9$ rhizobia mL$^{-1}$ in broth of pH 5.6, 5.8 or pH 7.0 which contained: 10 mM galactose, 10 mM arabinose, 3 mM glutamate, 10 mM MgSO$_4$.7H$_2$O, 10 mM FeCl$_3$.6H$_2$O, 7 mM Na$_2$SO$_4$, 1.5 mM KH$_2$PO$_4$, 1.5 mM K$_2$HPO$_4$, 1 M CaCl$_2$.2H$_2$O, 0.6 mM MnSO$_4$.4H$_2$O, 0.4 mM Na$_2$MoO$_4$.2H$_2$O, 0.37 mM ZnSO$_4$.7H$_2$O, 0.2 mM CuSO$_4$.5H$_2$O, 20 mg L$^{-1}$ biotin, 1 mg L$^{-1}$ thiamine, 1 mg L$^{-1}$ pantothenic acid, 40 µg mL$^{-1}$ chloramphenicol. The solutions were buffered with 10 mM MES (pH 5.6 and 5.8) or 10 mM N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES; pH 7.0). Rhizobia to be inoculated to root exudates of pH 5.6, 5.8 and 7.0 were washed and resuspended in fresh nutrient solution of corresponding pH 5.6, 5.8 and 7.0. Immediately before inoculation to root exudates, 10 mL of rhizobial culture was centrifuged (Eppendorf centrifuge 5810, Hamburg, Germany) at 10 000 rpm for 5 min. The supernatant was discarded and the pellet washed in 1 mL of fresh nutrient solution, centrifuged again and resuspended in 1 mL of fresh nutrient solution. Suspensions of rhizobia were prepared by diluting the culture in nutrient solution of pH 5.6, 5.8 or 7.0. A total of 100 µL of the rhizobial
suspension was inoculated to each tube of pH 5.6, 5.8 and 7.0 to give approximately 5.65×10^2, 2.00×10^3 or 1.12×10^4 rhizobia mL^{-1}, respectively. There was a difference in the numbers of rhizobia inoculated depending on the pH of the broth in which rhizobia were grown. All tubes were then placed on a platform shaker (Innova 2100, New Brunswick Co. Inc., Edison, New Jersey, USA) at 200 rpm at 28 °C.

3.2.2.4 Enumeration of rhizobia in root exudates

Rhizobia were counted on the media described in Section 2.2.3, which also contained 1.5% (w/v) agar (Grade J3, Leiner Davis Gelatin, Australia). Plates were incubated at 28 °C until single colonies were seen. The numbers of S. medicae were determined between 22 and 118 h after inoculation. At each sampling, 100 μL of the nutrient solution was aseptically removed, and the numbers of S. medicae was determined by either the Miles and Misra drop-plate method (Section 2.2.3), or the spread-plate method. The numbers of colony forming units (cfu) mL^{-1} was calculated and the data transformed to log_{10} (cfu+1) mL^{-1} values to avoid non-existent log_{10}(0) situations (Simons et al., 1996).

3.2.3 Statistical analysis

For both experiments, variation in the measured values among various replicates is indicated by standard deviations and standard errors. Significant differences between treatments were deduced from an analysis of variance (ANOVA) using Statistica for Windows 1999 (Kernal Release 5.5, StatSoft, Inc.,
Tulsa, Oklahoma, USA), and a $t$-test using Microsoft Excel 2000 (Microsoft Corporation, Washington, USA) at $P < 0.05$.

3.3 RESULTS

3.3.1 Experiment 3.1. Numbers of *S. meliloti* on roots of *Medicago*

3.3.1.1 Root length

In soil of pH$_{\text{CaCl}_2}$ 4.3 at 4 d after sowing, roots of *M. murex* were approximately 4.4 cm while roots of *M. sativa* were 3.2 cm (Table 3.1). At 24 d after sowing, *M. murex* roots had increased 6.8 cm to 11.2 cm, while *M. sativa* roots had increased 4.7 cm to 7.9 cm. In soil of pH$_{\text{CaCl}_2}$ 7.0 at 3 d after sowing, roots of both species were approximately 3.4 cm (Table 3.1). By 7 d after sowing, roots of *M. murex* were 4.6 cm and roots of *M. sativa* were 4.4 cm.
Table 3.1. Root length (mean value ± standard deviation) of *M. murex* and *M. sativa* roots in soil of pH\(_{\text{CaCl}_2}\) 4.3 and 7.0. Experiment 3.1.

<table>
<thead>
<tr>
<th>Days after sowing</th>
<th>Root length (cm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. murex</em></td>
<td><em>M. sativa</em></td>
<td></td>
</tr>
<tr>
<td>Soil pH(_{\text{CaCl}_2}) 4.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.4 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>7.5 ± 1.3</td>
<td>6.0 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8.1 ± 0.6</td>
<td>6.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>9.6 ± 0.7</td>
<td>6.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>11.2 ± 1.9</td>
<td>7.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soil pH(_{\text{CaCl}_2}) 7.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.4 ± 0.6</td>
<td>3.4 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4.6 ± 1.2</td>
<td>4.4 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

3.3.1.2 Rhizobial growth

All containers, including those carrying the uninoculated controls, were free from contamination. In soil of pH\(_{\text{CaCl}_2}\) 7.0 between 3 and 7 d after sowing, there were significantly higher numbers of *S. medicae* associated with the roots of *M. murex* than *M. sativa* (Figure 3.1). In soil of pH\(_{\text{CaCl}_2}\) 4.3 at 4 d after sowing, there was approximately equal numbers of rhizobia (1×10\(^2\) to 3×10\(^2\) cfu mL\(^{-1}\)) associated with the roots of both *Medicago* spp. (Figure 3.1). From 7 d after sowing, numbers of rhizobia associated with the roots of *M. murex* were significantly higher than those on *M. sativa* roots. Between 4 and 7 d after sowing, numbers of rhizobia increased 10-fold on the roots of *M. murex*, and then remained in the order of 10\(^3\) cfu mL\(^{-1}\) throughout the experiment. With *M. sativa* during the same period from 4 to 24 d after sowing, numbers of *S. medicae* decreased from 3.5×10\(^2\) to 11 cfu mL\(^{-1}\).
Figure 3.1. Numbers of *S. medicae* associated with roots of *M. murex* and *M. sativa* grown in soil of pH CaCl₂ 4.3 and 7.0. Bars represent standard error. Experiment 3.1.

### 3.3.2 Experiment 3.2. *Medicago* root exudates on the growth of *S. medicae*

#### 3.3.2.1 Plant biomass

After 72 h in nutrient solution of pH 5.6, *M. murex* produced significantly more dry weight than *M. sativa* (Table 3.2). Both species acidified the nutrient solution: *M. murex* by 0.68 pH-unit and *M. sativa* by 0.21 pH-unit (Table 3.2).
Table 3.2. Dry weight of *M. murex* and *M. sativa* seedlings and pH of nutrient solution (mean value ± standard deviation) after 72 h in nutrient solution of pH 5.6. Experiment 3.2.

<table>
<thead>
<tr>
<th></th>
<th>No plants</th>
<th><em>M. murex</em></th>
<th><em>M. sativa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Seedling dry weight (mg plant⁻¹)</td>
<td>—</td>
<td>3.95 ± 0.38*</td>
<td>1.89 ± 0.10</td>
</tr>
<tr>
<td>pH of nutrient solution</td>
<td>5.63 ± 0.08a</td>
<td>4.95 ± 0.27b</td>
<td>5.42 ± 0.09c</td>
</tr>
</tbody>
</table>

Mean values with different letters within a line are significantly different at *P* < 0.05.

* Significant difference in root dry weight between *M. murex* and *M. sativa* at *P* < 0.05.

3.3.2.2 Rhizobial growth

Numbers of rhizobia in the root exudates at pH 5.6 decreased between 1- to 2-fold, 46 h after inoculation (Figure 3.3a). However, there was no significant difference between the numbers of *S. medicae* in all treatments at 22 and 46 h after inoculation at pH 5.6. By 70 h after inoculation, *S. medicae* was not detected in any treatment at pH 5.6. In root exudates at pH 5.8, numbers of rhizobia decreased by approximately 1-fold 25 h after inoculation in all treatments, and there was no significant difference in the numbers between any treatment (Figure 3.3b). *S. medicae* could not be detected in root exudates at pH 5.8 after 25 h. In root exudates at pH 7.0 after 70 h, numbers of *S. medicae* increased 4-fold in treatments with root exudates, while numbers increased 3-fold in treatments without root exudates (Figure 3.3c). Numbers of rhizobia were significantly higher in nutrient solution containing root exudates, but there was no difference in numbers between *M. murex* and *M. sativa* exudates. By 118 h after inoculation, numbers of *S. medicae* reached approximately 10⁷ cfu mL⁻¹ (no exudates) and 10⁸ cfu mL⁻¹ (with exudates of both *Medicago* spp.).
Figure 3.3. Numbers of *S. medicae* growing in root exudates of *M. murex* and *M. sativa* collected at pH 5.6, and adjusted to (a) pH 5.6, (b) 5.8 and (c) 7.0. Bars represent standard error. Experiment 3.2.
In soil of both pH\textsubscript{CaCl\textsubscript{2}} 4.3 and 7.0, there were higher numbers of \textit{S. medicae} associated with the roots of \textit{M. murex} than \textit{M. sativa} despite similar relative root elongation rates. This data agrees with that of Barclay et al. (1994), who found that in an acidic soil, \textit{S. meliloti} colonised the roots of different \textit{Medicago} species differentially. If the size of the rhizobial population in the rhizosphere is regarded as an index of nodulation potential (Barclay et al., 1994), then the decrease in nodule numbers, and the delayed nodule appearance in \textit{M. sativa} growing in the acidic soil (Chapter 2) may be due to the smaller population of \textit{S. medicae} associated with the roots of \textit{M. sativa} compared to \textit{M. murex}.

Plants differ in their ability to nodulate in acidic conditions, and both host plant genotype and rhizobial strain can affect nodulation at low pH. Among different annual species of \textit{Medicago} and strains of \textit{S. meliloti}, \textit{M. murex} (Howieson & Ewing, 1989) and strain WSM419 (Howieson & Ewing, 1986) are both relatively acid-tolerant. In experiment 3.2, it was hypothesised that differences in compounds released by the roots of \textit{M. murex} and \textit{M. sativa} growing at low pH contribute to the potentially different numbers of \textit{S. medicae} in the rhizosphere of these species. However, this was not the case. At pH 5.6 and 5.8, the low numbers of \textit{S. medicae} in the exudates of both species suggested that low pH affected either the ability of rhizobia to utilise and metabolise substrates, or the substrates were not available to the rhizobia. Low pH altered the metabolism of glutamate and carbohydrate by \textit{R. trifolii} (Wood & Cooper, 1988). It may be that the concentration of compounds in root exudates collected hydroponically (experiment 3.2) was very low because of dilution of
compounds in the nutrient solution. In contrast, root exudates released by soil-grown plants (experiment 3.1) may have concentrated around the roots rather than diffused completely into the bulk soil. *S. medicae* may have grown and metabolised these root exudates along the root surface. At pH 7.0, the growth of *S. medicae* without root exudates indicated that a substrate source was indeed not limiting, which was unexpected since the nutrient solution in which root exudates were collected contained no C or N source. The only other possibility is that rhizobia utilised some C or N source from the containers’ material, or carried C or N over in the inoculant cells. If this was the case, then rhizobia growing at pH 5.6 and 5.8 would also have access to a similar substrate source since identical containers and method of inoculation were used. The similar numbers of *S. medicae* growing with the root exudates of *M. murex* and *M. sativa* at pH 7.0 indicated that there was no significant difference in the composition of root exudates between the *Medicago* spp. on the growth of *S. medicae*. These results suggest the availability of root substrates was not the limiting factor on the population size of *S. medicae* in the rhizosphere of *M. murex* and *M. sativa*. Plant roots themselves can contribute to pH changes in the rhizosphere and this may contribute to the differential numbers of *S. medicae* in the rhizosphere of the two *Medicago* spp.
CHAPTER 4. PROTON RELEASE AND RHIZOSPHERE pH CHANGES BY ROOTS OF *M. murex* AND *M. sativa* GROWING IN ACIDIC CONDITIONS

4.1 INTRODUCTION

The data from the experiment described in Chapter 2 showed that the nodulation of *M. murex* and *M. sativa* was affected differently by soil acidity. When grown in an acidic sandy soil of pH$_{CaCl_2}$ 4.3, *M. murex* produced fewer root nodules than when grown in soil of pH$_{CaCl_2}$ 7.0, but these nodules formed at a similar rate. In the same soil, *M. sativa* also produced fewer nodules when grown in the acidic soil, but the nodules appeared later compared to those on plants grown in soil of pH$_{CaCl_2}$ 7.0 (Chapter 2). These differences in nodulation response could be due to differential multiplication of *S. medicae* in the rhizosphere. After 24 d of growth in soil of pH$_{CaCl_2}$ 4.3 inoculated with *S. medicae*, there were over 100-fold more *S. medicae* associated with the roots of *M. murex* than *M. sativa* (Chapter 3). The survival and growth of *S. medicae* is highly sensitive to acidity (Reeve et al., 1993; Howieson & Ewing, 1986; Rice et al., 1977; Graham & Parker, 1964), and rhizosphere pH may affect the ability of rhizobia to survive and grow on the host root. The ability of different pasture legumes to modify rhizosphere pH varies within and between species (Tang et al., 1997). The average rhizosphere pH of *M. sativa* seedlings growing in soil of pH$_{H_2O}$ 5.2 increased from pH 5.1 (1 d after sowing) to pH 5.7 (12 d after sowing), but nodulation was not affected (Pijnenborg et al., 1990b). Localised changes in rhizosphere pH have also been observed along the roots of *M. sativa* (Blanchard & Lipton, 1986). In soil of pH$_{H_2O}$ 5.5, the rhizosphere pH...
of 10 d-old *M. sativa* seedlings at the older tap roots was more alkaline (pH 6.8), while the rhizosphere pH at the younger lateral roots was more acidic (pH 4.2) (Blanchar & Lipton, 1986). Modification of rhizosphere pH by *M. murex* roots has not been previously studied, and *M. murex* may modify the pH of the rhizosphere differently from *M. sativa*, thereby developing more favourable conditions for rhizobial survival and growth.

