ECOLOGICAL AND MOLECULAR STUDIES ON RHIZOBIAL RHIZOPINES

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TABLE OF CONTENTS

SUMMARY

The aims of this project were to investigate the role of rhizopines in rhizobial competition for nodulation, and to isolate the rhizopine synthesis (*mos*) genes in *Rhizobium leguminosarum* bv. *viciae*.

Rhizopines are simple sugar-like molecules which are synthesized and catabolized by approximately 10% of the strains of *R. leguminosarum* bv. *viciae* and *Sinorhizobium meliloti*. Two different rhizopines have been discovered, L-3-*O*-methyl-*scyllo*-inosamine (3-*O*-MSI) and *scyllo*-inosamine (SI). Rhizopines influence rhizobial competition for nodulation, but the mechanism by which this occurs, and the precise role which rhizopines play in the *Rhizobium*-legume symbiosis, are not fully understood.

Competition data presented in this thesis extend previous findings. *S. meliloti* L5-30, a rhizopine producing and synthesizing strain, was used in the competition experiments. It was shown that over a period of 10 months, a mutant of L5-30 unable to catabolize rhizopine (Moc$^-$) was at a competitive disadvantage in nodulating a compatible host plant compared to the wild-type (Gordon *et al.*, 1996). In this thesis, results are presented which extend findings from 10 months to four years, in environmental conditions which were suboptimal for both the plant and the bacteria. These conditions included water stress and nutrient deficiency. A steady state of competitiveness was achieved soon after inoculation, in which the catabolizing strain occupied more than 70% of the nodules. The proportion of nodules occupied by this strain remained constant throughout the four year period. These results suggested that even in a harsh and nutrient-poor environment, rhizopine is not a specific growth substrate.

When L5-30 was competed against a mutant defective for rhizopine synthesis (Mos$^-$), or a neutral mutant, it did not receive a similar nodulation advantage. The competitive benefit

was specific for ability to catabolize rhizopine. Nodule occupancy by L5-30 in these treatments was initially 50% but declined gradually to approximately 30% by the end of the first year. There was no further decline in the following years.

An individual nodulation study revealed that the Moc^- mutant does not have a reduced ability to nodulate a compatible host plant. The poor nodulation is a characteristic which only appeared in direct competition with a Moc^+ Mos^+ strain. This result justified an important underlying assumption of the competition experiments, that the Moc^- mutant is deficient only in nodulation competitiveness and does not have a reduced ability to nodulate a compatible host plant in the absence of a rhizopine-synthesizing and catabolizing competitor.

The competition experiments demonstrated that rhizopines exert a very rapid effect, influencing the initial nodulation dynamics. This implied firstly that rhizopines are not a growth substrate, and secondly that they must be produced at an extremely early stage in development. Previous genetic studies indicated that the *mos* genes are under symbiotic regulation, and therefore rhizopines would only be produced by bacteroids in nodules. However, it was shown here using gas chromatography-mass spectrometry (GC-MS) that 3-*O*-MSI could be detected in four day old lucerne plants which had been inoculated with *S. meliloti* strain L5-30. This was before any effective nodules were visible on the roots. Rhizopine synthesis remained at an extremely low level from four days until approximately 18 days, when there was a massive induction of synthesis. This indicated symbiotic regulation of *mos* by the common regulators NifA and NtrA. There is likely to be a low level of micro-aerobic expression of the *mos* genes, possibly in free-living rhizobia on the root surface or in the infection thread, prior to symbiotic induction of these genes in the bacteroids. Synthesis at such an early stage may explain the competition phenomenon, and suggests that rhizopines may perhaps influence intra-specific competition for nodulation by exerting a direct effect on an early event in the nodulation process.

The rhizopine synthesis genes have been sequenced in two strains of *S. meliloti*, however, those of *R. leguminosarum* bv. *viciae* have not yet been isolated. The final aim of this project was to locate and characterize the *mos* genes in strain 1a. Transconjugant strains were used to show that they are located on the Symbiotic (Sym) plasmid. When the native Sym plasmid of some non-rhizopine strains was replaced with the 1a Sym plasmid, these strains gained the ability to direct rhizopine synthesis in root nodules. All pSym1a transconjugants, with the exception of P342(pSym1a::Tn*5*-*mob*), formed an effective symbiosis. The ineffective nodules produced by P342(pSym1a::Tn*5*-*mob*) also contained some 3-*O*-M*S*I; however, they contained a smaller quantity of 3-*O*-M*S*I than transconjugants which nodulated effectively.

Subsequent work directed at isolating the *R. leguminsarum mos* genes involved two approaches. Cosmid clones and R-primes were made from the 1a Sym plasmid. These clones were transferred to several non-rhizopine strains of *R. leguminosarum* bv. *viciae*, and one non-rhizopine strain of *S. meliloti.* Screening was accomplished by analyzing for the phenotype of rhizopine synthesis in root nodules which had been produced by transconjugant rhizobia containing cosmids or R-prime plasmids.

Ten R-prime plasmids were examined, but none was able to direct the synthesis of rhizopine in root nodules after transfer to several different non-rhizopine strains of *Rhizobium.*

Four cosmid clones were found to synthesize a compound in their root nodules, which stained with silver nitrate, and migrated in the same direction as rhizopine in high voltage paper electrophoresis (HVPE). One of these, pKH104, was confirmed by GC-MS analysis to produce the rhizopine 3-*O*-M*S*I. Rhizobia containing pKH104 synthesized less rhizopine than the wild-type rhizopine-producing strain, 1a, indicating perhaps that chromosomal genotype can influence *mos* expression. pKH104 is 22kb in size, but has

not yet been characterized in detail. However, it does not have homology to the *S. meliloti mos* genes.

This study presents for the first time experiments describing the long term persistence of a competitive advantage in nodulation for a rhizopine catabolizing strain over a non-catabolizing competitor. This study has also shown the presence of the rhizopine 3-*O*-MSI in four day old plants before the appearance of effective nodules on the roots. Finally, this work also summarizes steps undertaken in the search for the rhizopine synthesis genes in *R. leguminosarum* bv. *viciae*, and describes the discovery of a 22 kb rhizopine-synthesizing cosmid clone obtained from the Sym plasmid of strain 1a.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Keith Heinrich

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ABBREVIATIONS

3-*O*-MSI	L-3-*O*-methyl *scyllo* inosamine
A	absorbance
amu	atomic mass units
Ap	ampicillin (sodium salt)
BSA	bovine serum albumin
bp	base pairs
bv.	biovar
°C	degrees Celsius
Cb	carbenicillin
Cm	chloramphenicol
cm	centimeter
cv.	cultivar
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
EDTA	ethylenediamine tetraacetic acid (di sodium salt)
EtBr	ethidium bromide
eV	electron volts
g	gram
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
Gm	gentamycin
HVPE	high voltage paper electrophoresis

kb	kilobase pairs
kg	kilogram
Km	kanamycin monosulfate
λ	lambda
L	liter
M	molar
m^3	cubic meter
mA	milliamp
mg	milligram
min	minute
mL	milliliter
mM	millimolar
mm	millimeter
mRNA	messenger RNA
MS	mass spectrometer
mw	molecular weight
m/z	mass to charge ratio
Nal	naladixic acid
ng	