The release of H\(^+\) to counterbalance an excess uptake of cations over anions is often the major source of root-induced changes of rhizosphere pH (Hinsinger et al., 2003). The source of N supplied to plants can affect rhizosphere pH change. Generally, plants supplied with NO\(_3^-\) release HCO\(_3^-\) or OH\(^-\) and alkaline the rhizosphere, while plants supplied with NH\(_4^+\) release H\(^+\) and acidify the rhizosphere (Marschner & Römheld, 1996). Approaches to measure H\(^+\) concentrations in the rhizosphere, and fluxes of H\(^+\) released by roots, include investigation of the whole root system or single roots (Gregory & Hinsinger, 1999). At the level of the whole root system, the ‘root mat’ approach has been used to measure pH gradients in the rhizosphere of *T. repens* (Li et al., 1991) and *T. subterranenum* (Hinsinger & Gilkes, 1996). Back-titration of the nutrient solution can also measure fluxes of H\(^+\) released by whole root systems in solution (McLay et al., 1997; Tang et al., 1997), but cannot provide localised pH values along a root. This localisation of pH changes along single roots may be semi-quantitatively measured by colorimetry coupled with the agar-indicator technique (Weisenseel et al., 1979), quantitatively by micropotentiometry (Yu et al., 2000; Blanchar & Lipton, 1986; Marschner & Römheld, 1983), or both these techniques combined (Pijnenborg et al., 1990b). Videodensitometry, based on the principles of colorimetry (Jaillard et al., 1996), was developed to quantitatively measure H\(^+\) flux using coloured pH indicator
with agar or agarose gels. It provides maps of pH values along intact roots (Jaillard et al., 2003; Gregory & Hinsinger, 1999; Jaillard et al., 1996), and simultaneously calculates temporal and spatial profiles of H⁺ flux (Plassard et al., 1999).

The acidifying effect of N₂-fixing legumes has been demonstrated in both solution culture (McLay et al., 1997; Tang et al., 1997) and soil (Tang et al., 1998; Jarvis & Robson, 1983a; Jarvis & Robson, 1983b). However, there appears to be limited research on rhizosphere pH changes in young plants prior to nodulation where rhizosphere pH may affect the pre-infection steps of the nodulation process. The aim of this chapter was to determine whether young, non-nodulated plants of *M. murex* and *M. sativa* induced pH changes in the rhizosphere differentially under acidic conditions. Experiments using the ‘root mat’ approach and videodensitometry tested the hypothesis that at low pH the rhizosphere of young *M. murex* roots were less acidic than those of *M. sativa*.

### 4.2 MATERIALS AND METHODS

#### 4.2.1 Experiment 4.1. ‘Root mat’ approach

4.2.1.1 Experimental design

Experiment 4.1 used the ‘root mat’ approach to measure changes in soil rhizosphere pH. The experiment consisted of two *Medicago* spp. (*M. murex* cv. Zodiac and *M. sativa* cv. Aquarius), two pH treatments (soil disks of initial soil pH of 4.49 placed over deionised water or nutrient solution at pH 4.5, and soil disks of initial soil pH of 6.71 placed over deionised water or nutrient solution at pH 6.0). Deionised water and nutrient solution were used to compare
the responses of the two species in the absence or presence of a cation/anion supply from the solution medium. Six replicates were prepared for each treatment, and one additional replicate was used to assess shoot and root dry weight at the commencement of treatment. Root mats of the two *Medicago* species were grown in a specially designed cropping device (see Chaignon et al., 2002) in nutrient solution of pH 6.0 for 13 d. Roots were then placed in contact with soil disks. Soil disks of pH 4.49 were placed above solution media of pH 4.5, and soil disks of pH 6.71 were placed above solution media of pH 6.0, for a further 4 d. Control soil disks without root contact were also used as references. Bulk $H^+$ or $OH^-$ release fluxes were calculated from pH changes in the soil disks between 13 and 17 d after sowing, relative to the soil without root contact.

4.2.1.2 Plants

For both experiments, seeds of *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius were surface sterilised in 6% (v/v) hydrogen peroxide solution (10 min) to remove seed-borne microorganisms, and then thoroughly rinsed with deionised water. The nutrient solution for plant culture contained: 500 iM $K_2SO_4$, 200 iM $MgSO_4$, 100 iM FeEDTA, 100 iM $Ca(NO_3)_2$, 10 iM $KH_2PO_4$, 3 iM $H_3BO_3$, 1 iM $MnCl_2$, 0.75 iM $ZnSO_4$, 0.2 iM $CuSO_4$ and 0.03 iM $Na_2MoO_4$. The pH was adjusted to 6.0 with 0.01 M KOH. The composition of the nutrient solution was based approximately on the chemical properties of the soil described in Chapter 2 (Table 2.1). Nitrate was used as the main source of N in the nutrient solution to avoid extreme acidification of the rhizosphere and to prevent N deficiency in plants. Plants were not inoculated with rhizobia. For both
experiments, plants were placed in a growth cabinet with a 16 h, 25 °C/8 h, 20 °C day/night regime and a photon fluence rate of 600 μmol photons m⁻² s⁻¹.

4.2.1.3 Soil

The soil was an acidic sand mixed with coarse river sand (3:2). The pH_{CaCl_2} values of 4.49 and 6.71 were achieved by liming the soil mix at two rates (0.12 and 1.5 g kg⁻¹) as described in Section 2.2.2 and Table 2.1. After liming, the soil was left overnight at room temperature in covered plastic tote-boxes. For transportation purposes, the soil was dried at 70 °C for 48 h, and sealed in plastic bags. The soil mix was wet to field capacity with deionised water (10% w/v) and incubated in the dark for 24 h before being used.

4.2.1.4 Cropping device and procedure

The cropping device was that described by Chaignon et al. (2002). Soil (2.5 g wet soil) was placed into shallow PVC containers (32 mm internal diameter) to produce disks approximately 1.5 mm thick. Soil disks of pH_{CaCl_2} 4.49 were then placed on shelves above 5 L of aerated deionised water or nutrient solution of pH 4.5 (adjusted with 0.01 M H₂SO₄), while soil disks of pH_{CaCl_2} 6.71 were placed above 5 L of aerated deionised water or nutrient solution of pH 6.0. Soil disks were kept moist by capillary rise through a filter paper strip. To prevent the transfer of H⁺ or OH⁻ ions between different treatments through the solution medium, the six replicates of the same treatment were placed in the same solution medium.

From a preliminary experiment (data not shown), 40 seeds/cropping device of both Medicago spp. were needed to form a dense, planar mat of roots within 13 d after sowing. Plant culture in this experiment was a 2-step
procedure. In the first step, root mats were made by growing *M. murex* and *M. sativa* in an aerated nutrient solution (pH 6.0; replaced after 7 d) for a period of 13 d (Figure 4.1a). In the second step, cropping devices containing 13 d-old plants were randomised, taped securely in contact with soil disks (Figure 4.1b) and placed over an aerated nutrient solution. Soil disks without plants were covered with an opaque plastic lid to minimise evaporation and algal growth. All soil disks were placed in the growth cabinet for a further 4 d.

![Diagram](image)

**Figure 4.1.** Cropping device used to produce root mats of *M. murex* and *M. sativa* in a 2-step process. (a) step 1, growth of seedlings in aerated nutrient solution for 13 d; (b) step 2, roots placed in contact with soil disk, and plants cultured in aerated nutrient solution for a further 4 d. Experiment 4.1.

4.2.1.5 Measurements and calculations

At the time of soil-plant contact (13 d after sowing), shoots and roots of plants from one additional replicate were separated, fresh weight recorded, dried at 105 °C for 24 h and re-weighed. After 4 d of treatment (at 17 d after sowing), plants placed on soil disks were removed from the soil disks. The shoots and
roots were separated, and fresh and dry weights recorded. Relative root growth rate (RRGR, (mg g$^{-1}$ dry weight) d$^{-1}$) was calculated by the equation:

$$RRGR = \frac{(W_2 - W_1)}{W_1 \times (t_2 - t_1)}$$

where $W_1$ and $W_2$ are the initial and final dry weight of roots at time $t_1$ and $t_2$, respectively. The pH of the wet soil at the commencement of root contact was measured by shaking soil for 1 h in 0.01 M CaCl$_2$ (1:5) (Metrohm 744 pH meter, Herisau, Switzerland). The pH of soil disks in contact with plants represents rhizosphere pH. As the moisture content of the soil disks may have changed during the course of the experiment, it was necessary to determine the exact volume of 0.01 M CaCl$_2$ to add to the soil to constitute a 1:5 suspension for pH measurements. This was done by the following procedure. Four soil disks with root contact and four disks without root contact were randomly chosen. The moisture content of 0.5 g of soil from each randomly chosen disk was determined after drying at 105 °C for 1 h. A total of 5 mL of 0.02 M CaCl$_2$ was added to the remaining 2 g of soil from each soil disk. Depending on the moisture content of the 0.5 g of soil, a volume of deionised water added to the 2 g soil suspended in 0.02 M CaCl$_2$ such that its dilution resulted in a 1:5 suspension in 0.01 M CaCl$_2$. The soil suspension was then shaken for 1 h and pH measured.

To estimate H$^+$ flux released by the plant roots, pH buffer curves of the soils were first determined. Soil of both low and high pH were wet to 10% moisture (w/v) with either 0.01 M CaCl$_2$ or 0.01 M CaCl$_2$ containing various concentrations of HCl or NaOH. The concentrations of HCl and NaOH ranged from 0.4-8.0 mM. Soil was incubated in the dark at 22 °C for 3 d, suspended in 0.01 M CaCl$_2$ (1:5), shaken for 1 h and the pH measured. The pH buffer
capacity of soil was calculated from the slope (\( \bar{a} \), in \( \text{\textmu} \text{mol (g soil}^{-1} \) (pH-unit)}^{-1} \)) of the linear regression of the amount of HCl or NaOH added versus the soil pH (Tang et al., 1998) (Figure 4.2). The soil pH values were 4.49 and 6.71 (0.01 M CaCl\(_2\)). The \( \bar{a} \) values were \(-8.1 \text{ \textmu mol (g soil}^{-1} \) (pH-unit)}^{-1} \) (soil of pH 4.49, \( r^2 = 0.96 \)), and \(-9.6 \text{ \textmu mol (g soil}^{-1} \) (pH-unit)}^{-1} \) (soil of pH 6.71, \( r^2 = 0.98 \)). Amount of proton released was calculated from total acid production, estimated as the difference in acidity between soils with plants and control soils without plants. Thus, flux of proton \( j_{H^+} \) (nmol (g root}^{-1} \text{s}^{-1} \)) released by roots was calculated as:

\[
j_{H^+} = \frac{\bar{a}(pH_o - pH) \times M_{soil}}{(M_{root} \times t)}
\]

where \( pH_o \) is pH of soil disks without plants; \( pH \) is pH of soil disk with plants; \( M_{soil} \) is mass of the soil disk; \( M_{root} \) is root dry weight, averaged from plants at the beginning of soil-plant contact, and from plants 4 d after soil-plant contact; and \( t \) is the time duration of soil-plant contact in seconds (4 d in this experiment, i.e. 345 600 s).
Figure 4.2. Soil pH buffering curves of an acidic sand mixed with river sand (3:2) at initial pH (CaCl₂) of 4.49 and 6.71, as obtained by addition of HCl or KOH. The pH values were achieved by liming at 0.12 and 1.5 g kg⁻¹. Slopes (\(\beta\)) of the linear regressions, i.e. H⁺ soil buffer capacities, were \(-8.1\) mol (g soil)⁻¹ (pH-unit)⁻¹ (soil pH (CaCl₂) 4.49, \(r^2 = 0.99\)), and \(-9.6\) mol (g soil)⁻¹ (pH-unit)⁻¹ (soil pH (CaCl₂) 6.71, \(r^2 = 0.98\)). Experiment 4.1.

4.2.2 Experiment 4.2. Videodensitometry

4.2.2.1 Experimental design

Experiment 4.2 used the videodensitometry technique designed by Jaillard et al. (1996). *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius were grown in nutrient solution of pH 6.0 for 13 d. In each of six measurements, three to five plants of each species were embedded in nutrient gel film of pH 4.5 or 6.0 containing pH indicator. Values of proton flux were calculated from images acquired with a video camera.
4.2.2.2 Plant culture

*M. murex* cv. Zodiac and *M. sativa* cv. Aquarius were grown in the cropping device as described by Chaignon et al. (2002) for experiment 4.1 (Section 4.2.1.4), except the lower nylon mesh (30 μm) was not used, and the upper nylon mesh (900 μm) was replaced by a cloth mesh of 900 μm. Roots grew through the cloth mesh into 5 L of an aerated nutrient solution (pH 6.0), which was changed after 7 d, for a period of 13 d.

4.2.2.3 Measurements

After 13 d of growth in nutrient solution of pH 6.0, plants were equilibrated in fresh nutrient solution. This was done 48 h before embedding in nutrient agarose gel. Plants for the pH 4.5 treatment were placed in fresh nutrient solution of pH 5.2 for 24 h, and then transferred to nutrient solution of pH 4.5, 24 h before embedding. This intermediate step decreased acid-shock and subsequent necrosis of root tips. Plants for the pH 6.0 treatment were transferred to fresh nutrient solution of pH 6.0, 24 h before embedding. By the time roots were embedded, plants were 15 d-old. A separate volume (100 mL) of each nutrient solution (pH 4.5 and 6.0) that was used for equilibrating plants was also placed in the growth cabinet for 24 h before embedding. These solutions were used for embedding plants.

Solutions of pH indicators bromocresol green (pK = 4.69 for measurements at pH 4.5) and bromocresol purple (pK = 6.40 for measurements at pH 6.0) were prepared first. Films were prepared by melting 1 g of agarose powder (Fluka, Buchs, Switzerland; see Calba et al. (1996)) in 20 mL of pH indicator solution and 80 mL of the nutrient solution used for plant culture (pH 4.5 or 6.0), yielding a final pH indicator concentration of 90 mg L⁻¹. The agarose
solutions were boiled for 30 min, cooled to 37 °C in a water bath and pH readjusted to 4.5 or 6.0 with 0.01 M KOH or H₂SO₄. Plants were gently removed from the cloth mesh so not to damage roots, roots blotted dry with tissue, and carefully placed between two glass sheets (100×200 mm) separated by a 1 mm thick strip of PVC. The agarose solution was then syringed between the glass sheets. After approximately 5 min when the agarose solution had cooled and set, the upper glass sheet was removed and a 3 mm PVC strip was placed between the glass sheets so as not to confine the roots, and to enable respired CO₂ to diffuse into the atmosphere. Plants were placed in the growth cabinet and images were acquired by videodensitometry 2 h after embedding. Two saturated calibration standards (pH 3.5 and 8.0 with bromocresol green, and pH 4.8 and 8.5 with bromocresol purple) were prepared for each series of measurements. Three to five plants of each species per treatment were embedded and measured in six separate experiments. Images were acquired using the methods described by Plassard et al. (1999). H⁺ flux profile were calculated at three defined locations along the root: 3, 20 and 50 mm from the root tip. These locations correspond with the emerging root hair, growing root hair and mature root hair zones of the root, respectively, with decreasing susceptibility to infection by rhizobia further away from the root tip (Wood & Newcomb, 1988; Bhuvaneswari et al., 1981).

4.2.3 Statistical analysis

For both experiments, variability in the measured values among the various replicates is indicated by standard deviations. Significant differences between treatments were deduced from an analysis of variance (ANOVA) and a t-test as described in Section 3.2.3.
4.3 RESULTS

4.3.1 Experiment 4.1. ‘Root mat’ approach

4.3.1.1 Root growth

After 13 d of growth in nutrient solution of pH 6.0, root dry weight (mg plant\(^{-1}\)) for *M. murex* was 0.360 (± 0.193), and for *M. sativa* was 0.128 (± 0.067). The greater initial weight of *M. murex* seeds probably contributed to the greater root dry weight of *M. murex* compared to *M. sativa* at 13 d after sowing. Between 13 and 17 d after sowing, RRGR was generally higher in *M. sativa* than *M. murex*, despite *M. sativa* having lower root dry weights in all treatments (Table 4.1). In nutrient solution of pH 4.5, RRGR was significantly higher (approximately 2-times) in *M. sativa* than *M. murex*. 
Table 4.1. Root dry weights (mean value of 6 replicates ± standard deviation) of *M. murex* and *M. sativa* cultured in deionised water or nutrient solution at pH 4.5 and 6.0 for 4 d, and their relative root growth rate (RRGR) between 13 and 17 d. Experiment 4.1.

<table>
<thead>
<tr>
<th>Solution media</th>
<th>pH</th>
<th>Root dry weight (mg plant⁻¹)</th>
<th>RRGR ((mg g⁻¹ dry weight) d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>17 d after sowing</td>
<td>13-17 d after sowing</td>
</tr>
<tr>
<td>Water</td>
<td>4.5</td>
<td>0.614 ± 0.354</td>
<td>0.248 ± 0.140</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>0.732 ± 0.379</td>
<td>0.380 ± 0.118</td>
</tr>
<tr>
<td>Nutrient solution</td>
<td>4.5</td>
<td>0.510 ± 0.096</td>
<td>0.380 ± 0.094</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>0.780 ± 0.261</td>
<td>0.602 (±0.619)</td>
</tr>
</tbody>
</table>

* Significant difference in RRGR between *M. murex* and *M. sativa* at P < 0.05.

4.3.1.2 Soil pH and H⁺ extrusion

For soil disks of pH\textsubscript{CaCl₂} 4.49 moistened with deionised water or nutrient solution of pH 4.5, the pH of soil in contact with *M. murex* roots did not differ from the pH of the control soil, while the pH of the soil in contact with *M. sativa* roots was significantly lower (Table 4.2). With soil disks of pH\textsubscript{CaCl₂} 6.71 moistened with deionised water of pH 6.0, both *Medicago* spp. decreased soil pH. With soil disks of pH\textsubscript{CaCl₂} 6.71 moistened with nutrient solution of pH 6.0, *M. murex* roots decreased soil pH while *M. sativa* roots did not significantly change soil pH relative to the control soil. For soil disks of pH\textsubscript{CaCl₂} 6.71 without root contact, increases in pH after moistening for 4 d with deionised water (pH increased by 0.52 pH-unit) and nutrient solution (pH increased by 0.37 pH-unit) (Table 2) may be due to the short time (overnight) in which lime equilibrated in the soil before the limed soil was dried.

For soil disks of pH\textsubscript{CaCl₂} 4.49 moistened with nutrient solution of pH 4.5, proton release was significantly higher from *M. sativa* than *M. murex* (Table 4.2), with the rate of proton release from *M. sativa* being 1.9 nmol (g root)⁻¹ s⁻¹.
compared to *M. murex* at \(-0.1\) nmol \((g\ root)^{-1}\ s^{-1}\). The negative value indicates net OH\(^-\) release from *M. murex*. In soil of pH\(_{CaCl2}\) 6.71, there was no significant difference in proton release fluxes between the two species. Except for *M. murex* grown in nutrient solution of pH 4.5 releasing OH\(^-\), H\(^+\) release was generally higher with plants grown in nutrient solution than in deionised water.
Table 4.2. Soil pH and proton extrusion rates (mean value of 6 replicates ± standard deviation) from roots of *M. murex* and *M. sativa* cultured in rhizosphere soil moistened with water or nutrient solution between 13 and 17 days after planting. Experiment 4.1.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Initial pH of soil disk (0.01 M CaCl$_2$) and solution</th>
<th>Mean control soil pH (0.01 M CaCl$_2$)</th>
<th>Mean rhizosphere soil pH (0.01 M CaCl$_2$)</th>
<th>Proton extrusion rate$^a$ (nmol (g root)$^{-1}$ s$^{-1}$)</th>
<th>Ratio <em>M. sativa</em>/<em>M. murex</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No plants</td>
<td><em>M. murex</em></td>
<td><em>M. sativa</em></td>
<td><em>M. murex</em></td>
<td><em>M. sativa</em></td>
</tr>
<tr>
<td>Water</td>
<td>4.49/4.5</td>
<td>4.49 ± 0.02b</td>
<td>4.45 ± 0.08b</td>
<td>4.31 ± 0.07a</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>6.71/6.0</td>
<td>7.23 ± 0.05c</td>
<td>6.55 ± 0.19a</td>
<td>6.70 ± 0.02b</td>
<td>2.6 ± 2.3</td>
</tr>
<tr>
<td>Nutrient</td>
<td>4.49/4.5</td>
<td>4.49 ± 0.05b</td>
<td>4.51 ± 0.04b</td>
<td>4.14 ± 0.15a</td>
<td>−0.1 ± 0.1$^*$</td>
</tr>
<tr>
<td></td>
<td>6.71/6.0</td>
<td>7.08 ± 0.17b</td>
<td>5.95 ± 0.50a</td>
<td>6.78 ± 0.04b</td>
<td>3.2 ± 1.3</td>
</tr>
</tbody>
</table>

Mean values with different letters within a line are significantly different at $P < 0.05$.

$^a$ Negative value represents net OH$^-$ extrusion.

$^*$ Significant difference in proton extrusion rate between *M. murex* and *M. sativa* at $P < 0.05$. 
4.3.2 Experiment 4.2. Videodensitometry

4.3.2.1 Proton flux

At 15 d after sowing, roots of both *M. murex* and *M. sativa* released OH\(^-\) (Table 4.3), with *M. murex* consistently exhibiting larger efflux than *M. sativa*. The rate of OH\(^-\) efflux was significantly higher (approximately 2-times) in *M. murex* than *M. sativa* at 20 mm from the root tip (at pH 4.5), and at 3 mm from the root tip (at pH 6.0). There was no significant difference in OH\(^-\) efflux rates between the two species at the other locations along the root. At pH 4.5, the highest rate of OH\(^-\) efflux occurred at 20 and 50 mm from the root tip of both species (Table 4.3). At pH 6.0, *M. murex* had a lower rate of OH\(^-\) efflux at 20 mm, compared to 3 and 50 mm from the root tip. In contrast, there was no significant difference in rates of OH\(^-\) efflux at the three locations of the *M. sativa* root. There was greater variation in proton efflux values from plants grown at pH 4.5 than pH 6.0.
Table 4.3. Proton efflux rates (mean value$^a$ ± standard deviation) from roots of 15 d-old *M. murex* and *M. sativa* 2 h after embedding in nutrient agarose gel of pH 4.5 and 6.0 containing 100 mM NO$_3^-$, Experiment 4.2.

<table>
<thead>
<tr>
<th>pH</th>
<th>Primary root, distance from root tip (mm)</th>
<th>Proton efflux (nmole (m root)$^{-1}$ s$^{-1}$)$^b$</th>
<th>Ratio <em>M. murex</em>/<em>M. sativa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>M. murex</em></td>
<td><em>M. sativa</em></td>
</tr>
<tr>
<td>4.5</td>
<td>50</td>
<td>$-0.085 \pm 0.065a$</td>
<td>$-0.061 \pm 0.019a$</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>$-0.097 \pm 0.046a^*$</td>
<td>$-0.048 \pm 0.022a$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$-0.005 \pm 0.037b$</td>
<td>$-0.003 \pm 0.036b$</td>
</tr>
<tr>
<td>6.0</td>
<td>50</td>
<td>$-0.008 \pm 0.003a$</td>
<td>$-0.005 \pm 0.003a$</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>$-0.005 \pm 0.007b$</td>
<td>$-0.004 \pm 0.004a$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$-0.011 \pm 0.005a^*$</td>
<td>$-0.006 \pm 0.006a$</td>
</tr>
</tbody>
</table>

Mean values with different letters within a column are significantly different at $P < 0.05$.

$^a$ Mean values from 7 and 11 plants (*M. murex*), and 8 and 7 plants (*M. sativa*) for pH 4.5 and 6.0, respectively.

$^b$ Negative values represent net OH$^-$ efflux.

$^*$ Significant difference in rate of proton efflux between *M. murex* and *M. sativa* at $P < 0.05$.

4.4 DISCUSSION

4.4.1 Changes in rhizosphere pH as affected by *Medicago* species

Roots of *M. sativa* released more protons than those of *M. murex* into a sandy soil of pH$_{CaCl_2}$ 4.49, while into a nutrient agarose gel of pH 4.5, *M. sativa* released fewer OH$^-$ ions compared to *M. murex*. As a result, the rhizosphere of *M. sativa* was consistently more acidic than the rhizosphere of *M. murex*. The differential ability of these species to acidify the root medium may be genetic, relating to species adaptation and tolerance to low pH. *M. murex* is commonly associated with acidic soils (Francis & Gillespie, 1981), nodulates consistently
at low pH (Howieson & Ewing, 1989), and is more tolerant of low pH than other annual *Medicago* spp. (Ewing, 1991).

Differences in the rate of H$^+$ release between *M. murex* and *M. sativa* may also be related to their different relative growth rates of root growth. *M. sativa* had a greater relative rate of root growth and a higher rate of H$^+$ release compared to *M. murex*, which had a slower relative rate of root growth, released less H$^+$, and acidified the rhizosphere less than *M. sativa*. In a previous study comparing Al-tolerant and Al-sensitive genotypes of *Zea mays* (maize), Calba and Jaillard (1997) similarly showed that the genotype which grew the fastest, also exhibited the largest nutrient requirements, and hence released the largest fluxes of protons. Different growth rates also affected rhizosphere pH changes in different genotypes of *T. subterraneum* (Jarvis & Robson, 1983a). Root exudation may be directly associated with root growth since almost no root exudation occurred in plants where almost no root growth was observed even though shoots were actively growing (Prikryl & Vancura, 1980).

*M. murex* and *M. sativa* induced pH changes in the rhizosphere differently, especially in younger parts of roots where the rhizosphere of *M. murex* was less acidic than that of *M. sativa*. In acidic soils where survival and growth of *S. meliloti* (Coventry & Evans, 1989; Robson & Loneragan, 1970c), and attachment to host roots (Howieson et al., 1993; Caetano-Anollés et al., 1989) are already challenged by low pH, root-induced acidification of the rhizosphere will further inhibit the nodulation process. The more acidic rhizosphere of *M. sativa* would certainly be less favourable for saprophytic growth and colonisation by *S. medicae*. Under acidic conditions, a decrease in even 0.1 pH-unit adversely affected the survival and growth of *S. meliloti*
Consequently, rhizobial populations of *S. medicae* on the roots of *M. sativa* may take a longer time to reach a sufficient population size to initiate infection (Hirsch, 1992). Rhizosphere acidification may be an important factor contributing to the lower numbers of rhizobia associated with the roots of *M. sativa* (Chapter 3) and delayed nodule initiation and development of *M. sativa* in soil of low pH (Chapter 2). Rhizosphere alkalinisation by seedlings of *M. sativa* growing in an acidic soil (Pijnenborg et al., 1990b) compared to rhizosphere acidification by the same species observed in experiment 4.1 of this chapter was probably due to soil properties contributing to different nutrient uptake and hence pH changes. Pijnenborg et al. (1990b) suggested that root-induced rhizosphere alkalinisation by *M. sativa* roots did not result in the formation of nodules on *M. sativa* since the 0.6 pH-unit increase occurred after the acid-sensitive period of the nodulation process. However, an equivalent decrease in pH would certainly affect nodulation in an acidic soil, especially during the acid-sensitive stages of rhizobial survival, growth and colonisation in the rhizosphere.

### 4.4.2 Changes in rhizosphere pH as affected by growth conditions

Alkalisation of rhizosphere soil when cultured at low pH, and acidification of rhizosphere soil when cultured at higher pH as displayed by *M. murex*, has previously been observed in *H. vulgare* and *G. max* (Youssef & Chino, 1989) and in *V. faba* (Schubert et al., 1990). The reason for this ‘plasticity’ in response to external pH is unclear, but may be related to nutrient availability, and the subsequent imbalance of cation-anion uptake.
In addition, the amount of $H^+$ released by roots of both *Medicago* spp. was higher in plants grown at higher pH than those grown under acidic conditions. Furthermore, the higher overall rate of ion exchange may result from higher nutrient uptake when plants were less acid-stressed. Decreased plant growth caused by low pH in acid-sensitive species has been related to a build-up of $H^+$ ions in the cytoplasm, because of a lack of $H^+$ release (Schubert et al., 1990), or an influx of protons into root cells because of higher membrane permeability (Yan et al., 1992). This might have occurred to a larger extent for *M. sativa* than for *M. murex* in the present experiment.

Compared to plants grown in soil moistened with deionised water, plants grown in soil moistened with nutrient solution are likely to have higher rates of nutrient uptake due to access to nutrients from both the soil disk and nutrient solution. This is consistent with the larger proton release fluxes observed in these plants relative to those grown in soil moistened with deionised water. Finally, the availability of nutrients (which is likely to vary considerably from soil to soil) will have a dramatic influence on both the direction (efflux or influx) and intensity of $H^+$ fluxes. Such considerations can help in understanding why plants grown in experiment 1 on soil disks decreased rhizosphere pH while plants of a similar age and past nutrient status exhibited net $OH^-$ efflux when grown in nutrient solution. Further work is needed to estimate whether the observed differences between these and other genotypes of *M. murex* and *M. sativa* are consistently found in a range of nutrient solutions of varying compositions, especially $NO_3^-/NH_4^+$ ratios, and in a range of acidic soils. In this respect, it should be stressed that plants were only relying on $NO_3^-$ in experiment 4.2 ($NH_4^+$ was not supplied in the agarose gel) and hence exhibited $OH^-$ efflux, while they might have derived some $NH_4^+$ from the soil in experiment 4.1,
thereby resulting in rhizosphere acidification. It is unclear why greater variation in proton efflux values were observed from plants grown at pH 4.5 than pH 6.0 (experiment 4.2), but may be related to the nutrient solutions being unbuffered against pH change.

4.4.3 Changes in rhizosphere pH as affected by the location along the root axis

Depending on plant species and N source, but also Fe and P status of the plant, root-induced changes in rhizosphere pH can vary considerably along the root (Marschner & Römheld, 1983; Marschner et al., 1982). Higher acidification at the root tips compared to basal parts of roots has often been reported, e.g. in Phleum pratense (timothy) (Zieschang et al., 1993) and Z. mays (Marschner & Römheld, 1983). In this study, the root tip did not behave differently at pH 6.0 (no significant differences were found with other locations along the root axis; Table 3). However at pH 4.5, root tips of both genotypes of Medicago spp. exhibited significantly lower efflux of OH\(^{-}\) and were thus more acidic than older regions of the roots. Different rates of ion release in various regions of a root may be related to age of roots cells, and hence affect the uneven pattern of cation and anion uptake along the root. It is possible that older parts of the root take up more NO\(_3^{-}\), while younger parts take up more cations (Blanchard & Lipton, 1986). This would need to be verified by the means of electrophysiological measurements, i.e. micropotentiometry with NO\(_3^{-}\) selective microelectrodes (Jaillard et al., 2003; Plassard et al., 1999).

Susceptibility of root hairs to infection by rhizobia decreases with root hair age. The highest frequency of susceptible root hairs is in the younger region of the root, although some root hairs are susceptible to infection in the
more mature region (Bhuvaneswari et al., 1981). At low pH, the more mature regions of roots of both *Medicago* species had higher rates of OH⁻ release, were less acidic, and therefore more favourable for rhizobial survival and growth than younger parts of the root. It would be useful to compare in situ colonisation of *S. medicae* at various zones along the roots of *M. murex* and *M. sativa*, and to relate any differences to the differential patterns of rhizosphere acidification between the two species.
CHAPTER 5. CONSTRUCTION AND CHARACTERISATION OF A S. MEDICAES STRAIN CARRYING A PLASMID OF THE GREEN FLUORESCENT PROTEIN pHC60

5.1 INTRODUCTION

Bacterial colonisation of roots is an essential step in many plant-microbe interactions. In legume-rhizobia symbioses, there are strong correlations between the numbers of rhizobia in the rhizosphere and plant nodulation (Young & Brockwell, 1992; Brockwell et al., 1989; Herridge et al., 1984). Root colonisation by rhizobia is important in the nodulation process as critical concentrations of rhizobial Nod factors are required for root hair deformation and cortical cell division in the host plant (Hirsch, 1992). The differential nodulation response between M. murex and M. sativa in an acidic soil (Chapter 2) may be related to the higher numbers of S. medicae associated with the roots of M. murex than M. sativa (Chapter 3). As roots of M. murex and M. sativa modified the pH of the rhizosphere differentially (Chapter 4), the more acidic rhizosphere of M. sativa may have been less favourable for the colonisation of S. medicae. Furthermore, successful nodulation depends on rhizobia infecting root hairs on parts of the root where root hairs are most susceptible to infection. In soil of pH\textsubscript{CaCl\textsubscript{2}} 4.3, both M. murex and M. sativa formed their uppermost nodules 4-5 cm below the hypocotyl, yet the nodule appeared on M. sativa almost 4 weeks later compared to M. murex (Chapter 2). Hence, a differential rate of colonisation by S. medicae may have had an effect on the rate of nodule appearance at that particular zone of the M. murex
and *M. sativa* roots. An assessment of the rate of *in situ* colonisation by *S. medicae* over time could reveal whether cells of *S. medicae* accumulate slower on the roots of *M. sativa* compared to *M. murex*.

There are various techniques for quantifying root colonisation by bacteria. Different methods of processing root samples are required depending on whether one is measuring external root colonisation, internal root colonisation, or both (Kloepper & Beauchamp, 1992). Indirect techniques require isolation and growth of bacteria on artificial media for enumeration and differentiation from indigenous microorganisms (e.g. plates counts with selective media) (Kloepper & Beauchamp, 1992). However, these methods tend to underestimate the actual number of bacteria as some cells may be physically attached to soil particles or roots, are killed by the dilution medium or do not grow on nutrient medium after ‘starvation’ conditions in the rhizosphere (Kloepper & Beauchamp, 1992). In contrast, microscopy with fluorescence technology allows the direct measurement of bacterial colonisation of the root. Green fluorescent protein (GFP), a small protein found in the jellyfish *Aequorea victoria* (Chalfie et al., 1994), has been used in biological studies.

Since GFP does not require cofactors to develop fluorescence (Stuurman et al., 2000; Chalfie et al., 1994), it has been used as a marker in rhizobia such as *S. meliloti* (Pistorio et al., 2002; Stuurman et al., 2000; Cheng & Walker, 1998; Gage et al., 1996), *S. fredii*, *R. leguminosarum*, *Mesorhizobium loti* (Stuurman et al., 2000), *R. etli* (Xi et al., 2001), *Psuedomonas* spp. (Unge & Jansson, 2001; Bloemberg et al., 2000; Ramos et al., 2000; Normander et al., 1999; Unge et al., 1999; Bloemberg et al., 1997), as well as the fungus *Fusarium oxysporum* (Lagopodi et al., 2002). GFP-marked bacteria have
been used to study their association with the roots of legumes such as *M. sativa* (Pistorio et al., 2002; Stuurman et al., 2000; Cheng & Walker, 1998; Gage et al., 1996), *Vicia sativa* (common vetch), *Lotus japonicus*, *Macroptilium atropurpureum* (siratro) (Stuurman et al., 2000), and *P. vulgaris* (Xi et al., 2001), and non-legumes such as *H. vulgare* (Ramos et al., 2000; Normander et al., 1999), *O. sativa* (Prayitno et al., 1999), *T. aestivum* (Unge & Jansson, 2001) and *Lycopersicon esculentum* (tomato) (Bloemberg et al., 2000). Among legume-rhizobia associations, GFP-marked strains of *S. meliloti* have been used to follow the initiation and elongation of infection threads in *M. sativa* (Pistorio et al., 2002; Cheng & Walker, 1998; Gage et al., 1996). These studies were done in nutrient solutions at neutral pH, and their results cannot be used to explain the differential nodulation response of *M. murex* and *M. sativa* observed in an acidic soil (Chapter 2). Therefore, it is important that the colonisation of *M. murex* and *M. sativa* by *S. meliloti* in soil of low pH is studied.

This chapter describes the insertion of the GFP carrying plasmid pHC60 (Cheng & Walker, 1998) into *S. medicae* strain WSM419. pHC60 constitutively expresses GFP and has been reported to be stable in cells of *S. meliloti* strain Rm1021 without antibiotic pressure (Cheng & Walker, 1998). GFP-marked Rm1021 fluoresced brightly under a fluorescent microscope and nodulated plants of *M. sativa* as efficiently as wild-type strain Rm1021 (Cheng & Walker, 1998). Furthermore, rhizobial cells recovered from pink nodules formed by GFP-marked Rm1021 on *M. sativa* expressed GFP (Cheng & Walker, 1998), indicating the retention of the genes in Rm1021 during passage through nodules. However, the mean generation time of GFP-marked Rm1021
relative to wild-type strain Rm1021 was not been determined, and its ability to survive, grow and colonise roots in soil is unknown. Due to stability of the plasmid carrying GFP in \textit{S. meliloti} Rm1021, and the ability of GFP-marked Rm1021 to nodulate \textit{M. sativa}, pHC60 was inserted into \textit{S. medicae} strain WSM419. This chapter describes the construction of a GFP-marked strain of \textit{S. medicae} strain WSM419, and experiments to determine the growth and nodulation properties of GFP-marked transconjugants WSM419(pHC60). Three characteristics of WSM419(pHC60) were compared with WSM419: (1) mean generation time; (2) survival in soil; and (3) nodulation of host \textit{Medicago} plants in an acidic soil. If the characteristics of WSM419(pHC60) were not significantly different to WSM419 under acid stress, the rate of colonisation by WSM419(pHC60) would be determined on the roots of \textit{M. murex} and \textit{M. sativa} growing in soil of low pH.

5.2 \textbf{MATERIALS AND METHODS}

5.2.1 \textbf{Construction of a GFP-marked strain of \textit{S. medicae} WSM419}

Different concentrations of antibiotics were used in the construction of a GFP-marked \textit{S. medicae} to overcome intrinsic wild-type resistance in \textit{Escherichia coli} and \textit{S. medicae} (R Tiwari, pers. comm., January 2001). \textit{E. coli} strain DH5\textasciitilde/pHC60 (from G Walker and B Pellock, Biology Department, Massachusetts Institute of Technology, USA) containing the plasmid pH60 that constitutively expresses the \textit{gfp} gene (Cheng & Walker, 1998); and strain
HB101/pRK2013 containing the helper plasmid pRK2013 (from R Tiwari, Centre for Rhizobium Studies, Murdoch University, Australia) were grown overnight at 37 °C in Luria-Bertani (LB) medium which contained: 10 g L⁻¹ tryptone peptone, 5 g L⁻¹ yeast, 80 mM NaCl, and either Tc₁₂.₅ (12.5 ìg mL⁻¹ tetracycline for DH5á/pHC60), or Km₅₀ (50 ìg mL⁻¹ kanamycin for HB101/pRK2013). 

S. medicae strain WSM419 (Howieson & Ewing, 1986) was grown overnight at 28 °C in TY medium which contained: 5 g L⁻¹ tryptone peptone; 7.5 g L⁻¹ yeast, 15 mM CaCl₂ and Cm₂₀ (20 ìg mL⁻¹ chloramphenicol). A total of 0.5 mL of each of the three cultures was then centrifuged at 14 000 rpm for 1 min (IEX MicroMax, International Equipment Company, Needham Heights, MA, USA), the supernatant removed and the pellet resuspended in 50 ìL fresh TY medium without any antibiotics. A total of 20 ìL of each suspension was then dropped on top of each other on TY medium containing 1.5% (w/v) agar (Grade J3, Leiner Davis Gelatin, Australia), allowed to dry and incubated overnight at 28 °C. Colonies were then subcultured on fresh TY agar medium containing Cm₂₀Tc₂₀ (20 ìg mL⁻¹ chloramphenicol, 20 ìg mL⁻¹ tetracycline) and incubated at 28 °C until colonies were visible. Eight single colonies were subcultured onto TY agar medium containing Cm₂₀, and Cm₄₀Tc₂₀ (40 ìg mL⁻¹ chloramphenicol, 20 ìg mL⁻¹ tetracycline).

Two of the eight transconjugants WSM419(pHC60), named YC2 and YC8, were selected for further studies. YC2 and YC8 were grown on TY agar medium which contained either Cm₄₀, or both Cm₄₀Tc₂₀. Colonies that grew on media with both chloramphenicol and tetracycline were considered to contain the pHC60 plasmid. YC2 and YC8 were then used for subsequent characterisation experiments.
5.2.2 Colony morphology and expression of GFP in YC2 and YC8

The morphology of YC2 and YC8 colonies cultured on TY agar media containing Cm$_{40}$Tc$_{20}$ were recorded. Cultures of YC2 and YC8 were grown overnight in 5 mL of nutrient media of pH 7.0 and 5.8 without antibiotics. Cultures were the streaked onto a glass slide and examined for GFP expression by fluorescence microscopy at 450-490 nm (Zeiss Photomicroscope III RS, Oberkochen, Germany).

5.2.3 Experiment 5.1. Mean generation time of WSM419, YC2 and YC8

5.2.3.1 Experimental design

Experiment 5.1 was done to measure the growth rate of *S. medicae* WSM419, YC2 and YC8. Rhizobia were grown in duplicate flasks of media of initial pH 5.8 and 7.0. Growth of cells was determined by periodically measuring the optical density (O.D.) of the media at $A_{600}$ over 24 h. The mean generation time (MGT) of strain WSM419, YC2 and YC8 were calculated.

5.2.3.2 Media

Rhizobia were grown in the medium described in Section 2.2.3. For growth studies at pH 5.8, 20 mM 2-[N-Morpholino]ethane-sulphonic acid (MES) was used At pH 7.0, 20 mM N2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) was used. The media were adjusted to either pH 5.8 or 7.0 with 0.1 M
KOH and sterilised at 121 °C for 20 min.

5.2.3.3 Mean generation time of WSM419, YC2 and YC8

Starter cultures of WSM419, YC2 and YC8 were grown to late log phase in 5 mL of nutrient medium at pH 7.0, which contained Cm$_{40}$ (for WSM419), or Cm$_{40}$Tc$_{20}$ (for YC2 and YC8). Immediately before inoculation to flasks to measure growth rate, starter cultures were centrifuged at 10 000 rpm for 5 min (Eppendorf 5810, Hamburg, Germany), and resuspended in 1 mL of sterile 0.89% (w/v) NaCl. An aliquot of this suspension was then inoculated to duplicate growth flasks containing 50 mL of fresh medium, to give an initial absorbance at 600 nm of 0.05 (Hitashi U-1100, Tokyo, Japan). For growth measurements, rhizobia were grown in media without antibiotics. Flasks were aerated on a platform shaker (Innova 2100, New Brunswick Co. Inc., Edison, New Jersey, USA) at 200 rpm. The rate of cell growth was determined by periodically taking duplicate 1-mL samples to measure absorbance at 600 nm over 24 h. Growth curves of WSM419, YC2 and YC8 were plotted, and the mean generation time (MGT; h) of each culture was calculated.

5.2.4 Experiment 5.2. Recovery of WSM419, YC2 and YC8 from soil

5.2.4.1 Experimental design

Experiment 5.2 was a randomised design with three replications. Strain WSM419, YC2 and YC8 were inoculated to sterile soil of pH$_{CaCl_2}$ 4.3 and 7.0. After 7 d, 1 g of soil was removed and the numbers of rhizobia in the sample
was determined by the Miles and Misra drop-plate method described in Section 2.2.3.

5.2.4.2 Soil and rhizobia

The soil used was described in Section 2.2.2 and Table 2.1. The soil was wet to field capacity (10% w/v) with deionised water, and 25 g of soil was placed in screw-top tubes (2.5 cm diameter, 8 cm height). Tubes were then sterilised at 121 °C for 20 min.

Cultures of *S. medicae* strain WSM419, YC2 and YC8 were grown to approximately $10^9$ rhizobia mL$^{-1}$ in the nutrient media described in Section 2.2.3. Immediately before inoculation to tubes, suspensions containing approximately $10^7$ rhizobia mL$^{-1}$ were prepared by diluting the culture with sterile deionised water. A total of 1 mL of this suspension was then aseptically syringed into soil of each tube, and thoroughly mixed by inverting the tube 3-4 times. Viable counts using the Miles and Misra drop-plate method (Section 2.2.3) were made to determine the initial numbers of rhizobia added to each tube. Tubes were then placed in an air-conditioned glasshouse maintained at 22 °C for 7 d.

5.2.4.3 Recovery of rhizobia from soil

After 7 d, one 1-g sample of soil was aseptically removed from each tube and suspended in 9 mL of sterile 0.89% (w/v) NaCl. Tubes were placed on a platform shaker (Innova 2100, New Brunswick Co. Inc., Edison, New Jersey, USA) at 150 rpm for 1 h. Rhizobial counts from the soil suspension were made by the Miles and Misra drop-plate method onto media, described in Section 2.2.3, which also contained 1.5% (w/v) agar (Grade J3, Leiner Davis Gelatin,
Australia), and either Cm$_{40}$ (for WSM419), or Cm$_{40}$Tc$_{20}$ (for YC2 and YC8). Plates were incubated at 28 °C until single colonies were visible. The number of colony forming units were counted, and the rate of recovery of rhizobia from soil was expressed as a percentage of the initial numbers of rhizobia inoculated into the soil.

5.2.5 Experiment 5.3. Nodulation of *M. murex* and *M. sativa* by WSM419, YC2 and YC8 in soil

5.2.5.1 Experimental design

Experiment 5.3 was a split-plot design with five replications. Strain WSM419, YC2 and YC8 were inoculated to soil (pH$_{\text{CaCl}_2}$ 4.3 and 7.0) sown with *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius. An uninoculated control without *S. medicae* was also used. Plants were harvested after 6 weeks and assessed for dry matter production and nodulation.

5.2.5.2 Soil and rhizobia

The soil used was described in Section 2.2.2 and Table 2.1. Pots (11 cm diameter, 11.5 cm height) were sterilised and prepared as described in Section 2.2.2. A total of 1.5 kg of soil was placed in each pot, which were covered with a plastic sheet until plants were sown.

*S. medicae* strain WSM419, YC2 and YC8 were grown on media (pH 7.0) described in Section 3.2.2.3, which also contained 1.5% (w/v) agar (Grade J3, Leiner Davis Gelatin, Australia). The media was buffered with 10 mM HEPES. Immediately before inoculation to pots, colonies from one plate of
each strain were suspended in 100 mL of sterile 10% (w/v) sucrose solution, and approximately 2 mL of the suspension was inoculated on top of each sown seed with a sterile syringe. Approximately 2 mL of sterile 10% (w/v) sucrose solution was syringed on each seed sown in the uninoculated treatment.

5.2.5.3 Plants

Seeds of *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius were sorted for uniformity in colour, shape and size. Seeds were washed in 70% (v/v) ethanol (1 min), soaked in 3% (v/v) hypochlorite solution (30 s), rinsed in six changes of sterile deionised water and left to imbibe in the last change of water overnight. Imbibed seeds were then placed on 1.5% (w/v) water agar (Grade J3, Leiner Davis Gelatin, Australia) and incubated at 20 °C in the dark until radicles were approximately 1 cm long. Six seedlings of each species were sown into each pot, and each pot was inoculated with either *S. medicae* strain WSM419, YC2 or YC8. A sterile watering tube with cap was inserted in the centre of each pot and the soil surface was covered with sterile plastic beads to minimise contamination. Approximately 30 mL of sterile deionised water was added to each pot daily. One week after planting, plants were thinned to three plants/species/pot.

5.2.5.4 Assessment of dry matter production and nodulation

Assessment of nodulation was done 31 d after sowing. Plants were carefully removed from the soil and roots were gently rinsed in running water. Each plant was cut at the hypocotyl and assessed for the presence or absence of nodules. Plant nodulation scores were determined by the system described in Section
2.2.7. Shoots and roots from all plants were dried at 70 °C for 48 h and weighed.

5.2.5.5 Recovery of YC2 and YC8 from nodules

To determine whether rhizobia in the nodules of *M. murex* and *M. sativa* inoculated with YC2 and YC8 carried the pHc60 plasmid, two nodules from two plants/species from replicate 1 were carefully removed from the root with a sterile scalpel. Nodules were then sterilised in 70% (v/v) ethanol for 30 s, 3% (v/v) NaOCl for 2 min and rinsed in six changes of sterile deionised water. Each nodule was crushed, and the contents plated on media (described in Section 2.2.3) which contained either Cm\(_{40}\), or Cm\(_{40}\)Tc\(_{20}\). Plates were incubated at 28 °C until colonies were visible.

5.2.6 Statistical analysis

For all experiments, variability in the measured values among the various replicates is indicated by standard deviations. Significant differences between treatments were deduced from an analysis of variance (ANOVA) as described in Section 3.2.3.

5.3 RESULTS

5.3.1 Colony morphology and expression of GFP in YC2 and YC8

Colonies of YC2 and YC8 were greenish-yellow in colour. Cells of YC2 and YC8
fluoresced bright green under a fluorescent microscope.

5.3.2 Experiment 5.1. Mean generation time of WSM419, YC2 and YC8

Growth curves of WSM419, YC2 and YC8 are shown in Figure 5.1.

**Figure 5.1.** Growth curves of *S. medicae* WSM419, YC2 and YC8 in media of pH 5.8 and 7.0. Experiment 5.1.

In media of both initial pH 5.8 and 7.0, there was no significant difference in MGT between WSM419, YC2 and YC8 (Table 5.1). The MGT of all rhizobia was 0.7-1 h slower at pH 5.8 compared to pH 7.0 (Table 5.1).
Table 5.1. Mean generation time of *S. medicae* WSM419, YC2 and YC8 in media with initial pH of 5.8 and 7.0. Experiment 5.1.

<table>
<thead>
<tr>
<th>Initial pH</th>
<th>Mean generation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WSM419</td>
</tr>
<tr>
<td>5.8</td>
<td>4.0 ± 0.1a</td>
</tr>
<tr>
<td>7.0</td>
<td>3.3 ± 0.1a</td>
</tr>
</tbody>
</table>

Mean values with different letters within a line are significantly different at *P* < 0.05.

5.3.3 Experiment 5.2. Recovery of WSM419, YC2 and YC8 from soil

The initial numbers of rhizobia inoculated into soil were similar for all strains (Table 5.2). After 7 d, WSM419, YC2 and YC8 were not recovered from soil of pH\(_{\text{CaCl}_2} 4.3\) (Table 5.2). From soil of pH\(_{\text{CaCl}_2} 7.0\), 81% of WSM419 was recovered. The percentage recovery of YC2 and YC8 were considerably lower: 23% for YC2 and 48% for YC8.

Table 5.2. Initial numbers of rhizobia, and percentage of initial number (mean of 3 replicates ± standard deviation) of *S. medicae* WSM419, YC2 and YC8 recovered from soil of pH\(_{\text{CaCl}_2} 4.3\) and 7.0 after 7 d. Experiment 5.2.

<table>
<thead>
<tr>
<th>S. medicae strain</th>
<th>WSM419</th>
<th>YC2</th>
<th>YC8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial numbers of rhizobia (rhizobia g soil(^{-1}))</td>
<td>3.5×10(^5)</td>
<td>8.5×10(^5)</td>
<td>1.2×10(^6)</td>
</tr>
<tr>
<td>Soil pH (0.01 M CaCl(_2))</td>
<td>Percentage of initial number recovered from soil after 7 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.0</td>
<td>81 ± 2a</td>
<td>23 ± 9b</td>
<td>48 ± 5b</td>
</tr>
</tbody>
</table>

Mean values with different letters within a line are significantly different at *P* < 0.05.
5.3.4  Experiment 5.3. Dry matter production and nodulation of *Medicago* spp.

5.3.4.1  Dry matter production

In soil of both pH$_{\text{CaCl}_2}$ 4.3 and 7.0, *M. murex* and *M. sativa* grown without inoculated rhizobia produced significantly less dry matter than plants inoculated with rhizobia (Table 5.3). Except in soil of pH$_{\text{CaCl}_2}$ 4.3 where *M. murex* inoculated with YC8 produced less dry matter compared to plants inoculated with WSM419, there was no significant difference in dry matter production in plants of *M. murex* and *M. sativa* inoculated with WSM419, YC2 or YC8 (Table 5.3). Except in the uninoculated soil, *M. murex* grown in soil of pH$_{\text{CaCl}_2}$ 7.0 produced approximately the same amount of dry matter as plants grown in the acidic soil, while *M. sativa* grown in soil of pH$_{\text{CaCl}_2}$ 4.3 produced approximately half the dry matter of plants grown in the neutral soil.

*Table 5.3.* Dry matter production (mean of 5 replicates ± standard deviation) *M. murex* and *M. sativa* grown in soil of pH$_{\text{CaCl}_2}$ 4.3 and 7.0 inoculated with *S. medicae* WSM419, YC2 and YC8, 31 d after sowing. Experiment 5.3.

<table>
<thead>
<tr>
<th><em>Medicago</em> spp.</th>
<th>Soil pH (0.01 M CaCl$_2$)</th>
<th>Dry matter production (g plant$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninoculated</td>
<td>WSM419</td>
</tr>
<tr>
<td><em>M. murex</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>0.02 ± 0.01a</td>
<td>0.13 ± 0.03b</td>
</tr>
<tr>
<td>7.0</td>
<td>0.07 ± 0.04a</td>
<td>0.12 ± 0.02b</td>
</tr>
<tr>
<td><em>M. sativa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>0.02 ± 0.03a</td>
<td>0.05 ± 0.02b</td>
</tr>
<tr>
<td>7.0</td>
<td>0.05 ± 0.02a</td>
<td>0.13 ± 0.02b</td>
</tr>
</tbody>
</table>

Mean values with different letters within a line are significantly different at $P < 0.05$. 

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5.3.4.2 Nodulation score

In soil of pH$_{\text{CaCl}_2}$ 4.3 with plants of *M. murex*, there was no significant difference in the nodulation scores produced by strains of *S. medicae* (Table 5.4). In the same soil, *M. sativa* achieved the highest nodulation score with YC8, but there was no difference between WSM419 and YC2. In soil of pH$_{\text{CaCl}_2}$ 7.0, *M. murex* achieved the lowest nodulation scores with YC8, and there was no difference between WSM419 and YC2 (Table 5.4). However, YC2 produced the highest nodulation score for plants of *M. sativa* in the same soil. Generally, plants grown in soil of pH$_{\text{CaCl}_2}$ 7.0 achieved higher nodulation scores than plants grown in soil of pH$_{\text{CaCl}_2}$ 4.3.

Table 5.4. Nodulation score\(^a\) (mean of 5 replicates ± standard deviation) of *M. murex* and *M. sativa* grown in soil of pH$_{\text{CaCl}_2}$ 4.3 and 7.0 inoculated with *S. medicae* WSM419, YC2 and YC8, 31 d after sowing. Experiment 5.3.

<table>
<thead>
<tr>
<th><em>S. medicae</em> strain</th>
<th>Nodulation score(^a)</th>
<th>[\text{Soil pH 4.3 (0.01 M CaCl}_2)]</th>
<th>[\text{Soil pH 7.0 (0.01 M CaCl}_2)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. murex</em></td>
<td><em>M. sativa</em></td>
<td><em>M. murex</em></td>
</tr>
<tr>
<td>WSM419</td>
<td>48 ± 36a</td>
<td>15 ± 16b</td>
<td>121 ± 52a</td>
</tr>
<tr>
<td>YC2</td>
<td>57 ± 46a</td>
<td>6 ± 12b</td>
<td>126 ± 68a</td>
</tr>
<tr>
<td>YC8</td>
<td>49 ± 55a</td>
<td>34 ± 40c</td>
<td>85 ± 50b</td>
</tr>
</tbody>
</table>

Mean values with different letters within the same soil pH are significantly different at \(P < 0.05\).

\(^a\) Described in Section 2.2.7 and Table 2.2.

5.3.4.3 Recovery of YC2 and YC8 from *M. murex* and *M. sativa* nodules

From soil of pH$_{\text{CaCl}_2}$ 4.3, isolates from 67% of the nodules crushed from *M. murex* and *M. sativa* inoculated with YC2 and YC8 grew on plates containing both chloramphenicol and tetracycline. From soil of pH$_{\text{CaCl}_2}$ 7.0, isolates from 91% of the nodules crushed from plants inoculated with YC2 and YC8 showed
growth on media containing both chloramphenicol and tetracycline. The remaining 23% and 9% of crushed nodules from soil of pH\textsubscript{CaCl\(_2\)} 4.3 and 7.0, respectively, did not grow on plates containing chloramphenicol and tetracycline, or chloramphenicol alone.

5.4 DISCUSSION

A plasmid, pHC60, that constitutively expresses the \textit{gfp} gene was inserted into two transconjugants of the acid-tolerant \textit{S. medicae} strain WSM419. One of the two transconjugants is to be used to quantitatively measure the rate of rhizobial colonisation at various zones along the primary roots of \textit{Medicago} growing in an acidic soil. The similar growth rates of transconjugants YC2 and YC8 compared to strain WSM419 meant that the pHC60 plasmid did not place a metabolic burden on the cells carrying it. Hence, root colonisation by the transconjugant should reflect that by WSM419 at both acidic and neutral pH.

GFP did not impair plant dry matter production or nodulation by YC2 and YC8 at pH 7.0 in comparison with strain WSM419. Furthermore, both transconjugants retained the pHC60 plasmid in media of low pH after 24 h, and from the nodules of plants grown in soil of low pH when harvested 31 d after sowing. Although the pHC60 plasmid was retained by cells of YC2 and YC8 (this study), and was stable in Rm1021 in media at neutral pH (Cheng & Walker, 1998), the environment which cells will encounter in an acidic soil will be very different. As the survival of \textit{S. meliloti} is highly sensitive to acidity (Reeve et al., 1993), it was considered possible that rhizobia under acid stress would ‘drop’ the pHC60 plasmid. Furthermore, the plasmid could be dispersed by conjugal transfer to other soil bacteria. If other soil bacteria also carried
the GFP plasmid, then the strategy to specifically detect GFP-marked rhizobia on roots would be undermined. As cells of YC2 and YC8 retained the plasmid and fluoresced after growth in media without antibiotics, cells inoculated into soil without absence of antibiotics should also retain the plasmid. To eliminate the occurrence of conjugal transfer of pHC60 to other bacteria, sterile soil will be used for subsequent colonisation experiments.

A combination of very low numbers of rhizobia in the soil after 7 d, and the relatively insensitive method used to enumerate them, may have contributed to failure to detect *S. medicae* cells in soil of pH$_{CaCl_2}$ 4.3. It is unlikely that there were no cells of *S. medicae* in the soil. Using the more sensitive plant infection test (Brockwell, 1982), very low numbers of rhizobia (as low as 5.02 rhizobia g$^{-1}$ soil) could be detected in soil of pH$_{CaCl_2}$ 4.3 after 7 d, despite 100-fold less rhizobia initially inoculated (Chapter 2). Furthermore, the fact that plants grown in soil of pH$_{CaCl_2}$ 4.3 formed nodules with all strains of *S. medicae* (experiment 5.3) confirms that cells of both YC2 and YC8 survived in acidic soil in a plant rhizosphere.

Generally, there was no significant difference between the two transconjugants YC2 and YC8. Since the recovery rate of YC8 from soil of pH$_{CaCl_2}$ 7.0 was higher than YC2, YC8 was selected for use in subsequent colonisation studies with roots of *M. murex* and *M. sativa* growing in soil. It is envisaged that cells of YC8 will be visualised on *Medicago* roots by confocal laser scanning microscopy (CLSM). CLSM has been used to localise *R. etli* in the infection threads and cells of *P. vulgaris* (Xi et al., 2001), and to study *in situ* root colonisation of GFP-marked *Pseudomonas fluorescens* on soil-grown plants of *H. vulgare* (Normander et al., 1999), *L. esculentum* (Bloemberg...
et al., 2000) and *T. aestivum* (Unge & Jansson, 2001). As a light microscope, CLSM eliminates the dehydration problems associated with scanning electron microscopy and transmission electron microscopy. Furthermore, it is possible to make three-dimensional reconstructions of the recordings with minimum interference with autofluorescence (Assmus et al., 1995). Therefore, CLSM was considered suitable to assess *in situ* colonisation of *Medicago* roots by YC8 in an acidic soil.
CHAPTER 6. COLONISATION OF *M. murex* AND *M. sativa* ROOTS BY *S. medicae* IN AN ACIDIC SOIL

6.1 INTRODUCTION

Root colonisation may be defined as an active process which involves the increase in numbers of an introduced microorganism on or around roots, and not simply a passive chance encounter of a soil microorganism with a growing root (Kloepper & Beauchamp, 1992). Colonisation of legume roots by rhizobia is important because the establishment of the legume-rhizobia symbiosis is preceded by rhizobial attachment to roots and rhizobial growth in the rhizosphere. Although the data was equivocal, acid-tolerance in both host legume and rhizobia appears to improve rhizobial colonisation of the host roots in acidic soil (Barclay et al., 1994). Root colonisation by rhizobia is an important parameter to study as an indicator of nodulation potential under low pH stress.

As demonstrated in Chapter 2, *M. murex* produced fewer nodules in an acidic sandy soil of pH$_{CaCl_2}$ 4.3 compared to plants grown in soil of pH$_{CaCl_2}$ 7.0. The rate of nodulation by *M. murex* was not affected by acidity. In the same soil of pH$_{CaCl_2}$ 4.3, *M. sativa* formed fewer nodules than plants grown in soil of pH$_{CaCl_2}$ 7.0, and these nodules formed later compared to plants grown in soil of pH$_{CaCl_2}$ 7.0. Furthermore, in soil of pH$_{CaCl_2}$ 4.3, the uppermost nodule of both *M. murex* and *M. sativa* formed 4-5 cm below the hypocotyl, but this nodule appeared almost four weeks later on *M. sativa* than *M. murex*. As higher numbers of *S. medicae* were associated with the roots of *M. murex* than *M. sativa* in soil of low pH (Chapter 3), and the rhizosphere of *M. murex* was
less acidic than that of *M. sativa* (Chapter 4), it was hypothesised that *S. medicae* took longer to colonise the roots of *M. sativa* compared to *M. murex*. The subsequently smaller population of *S. medicae* on *M. sativa* roots resulted in delayed root hair infection, which consequently led to the observed delay in nodule appearance of this species (Chapter 2).

The experiments described in this chapter tested the hypothesis that root colonisation by *S. medicae* was slower for *M. sativa* than for *M. murex*. This hypothesis was tested in two parts. It was determined whether: (1) there were any differences in the density and location of root hairs on *M. murex* and *M. sativa* roots growing in soil of low pH, and (2) rhizobial colonisation could be related to any differential densities and location of root hairs between the two species. Depending on the density and location of root hairs, zones on the primary roots of *M. murex* and *M. sativa* could then be assessed for the temporal and spatial pattern of colonisation by *S. medicae*.

Root colonisation can be assessed using GFP-marked rhizobia. Thus, a green fluorescent protein (GFP)-marked transconjugant of *S. medicae* WSM419, YC8, was constructed (Chapter 5) for the study of *in situ* temporal and spatial colonisation of *Medicago* roots. An acidic soil was inoculated with YC8, whose fluorescence was used to determine whether differential rhizobial colonisation of the root zone 4-5 cm below the hypocotyl contributed to the delayed nodule appearance on *M. sativa* observed in Chapter 2. Fluorescence was measured at the zone 2-3 mm from the root tip to determine the extent of rhizobial colonisation at a root zone where root hairs are most susceptible to rhizobial infection (Bhuvaneswari et al., 1981).
6.2 MATERIALS AND METHODS

6.2.1 Experiment 6.1. Density and location of root hairs on *M. murex* and *M. sativa*

6.2.1.1 Experimental design

Experiment 6.1 was a split-plot design with two replications. *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius were grown in soil of pH$_{\text{CaCl}_2}$ 4.3 and 7.0. At 3, 7 and 9 d after sowing, the length of the primary root of each plant, and the distance from the root tip to the youngest visible root hair of the primary root was measured. The numbers of root hairs mm$^{-1}$ root were determined by counting root hairs at three zones along the primary root: (1) where the youngest root hairs were located near the root tip; (2) mid-length along the root; and (3) where the oldest root hairs were located at the more mature region of the root.

6.2.1.2 Plants and soil

Seeds of *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius were surface sterilised as described in Section 4.2.1.2. Seeds were left to imbibe overnight in the last change of water. The following day, seeds had fully imbibed and radicles were approximately 0.2 cm long.

The soil used was that described in Section 2.2.2 and Table 2.1. A total of 200 g of soil of pH$_{\text{CaCl}_2}$ 4.3 or pH$_{\text{CaCl}_2}$ 7.0 was placed in undrained containers (6.5 cm diameter, 8 cm height), and wet to field capacity (10% w/v) with
deionised water. Eight plants each of *M. murex* and *M. sativa* were sown into each container and all containers were then placed in an air-conditioned glasshouse maintained at 22 °C. Field capacity of the soil was maintained throughout the experiment by weighing each container daily and adding the required volume of deionised water.

6.2.1.3 Measuring root hair density and location

At 2, 3 and 9 d after sowing, plants in two replicates were carefully removed from the soil using a small spatula to avoid damage to root hairs. Roots were then gently rinsed in two changes of deionised water (10 s each) to remove excess soil. The length of the primary root of each seedling was measured, roots mounted in deionised water on a glass slide and covered with a glass slip. Each root was then examined under a dissecting microscope (Olympus SZX12, Olympus Optical Co. Ltd, Tokyo, Japan) to measure the distance from the root tip to the first visible root hair nearest to the root tip. The numbers of root hairs mm⁻¹ root were recorded at three zones along the primary root axis: (1) zone S1: from the first visible root hair from the root tip, for 1 mm up the root towards the middle of the root, (2) zone S2: mid-distance along the primary root, for 1 mm up the root towards the hypocotyl, and (3) zone S3: from the first visible root hair from the hypocotyl, for 1 mm down the root towards the middle of the root. Location of the youngest root hair in relation to the length of the primary root was expressed as a ratio (Location Ratio) of the distance from the root tip to the youngest root hair to length of the primary root to give an indication of where the youngest root hair was located in relation to the length of the primary root. Relative root elongation rate (RRER; (mm cm⁻¹ root) d⁻¹) was calculated by
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the equation:

\[
RRER = \frac{(L_2 - L_1)}{L_1 \times (t_2 - t_1)}
\]

where \(L_1\) and \(L_2\) are the initial and final root length at time \(t_1\) and \(t_2\), respectively.

6.2.2 Experiment 6.2. Colonisation by YC8 at mature root zones of \(M. murex\) and \(M. sativa\)

6.2.2.1 Experimental design

Experiment 6.2 was a split-plot design with four replications. \(M. murex\) cv. Zodiac and \(M. sativa\) cv. Aquarius were sown into soil of pH\(\text{CaCl}_2\) 4.3 inoculated with YC8. At 5 d after sowing, the length of the primary root from the hypocotyl to the root tip of each plant was measured. Pixel intensity units (PIU), an indicator of the intensity of fluorescence from root images, were measured at 2-3 mm from the root tip with a confocal laser scanning microscope (CLSM). At 7 d after sowing, PIU was measured at a zone on the root the same distance from the hypocotyl that was the root length on 5 d. Plants grown in soil not inoculated with YC8 were used to detect for autofluorescence from the roots and soil.

6.2.2.2 Soil, rhizobia and plants

The soil used was that described in Section 2.2.2 and Table 2.1. The soil was moistened to field capacity (10% w/v) with deionised water. A total of 25 g of slightly moistened soil of pH\(\text{CaCl}_2\) 4.3 was placed in screw-top containers (2.5 cm diameter, 8 cm height) and sterilised at 121 °C for 20 min.

Transconjugant of \(S. medicae\) WSM419, YC8, was grown in TY medium
containing \( \text{Cm}_{40}\text{Tc}_{20} \) (40 \( \mu \text{g mL}^{-1} \) chloramphenicol, 20 \( \mu \text{g mL}^{-1} \) tetracycline), described in Section 5.2.1. Two cultures (50 mL each) of YC8 were grown overnight and combined immediately before inoculation to soil. A suspension of the combined cultures of YC8 was then prepared by diluting with sterile deionised water. Numbers of rhizobia in the suspension was quantified by the Miles and Misra drop-plate method (Section 2.2.3) using TY agar medium containing \( \text{Cm}_{40}\text{Tc}_{20} \) (Section 5.2.1). A total of 2.5 mL of the diluted suspension was added to each container to give approximately \( 3.95 \times 10^7 \) rhizobia g\(^{-1}\) soil, and 2.5 mL of sterile deionised water was added to each container in the uninoculated treatment.

Seeds of *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius were surface sterilised and germinated as described in Section 2.2.4, except seeds were treated in 4.5% (v/v) \( \text{H}_2\text{O}_2 \) for 2 min after imbibing overnight in sterile deionised water. One seedling each of *M. murex* and *M. sativa* were sown into the soil of each container, with lids loosely screwed to allow for air exchange. All containers were then placed in an air-conditioned glasshouse maintained at 22 °C.

6.2.2.3 Measurement of fluorescence from mature root zones

Levels of fluorescence to determine the numbers of YC8 on the root of each plant were measured *in situ* with a Bio-Rad MRC-1000 confocal laser scanning microscope (CLSM) equipped with an argon/krypton ion laser (excitation wavelengths 488, 568 and 647 nm). Plants were gently removed from soil at 5 and 7 d after sowing with a small spatula to avoid breaking or damaging roots. Each plant was cut at the hypocotyl with small scissors, roots rinsed in
sterile phosphate buffered saline (PBS) solution (Section 3.2.1.5) for 1 min, and mounted in PBS solution on a glass slide. Filters were used to discriminate between fluorescence emitted by YC8 and autofluorescence of the root and soil. An excitation wavelength of 488 nm, and filters to emit wavelengths 506-538 nm were used. Images were obtained by averaging five scans from 0-10 μm from the root surface with a Kalman collection filter (Zstep of 2 μm) at ×4 magnification. PIU of root images were used to give an indication of the population size, and hence the extent of colonisation, on the root by YC8. PIU were measured by making transects perpendicular to the root axis at various distances along the primary root.

As *M. murex* and *M. sativa* had formed the uppermost nodules approximately 4-5 cm below the hypocotyl in soil of pH$_{\text{CaCl}}_2$ 4.3 (Chapter 2), PIU transects were made at this root zone at 5 and 7 d after sowing. This was done by measuring the length of primary roots at 5 d after sowing when the average root lengths were 46 mm for *M. murex*, and 47 mm for *M. sativa* (Figure 6.1). At 7 d after sowing, PIU transects were made at 200 μm intervals at 3646 mm (*M. murex*) and 37-47 mm (*M. sativa*) below the hypocotyl (Figure 6.1). The roots of both *Medicago* spp. grew poorly, and by 7d, some plants of *M. sativa* had root lengths less than 47 mm. This was probably due to poor light rather than a decrease in soil pH (uninoculated soil had pH$_{\text{CaCl}}_2$ 4.41 and inoculated soil had pH$_{\text{CaCl}}_2$ 4.52, measured at 7 d after sowing). Since the roots of both species grew rather slowly, comparisons could still be made between the two species. For plants of *M. sativa* that had roots shorter than 47 mm, PIU transects were made at 200 μm intervals at 23 mm from the root tip.
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<table>
<thead>
<tr>
<th>Days after sowing</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>46 mm (M. murex)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47 mm (M. sativa)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Location of PIU measurements on primary root

Figure 6.1. Location of pixel intensity unit (PIU) measurements on the primary roots of *M. murex* and *M. sativa* grown in soil of pH CaCl₂ 4.3 inoculated with YC8, transconjugant of *S. medicae* WSM419 by confocal scanning laser microscopy. Not to scale. Experiment 6.2.

All image processing and extraction was done with Confocal Assistant 4.2 software package (© Todd Clark Brelje), and the average PIU for each root zone were determined after transferring confocal microscope data files to Microsoft Excel 2000 (Microsoft Corporation, Washington, USA).

6.2.3 Experiment 6.3. Colonisation by YC8 at young root zones of *M. murex* and *M. sativa*

6.2.3.1 Experimental design

Experiment 6.3 was a split-plot design with four replications. *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius were sown into soil of pH CaCl₂ 4.3 inoculated with YC8. At 2, 3 and 5 d after sowing, PIU was measured at 2-3 mm from the root tip with a CLSM.
6.2.3.2 Soil, rhizobia and plants

The soil used was that described in Section 2.2.2 and Table 2.1. The soil was moistened to field capacity (10% w/v) with deionised water. A total of 20 g of moistened soil of pH \( \text{CaCl}_2 \) 4.3 or pH 7.0 was placed in screw-top containers (2.5 cm diameter, 8 cm height) and sterilised at 121 °C for 20 min.

Cultures of transconjugant of \( S. \text{medicae} \) WSM419, YC8, were grown, combined, suspension diluted and the numbers of rhizobia in the suspension enumerated as described in Section 6.2.2.2. Immediately before inoculation to soil, 2 mL of the diluted suspension was added to each container to give approximately \( 6.78 \times 10^8 \) rhizobia g\(^{-1}\) soil.

Seeds of \( M. \text{murex} \) and \( M. \text{sativa} \) were surface sterilised, germinated and sown as described in Section 6.2.2.2.

6.2.3.3 Measurement of fluorescence from young root zones

At 2, 3 and 5 d after sowing, plants of \( M. \text{murex} \) and \( M. \text{sativa} \) were carefully removed from soil, roots cut at the hypocotyl, rinsed and mounted in PBS solution as described in Section 6.2.2.3. PIU were measured by transects at 200 \( \mu \)m intervals at 2-3 mm from the root tip of each plant using CLSM. Image processing and extraction was done as described in Section 6.2.2.3.

6.2.4 Statistical analysis

For all experiments, variability in the measured values among the various replicates is indicated by standard deviations and standard errors. Significant differences between treatments were deduced from a \( t \)-test as described in
Section 3.2.3.

6.3 RESULTS

6.3.1 Experiment 6.1. Density and location of root hairs

In soil of pH_{CaCl$_2$} 4.3 between 3 and 9 d after sowing, there was no significant difference in the location of the youngest root hair in relation to the length of the primary root (Location Ratio) between *M. murex* and *M. sativa* (Table 6.1). For both species, the youngest root hair was located between 1-4 mm from the root tip.

In soil of pH_{CaCl$_2$} 7.0 at 3 and 7 d after sowing, there was no significant difference in the Location Ratio between the two species. However at 9 d after sowing, the youngest root hair on *M. sativa* was 3-times further from the root tip compared to *M. murex*. RRER was approximately 2-times higher in *M. sativa* than *M. murex* (Table 6.1).
Table 6.1. Length of primary root, distance from root tip to youngest root hair (mean ± standard deviation) of *M. murex* and *M. sativa* grown in soil of pH$_{\text{CaCl}_2}$ 4.3 and 7.0 at 3, 7 and 9 d after sowing, and their relative root elongation rate (RRER; (mm cm$^{-1}$ root d$^{-1}$)) between 3 and 9 d. Experiment 6.1.

<table>
<thead>
<tr>
<th>Soil pH (0.01 M CaCl$_2$)</th>
<th>Days after sowing</th>
<th>Length of primary root (mm; A)</th>
<th>Distance from root tip to youngest root hair (mm; B)</th>
<th>Location Ratio (B/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>M. murex</em></td>
<td><em>M. sativa</em></td>
<td><em>M. murex</em></td>
</tr>
<tr>
<td>4.3</td>
<td>3</td>
<td>46 ± 5</td>
<td>26 ± 14</td>
<td>4 ± 1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>74 ± 7</td>
<td>74 ± 16</td>
<td>3 ± 1</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>80 ± 7</td>
<td>98 ± 29</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>RRER</td>
<td>0.13</td>
<td>0.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>3</td>
<td>39 ± 6</td>
<td>24 ± 13</td>
<td>5 ± 1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>73 ± 6</td>
<td>70 ± 20</td>
<td>3 ± 1</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>76 ± 8</td>
<td>85 ± 16</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>RRER</td>
<td>0.16</td>
<td>0.41</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference in Location Ratio between *M. murex* and *M. sativa* at *P* < 0.05.

In soil of pH$_{\text{CaCl}_2}$ 4.3 at 3 d after sowing, there was no significant difference between *M. murex* and *M. sativa* in the density of root hairs at the three zones of the primary root where root hair density were measured (Figure 6.2). At 7 and 9 d after sowing, there were approximately 0.6-fold more root hairs mm$^{-1}$ root at all three zones along the primary root of *M. murex* compared to *M. sativa*. In soil of pH$_{\text{CaCl}_2}$ 7.0 at 3 and 7 d after sowing, there was no difference in the densities of root hairs at zones S1 and S3 between *M. murex* and *M. sativa* (Figure 6.2). However at mid-length along the primary root, *M. murex* had approximately 0.7-fold more root hairs mm$^{-1}$ root than *M. sativa*. At 9 d after sowing, there were significantly more root hairs mm$^{-1}$ root on *M. murex* compared to *M. sativa* at zones S1 and S3. At mid-length along the root (38-42 mm from the root tip), there was no difference between *M. murex*
and *M. sativa* as both species had approximately 49-52 root hairs mm\(^{-1}\) root. Roots of both *M. murex* and *M. sativa* grown in soil of pH\(_{\text{CaCl}_2}\) 7.0 generally had more root hairs mm\(^{-1}\) root than roots of both species grown in soil of pH\(_{\text{CaCl}_2}\) 4.3. Furthermore, *M. murex* consistently had similar or more root hairs mm\(^{-1}\) than *M. sativa* in soil of both pH\(_{\text{CaCl}_2}\) 4.3 and 7.0. The difference in root hair densities between *M. murex* and *M. sativa* was greater in soil of pH\(_{\text{CaCl}_2}\) 4.3 than soil of pH\(_{\text{CaCl}_2}\) 7.0 (Figure 6.2).
Figure 6.2. Number of root hairs (mm\(^{-1}\) root) on the primary root of *M. murex* and *M. sativa* grown in soil of pH\(_{\text{CaCl}_2}\) 4.3 and 7.0, at 3, 7 and 9 d after sowing. Numbers of root hairs measured at three zones along the primary root: S1 = from the first visible root hair from the root tip, for 1 mm up the root towards the middle of the root, S2 = mid-distance along the primary root, for 1 mm up the root towards the hypocotyl, and S3 = from the first visible root hair from the hypocotyl, for 1 mm down the root towards the middle of the root. Bars represent standard error. Experiment 6.1
6.3.2 Experiment 6.2. Colonisation of mature root zones by YC8

Fluorescence was emitted from roots of *M. murex* and *M. sativa* grown in soil without *S. medicae*, indicating root autofluorescence (Figure 6.3). As the intensity of fluorescence emitted from uninoculated roots was consistently lower than the intensity of fluorescence emitted from inoculated roots, autofluorescence was not considered to interfere with the detection of fluorescence emitted by GFP-marked YC8 cells colonised on roots. At 5 d after sowing, the intensity of fluorescence emitted by *M. sativa* roots colonised by YC8 was significantly (1.7-fold) higher than that emitted by *M. murex* roots. At 7 d after sowing, fluorescence emitted by the roots of *M. sativa* colonised by *S. medicae* decreased 1.2-fold to 8.74 PIU, while that of *M. murex* increased 1.6-fold to 9.76 PIU (Figure 6.3).
Figure 6.3. Intensity of fluorescence (pixel intensity units) emitted from roots of *M. murex* and *M. sativa* grown in soil of pHCaCl$_2$ 4.3 uninoculated or inoculated with YC8, a transconjugant of *S. medicae* WSM419. Intensity of fluorescence was measured at 36-46 mm (*M. murex*) and 37-47 mm (*M. sativa*) below the hypocotyl of the primary root. Bars represent standard error. Experiment 6.2.

If root autofluorescence was taken into consideration by substracting PIU values of roots without YC8 from the PIU values of roots with YC8, the intensity of fluorescence from roots was not significantly different between the two *Medicago* species at 5 d after sowing (Figure 6.4). However by 7 d after sowing, PIU of *M. murex* increased 3.2-fold to 3.28 PIU, while that of *M. sativa* decreased by 1 PIU to 1.7 PIU. By 7 d, the intensity of fluorescence emitted from *M. murex* roots colonised by YC8 was significantly higher (by 1.8-fold) than that emitted from *M. sativa*. 
Figure 6.4. Intensity of fluorescence (pixel intensity units) emitted from roots of *M. murex* (36-46 mm below the hypocotyl of the primary root), and *M. sativa* (36-47 mm below the hypocotyl of the primary root) grown in soil of pH$_{\text{CaCl}_2}$ 4.3 inoculated with YC8, a transconjugant of *S. medicae* WSM419. Values represent PIU values of roots without YC8 subtracted from the PIU values of roots with YC8, and takes into account differential levels of autofluorescence from *M. murex* and *M. sativa* roots. Bars represent standard error. Experiment 6.2.

Representative confocal images of *M. murex* and *M. sativa* roots grown in soil of pH$_{\text{CaCl}_2}$ 4.3 inoculated with YC8 are shown in Figure 6.5.
Figure 6.5. Confocal images of roots of (a) *M. murex* at 36-46 mm below the hypocotyl of the primary root, and (b) *M. sativa* at 37-47 mm below the hypocotyl of the primary root, grown in soil of pH$_{\text{CaCl}_2}$ 4.3 inoculated with YC8, a transconjunct of *S. medicae* WSM419. Images acquired 5 d after sowing. Bar, 267 μm. Experiment 6.2.

6.3.3 Experiment 6.3. Colonisation of young root zones by YC8

In soil of pH$_{\text{CaCl}_2}$ 4.3 at 2 d after sowing, there was a significantly higher intensity of fluorescence emitted from the roots of *M. sativa* than *M. murex* colonised by YC8 (Figure 6.6). At 3 d after sowing, the intensity of fluorescence from YC8 colonised on *M. murex* roots remained at the same level (approximately 7 PIU), but that on *M. sativa* roots decreased by 1 PIU to 8 PIU. By 5 d after sowing, fluorescence intensity from *M. murex* roots colonised by YC8 increased 1.5-fold, to approximately 11 PIU, while that from *M. sativa* increased only 1-fold to 9 PIU. At 5 d after sowing, YC8 on the roots of *M. murex* emitted a significantly higher intensity of fluorescence than those on the roots of *M. sativa* (Figure 6.6).

In soil of pH$_{\text{CaCl}_2}$ 7.0, the intensity of fluorescence emitted by YC8 on the roots of both species were similar to the levels of those emitted from roots grown in soil of pH$_{\text{CaCl}_2}$ 4.3 up to 3 d after sowing. At 5 d after sowing, roots of *M. sativa* colonised by YC8 emitted a significantly higher level of fluorescence...
(almost 2-times higher) than roots of *M. murex* (Figure 6.6). Fluorescence emitted by YC8 on roots grown in soil of pH\textsubscript{CaCl\textsubscript{2}} 7.0 was significantly higher than those emitted by YC8 on roots grown in soil of pH\textsubscript{CaCl\textsubscript{2}} 4.3.

![Graph showing intensity of fluorescence for *M. murex* and *M. sativa* roots grown in soil of pH\textsubscript{CaCl\textsubscript{2}} 4.3 or 7.0 inoculated with YC8, a transconjugant of *S. medicae* WSM419. Intensity of fluorescence was measured at 2-3 mm below the hypocotyl of the primary root. Bars represent standard error. Experiment 6.3.](image)

**Figure 6.6.** Intensity of fluorescence (pixel intensity units) emitted from roots of *M. murex* and *M. sativa* grown in soil of pH\textsubscript{CaCl\textsubscript{2}} 4.3 or 7.0 inoculated with YC8, a transconjugant of *S. medicae* WSM419. Intensity of fluorescence was measured at 2-3 mm below the hypocotyl of the primary root. Bars represent standard error. Experiment 6.3.

Representative confocal images of *M. murex* and *M. sativa* roots grown in soil of pH\textsubscript{CaCl\textsubscript{2}} 7.0 are shown in Figure 6.7.
Figure 6.7. Confocal images of roots of (a) *M. murex* and (b) *M. sativa* at 23 mm below the hypocotyl of the primary root, grown in soil of pH\textsubscript{CaCl\textsubscript{2}} 7.0 inoculated with YC8, a transconjugant of *S. medicae* WSM419. Images acquired 5 d after sowing. Bar, 267 \textmu m. Experiment 6.3.

6.4 DISCUSSION

6.4.1 Location and density of *Medicago* root hairs

In soil of pH\textsubscript{CaCl\textsubscript{2}} 4.3, the youngest root hairs developed at approximately the same relative positions on the primary roots of *M. murex* and *M. sativa*. The young root hairs located near the root tip are the most susceptible to infection (Bhuvaneswari et al., 1981) by *S. medicae*. The plants in this experiment were grown under similar conditions to those described in Chapter 2. Therefore, the roots of *M. murex* and *M. sativa* growing in acidic soil seem to have the same potential to be infected by rhizobia. As root hairs were present at the same relative locations on the primary roots of both *M. murex* and *M. sativa*, the 3-4 week delay in the appearance of nodules on *M. sativa* roots observed in Chapter 2 was due neither to an absence of root hairs, nor a differential location of the youngest root hair. Therefore, other factors relating to root physiology or rhizobial growth in the rhizosphere contributed to the delayed nodule formation.
appearance in *M. sativa* in acidic soil. In this experiment with soil-grown plants, acidity decreased the density of root hairs in both *Medicago* spp., but more so in *M. sativa* than *M. murex*. There has not been previous research on the effect of low pH on root hair growth on *M. murex*. However, a previous study showed that in solution culture of pH < 5.0, acidity did not inhibit the growth or production of root hairs in *M. sativa* (Munns, 1968). The differential effect of acidity on root hair production was probably due to the different rhizosphere effects between soil and solution media. In solution culture of pH 4.3 and 4.7, acidity adversely affected root hair formation in *T. repens* unless enough Ca (500 or 1000 µM) was supplied (Wood et al., 1984). How acidity affects root hair growth and development is unclear. Just as H⁺ and Ca can affect the cell wall integrity of rhizobial cells (Munns, 1978; Humphrey & Vincent, 1962), high concentrations of H⁺ may also adversely affect the cell wall of root epidermal cells that project into root hairs, and the effect of H⁺ on root hair production can be ameliorated by Ca (Wood et al., 1984).

Previous research correlating the location of root hairs to nodules is limited. Munns (1970) observed the distribution of nodules and root hairs on *M. sativa* roots grown in nutrient solution of pH 5.2 and 5.6 and found that root hair emergence near the root tip continued only if nodulation had been prevented by low Ca concentrations or low pH. It was suggested that further root hair emergence stopped due to the suppressive effect of prior nodulation rather than acidity (Munns, 1968), since acidity did not affect the growth or production of root hairs of *M. sativa* (Munns, 1968). However at the time of observation, plants were about 15 d old and nodulated, making it difficult to compare the findings of Munns (1970) with the results from this study where
much younger, non-nodulated plants were used. The lack of research in this field is rather surprising since the location of root hairs and nodule development are directly related in the many species of legumes that are infected by rhizobia via root hairs (Sprent, 2001).

Two possibilities may explain why the youngest root hair of *M. sativa* was 2-times further from the root tip compared to *M. murex* in soil of pH\(^{\text{CaCl}_2}\) 7.0: (1) a faster rate of root elongation in *M. sativa*, or (2) a slower rate of root hair development in *M. sativa*. Results from this study support the first possibility since the roots of *M. sativa* elongated 3-times faster than *M. murex*. A faster rate of root elongation in *M. sativa* was also observed in Chapter 4 (Section 4.3.1.1, Table 4.1). Hence, the longer roots of *M. sativa* resulted in the youngest root hair being located further away from the root tip relative to root length compared to *M. murex*. *M. murex* consistently had similar or greater density of root hairs than *M. sativa*. Root hairs increase the surface area of plant roots for the absorption of water and nutrients (Bailey, 1999) and for the release of exudates (Brimecombe et al., 2001), and would also affect the surface area on which rhizobia can attach and colonise the root. *M. murex*, with more root hairs, would certainly provide a larger root surface area for the attachment of *S. medicae*, and with root exudates as substrates, rhizobial growth resulted in the higher numbers of *S. medicae* associated with *M. murex* roots compared to *M. sativa* (Chapter 3).

Genotypic differences may explain the higher density of root hairs on *M. murex*. As an annual species, *M. murex* may develop more root hairs in order to explore the surface layers of soil and extract as much water and nutrients as possible before flowering and senescence. In contrast, the
perennial species *M. sativa* may invest more photosynthate in the development of the tap root for survival and growth year round.

### 6.4.2 Colonisation of *Medicago* roots by *S. medicae*

In soil of pH$_{\text{CaCl}_2}$ 4.3, there were initially similar densities of *S. medicae* on both the young and mature regions of *M. murex* and *M. sativa* roots. This was probably related to the initially similar root hair densities between *M. murex* and *M. sativa* at both the root tip and near the hypocotyl of the root. It appears that during the first 2-5 d after sowing, the similar root hair densities of *M. murex* and *M. sativa* enabled the roots of both species to have an equal potential to be colonised by *S. medicae*. However as roots elongated, the root hair density of *M. murex* increased relative to that of *M. sativa*, resulting in *M. murex* having a relatively larger root surface area, and increasing its potential to be colonised by higher numbers of *S. medicae*. Indeed by 5 d after sowing, a higher density of *S. medicae* was detected on the roots of *M. murex* compared to *M. sativa*. Although root hair density affects the root surface area which rhizobia colonises, root-induced chemical changes in the rhizosphere may also determine the numbers of rhizobia associated with roots. For example, the higher rates of proton extrusion from the roots of *M. sativa* (Chapter 4) would certainly create a less favourable environment for the growth and colonisation of *S. medicae* compared to *M. murex*.

In soil of pH$_{\text{CaCl}_2}$ 7.0, the density of *S. medicae* on roots appeared unrelated to the root hair density, as despite having fewer root hairs, a higher density of *S. medicae* were detected on the roots of *M. sativa* compared to *M. murex*. The less acidic rhizosphere pH of *M. murex* compared to
*M. sativa* (Chapter 4) was probably more favourable for the colonisation of *S. medicae* and contributed to the significantly higher density of *S. medicae* on the roots of *M. sativa* compared to the roots of *M. murex* at 5 d after sowing.
CHAPTER 7. GENERAL DISCUSSION

Previous research identified three early acid-sensitive steps in legume nodulation: (1) the poor survival (Rice et al., 1977) and (2) poor growth of rhizobia in acidic soils (Carter et al., 1995; Evans et al., 1993; Pijnenborg et al., 1990a; Hartel & Alexander, 1983; Robson & Loneragan, 1970c), and (3) the inhibition of root hair curling by low pH (Wood et al., 1984; Franco & Munns, 1982; Munns, 1970; Lie, 1969; Munns, 1968). The connection between rhizobial growth and root hair curling at low pH is not known although it has been established that non-growing rhizobia do not attach to host roots (Lodeiro & Favelukes, 1999; Wisniewski & Delmotte, 1996; Lodeiro et al., 1995), and the attachment of rhizobia to host roots and Nod factor production are both required for root hair curling (Lhuissier et al., 2001). Although previous research has provided some explanation of the causes for decreased nodulation at low pH, there has not been an investigation of how plant mechanisms contribute to these causes.

The research in this thesis investigated plant mechanisms contributing to decreased nodulation of *M. sativa* at low pH, and was successful in meeting the aims of the thesis. In soil of pH$_{\text{CaCl}_2}$ 4.3, *M. murex* had higher numbers of *S. medicae* associated with its roots and nodulated earlier compared to *M. sativa*. Based on the results described in this thesis, two plant mechanisms were identified by which greater numbers of *S. medicae* were found in the *M. murex* rhizosphere compared to the *M. sativa* rhizosphere when plants were grown in an acidic soil. The first mechanism was physical: roots of 7 d-old *M. murex* growing in soil of pH$_{\text{CaCl}_2}$ 4.3 had a higher density of root hairs, and
thus provided a larger root surface area for the colonisation by \textit{S. medicae} compared to the roots of \textit{M. sativa}. The second mechanism was chemical: roots of \textit{M. murex} acidified their rhizosphere less when grown in acidic soil, and thus provided more favourable conditions for colonisation by \textit{S. medicae}. Models, based on the data in this thesis, providing a schematic view of the nodulation of \textit{M. murex} and \textit{M. sativa} with \textit{S. medicae} in soil of pH_{CaCl_2} 4.3 and 7.0 are presented in the following sections.

\textbf{7.1 THE ACID-STRESS MODEL}

The similar densities of root hairs at 1-2 mm above the root tip of \textit{M. murex} and \textit{M. sativa} at 3 d after sowing (Figure 7.1A) provided a similar root surface area for the attachment of \textit{S. medicae}. By 5 d after sowing, a higher density of \textit{S. medicae} was detected on the roots of \textit{M. murex} compared to \textit{M. sativa} (Figure 7.1B, C). However, from 7 d after sowing, changes occurred in the \textit{M. murex} root which, from this point, allowed this species to nodulate earlier in soil of low pH. One important feature distinguishing the two \textit{Medicago} spp. is their difference in root hair density. At 7 d after sowing, an increase in the density of root hairs on \textit{M. murex} provided the root surface area to support increasing numbers of \textit{S. medicae}, as measured on the root region 4-5 cm below the hypocotyl (Figure 7.1D). Infection of the \textit{M. murex} root by \textit{S. medicae} occurred prior to 7 d since nodule initials were seen 7 d after sowing (Figure 7.1D). In contrast, a decrease in the root hair density of \textit{M. sativa} (Figure 7.1E) limited the root surface area available for the colonisation by \textit{S. medicae}, which resulted in a decrease in the density of \textit{S. medicae} on the roots of \textit{M. sativa} (Figure 7.1E). Due to lower numbers of rhizobia, infection of the \textit{M. sativa}
root by *S. medicae* did not occur at this time. By 13 d after sowing, *M. murex* had formed a nodule at 4-5 cm below the hypocotyl (Figure 7.1F). Although rhizosphere pH values were not measured until 13 d after sowing (using the ‘root mat’ approach, see Chapter 4), differences in root-induced rhizosphere changes between *M. murex* and *M. sativa* could have contributed to the differential rhizobial colonisation of the rhizosphere before 13 d. At 13 d, the less acidic rhizosphere of *M. murex* (pH 4.51; Figure 7.1G) provided more favourable conditions for the growth and colonisation of *S. medicae*, and hence more rapid rhizobial infection of *M. murex*, compared to the more acidic rhizosphere of *M. sativa* (pH 4.14; Figure 7.1H). Nodulation of *M. sativa* was delayed because with lower numbers of *S. medicae* and a more acidic rhizosphere, rhizobia infected the root of *M. sativa* sometime after 13 d because nodule initials were not seen on this species at 13 d (Figure 7.1I). *M. sativa* was nodulated when plants were harvested at 41 d (Figure 7.1J).

The time-sequence of the nodulation events described in the acid-stress model is plausible given that in previous studies, nodules were seen on *M. murex* grown in soil of pH<sub>CaCl</sub><sub>2</sub> 4.9 when plants were harvested 56 d (8 weeks) after sowing (Howieson & Ewing, 1989). For *M. sativa*, the slower rate of nodule appearance described in the acid-stress model was probably due to the more acidic soil and smaller inoculum of *S. medicae* used in this study compared to previous work where nodules were present on *M. sativa* at 12 d (Pijnenborg et al., 1990b) and 14 d (Pijnenborg & Lie, 1990; Pijnenborg et al., 1990c) after sowing inoculated seed into soil of pH<sub>H</sub><sub>2</sub> 5.2.
Figure 7.1. Model presenting the nodulation events of *M. murex* and *M. sativa* with *S. medicae* in soil of pH$_{CaCl_2}$ 4.3. Underlined text are findings from this thesis. The days on which measurements and observations were made provide a sequence of events over time and does not imply that the events measured and observed occurred on those precise days. RH = root hairs; PIU = pixel intensity units, an estimate of rhizobial population size (see Chapter 6).
7.2 **The Neutral-pH Model**

Since rhizobia were detected at the root tip of both *M. murex* and *M. sativa* at 2 d after sowing into soil of pH$_{CaCl_2}$ 7.0, *S. medicae* would have been associated with the root hairs at this region of the root for both *Medicago* spp. between 0 and 2 d after sowing, when roots were approximately 0.2-0.3 cm long (Figure 7.2A). At 2 d after sowing, a similar root surface area was provided by the almost identical root hair density on *M. murex* (22 root hairs mm$^{-1}$ root; Figure 7.2B) and *M. sativa* (20 root hairs mm$^{-1}$ root; Figure 7.2C) at 1-2 mm above the root tip. Similar numbers of *S. medicae* were also detected at 2-3 mm above the root tip of both species (Figure 7.2B, C) at this time. At 5 d after sowing when roots were approximately 4-5 cm long, the numbers of *S. medicae* on the roots of both species increased (Figure 7.2D, F). However, this is where the similarity between *M. murex* and *M. sativa* stops. This increase in rhizobial numbers resulted in a larger population of rhizobia on the root of *M. sativa* (Figure 7.2F) compared to *M. murex* (Figure 7.2D). Since both species had a similar density of root hairs at 5 d after sowing, rhizosphere pH probably contributed to this difference in numbers: the rhizosphere pH of *M. sativa* was more favourable for the growth of *S. medicae* compared to the rhizosphere of *M. murex*. Indeed by 13 d after sowing, the rhizosphere of *M. sativa* was almost at neutral-pH (pH 6.78; Figure 7.2J) compared to the more acidic rhizosphere of *M. murex* (pH 5.95; Figure 7.2H). This difference of approximately 0.8 pH-unit will significantly affect on the growth rate of *S. medicae* in the rhizospheres of *M. murex* and *M. sativa*. Sometime before 7 d, *S. medicae* infected the roots of *M. murex* and *M. sativa* since nodule initials were seen on both species at 7 d (Figure 7.2)
D, F). Concurrently, numbers of *S. medicae* increased at 0.2-0.3 cm below the hypocotyl of the *M. murex* root (Figure 7.2E), and between 5 and 13 d after sowing infected the root of *M. murex* because a nodule was seen 0.2-0.3 cm below the hypocotyl 13 d after sowing (Figure 7.2G). However, rhizobial infection of the *M. sativa* root at this time did not result in the formation of nodules as none were present at 13 d.

It is difficult to explain why despite having higher numbers of rhizobia and a more favourable rhizosphere pH, *M. sativa* still nodulated later than *M. murex* in soil of pH\(_{\text{CaCl}_2}\) 7.0. Differential patterns of root-induced pH changes along the root axis cannot explain this difference. Cortical cell division in the root may explain the delay in the appearance of nodules on *M. sativa* until sometime after 13 d (Figure 7.2I), despite the presence of nodule initials at 7 d (Figure 7.2F). Compared to *M. murex*, cortical cells near the root tip of *M. sativa* may respond differently to rhizobial Nod factors by dividing at a slower rate. Consequently, *M. sativa* were not nodulated until plants were harvested at 19 d, when a nodule was seen at 4-5 cm below the hypocotyl on the root (Figure 7.2K).

By limiting root-induced rhizosphere acidification and producing more root hairs, it appears that *M. murex* has evolved adaptations for rapid nodulation that suit it for growth in the acidic and relatively infertile soils, with low N, in which it is found (Francis & Gillespie, 1981). Similarly, in soils of neutral-pH, *M. murex* also nodulates rapidly because its relationship with *S. medicae* has evolved to establish symbiosis quickly. In contrast, *M. sativa* has evolved in less acidic, more fertile soils (Lesins & Lesins, 1979), and because of the availability of soil N, does not need to establish symbiosis as rapidly as *M. murex*. In addition, difference in the life cycle may explain the different nodulation
responses between the two species: the perennial species *M. sativa* possibly invests more photosynthate in tap root development and less in root hair development to survive year round, while the annual species *M. murex* invests more photosynthate in the development of more root hairs in order to explore surface soil layers and extract water and nutrients during its shorter life cycle. Such difference in root architecture between *M. murex* and *M. sativa* may explain the delay in infection and nodule development in *M. sativa* in soil of pH\(_{\text{CaCl}_2}\) 7.0.

The results from this thesis indicate that differences in root architecture between *M. murex* and *M. sativa* may also affect the ability of the two species to nodulate in soils of low pH. Compared to *M. murex*, the faster rate of root growth and the more extensive tap root of *M. sativa* help its survival in relatively arid environments. However, these features of the *M. sativa* root compromise the ability of this species to nodulate effectively in acidic soil. The higher rate of H\(^+\) extrusion from *M. sativa* roots compared to *M. murex*, (which may benefit *M. sativa* by solubilising P), but further compromises the nodulation of *M. sativa* in acidic soils. In contrast, roots of *M. murex* develop a more lateral root system with a higher density of root hairs and exude less H\(^+\) when grown in acidic soils. Collectively, these features of the *M. murex* root enable this species to nodulate efficiently in acidic soils, and contribute to its symbiotic competency under acidic conditions.
Figure 7.2. Model presenting the nodulation events of *M. murex* and *M. sativa* with *S. medicae* in soil of pH_{CaCl_2} 7.0. Underlined text are findings from this thesis. The days on which measurements and observations were made provide a sequence of events over time and does not imply that the events measured and observed occurred on those precise days. RH = root hairs; PIU = pixel intensity units, an estimate of rhizobial population size (see Chapter 6).
7.3 Use of GFP-marked *S. medicae* and CLSM in Root Colonisation Studies

The colonisation of *S. medicae* on *Medicago* roots was successfully measured by the use of a GFP-marked *S. medicae* and CLSM in the experiments described in Chapter 6. However, there were two disadvantages associated with this technique. Firstly, the thickness of the root at the root zone of interest in 7 d-old plants limited the use of magnifications greater than ×4. Secondly, low magnification (×4) limited the detection of small populations of GFP-marked *S. medicae* on *Medicago* roots, especially those on roots in soil of pH\(\text{CaCl}_2\) 4.3. These problems may be overcome by sectioning root zones of interest and counting bacteria attached to root hairs (e.g. Prayitno et al., 1999). Autofluorescence from the root epidermal cells of *M. murex* and *M. sativa* was useful for orientation around the root surface (Hansen et al., 1997). It appears that the experiments reported in Chapter 6 were the first to use CLSM to measure root colonisation by a GFP-marked rhizobia in an acidic soil. In previous root colonisation studies using GFP-marked microorganisms and CLSM (e.g. Xi et al., 2001), the GFP-marked microorganisms were relatively easy to detect due to their high density as experiments were done using plants grown without acid stress. In contrast, unless the YC8 cells were densely localised and the GFP highly expressed on and around roots, as was the case with roots grown in soil of pH\(\text{CaCl}_2\) 7.0, the fluorescence signal from GFP was low and obscured by endogenous cellular fluorescence (Billinton & Knight, 2001).

If YC8 is to be used for further studies, its growth rate needs to be considered. Growth rate has a direct impact on GFP content – the faster
cells divide, the faster GFP is diluted in newly divided cells (Leveau & Lindow, 2001). In complex environments, such as soil, where rhizobial growth is different from one location to another, microsites that permit faster growth will contain dimmer bacteria so that GFP content is underestimated (Leveau & Lindow, 2001). In contrast, cells growing slower will have comparatively higher GFP levels that might be misinterpreted as greater cell density (Leveau & Lindow, 2001).

### 7.4 Future Research

With a more complete understanding of the mechanisms contributing to the poor nodulation of *M. sativa* in acidic soils it may now well be possible to improve the nodulation of *M. sativa* by developing genotypes of *M. sativa* with root systems more favourable for the growth and colonisation of *S. medicae*. It will be instructive to determine whether the differences in root hair density and root-induced rhizosphere pH changes observed between *M. murex* cv. Zodiac and *M. sativa* cv. Aquarius are consistently found in other genotypes of *M. murex* and *M. sativa* grown at low pH.

In additional to selecting and breeding genotypes of *M. sativa* with more root hairs by traditional plant breeding methods, genes such as those identified in *A. thaliana* for root hair initiation (Schneider et al., 1998) and root hair development (Shi & Zhu, 2002; Grierson et al., 2001) might similarly be identified in *M. murex* and *M. sativa*, with the aim of developing genotypes of *M. sativa* with higher densities of root hairs. Recent technology in functional gene analysis allows the identification of many genes and the different constituents of a cell (e.g. transcripts, proteins and metabolites) that help to
deduce gene function (Holtorf et al., 2002). For *M. murex* and *M. sativa*, it will be useful to extract the proteins coded by the genes responsible for root hair initiation and development (see review by Jacobs et al., 2000), separate them by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), identify them by peptide fingerprinting by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) (Jacobs et al., 2000), and measure the quantity of each protein (Thiellement et al., 1999). Similarly, this technology may be used to identify the genes responsible for proton extrusion in *M. murex* and *M. sativa* roots, with the aim of developing *M. sativa* roots that exude less protons when grown in acidic soils.

Differential root hair development and root-induced rhizosphere pH changes are two mechanisms contributing to the different nodulation response between *M. murex* and *M. sativa* in acidic soil. If the roots of *M. sativa* are modified to make them more favourable for the growth and colonisation of *S. medicae*, it will be an important step in improving the nodulation of this species in acidic soils, enabling the widespread use of *M. sativa* for the control of groundwater recharge in south-western Australia.
REFERENCES


**Watkin ELJ, O'Hara GW, Glenn AR. 1997.** Calcium and acid stress interact to affect the growth of *Rhizobium leguminosarum* bv. *trifolii*. *Soil Biology and Biochemistry* 29: 1427-1432.


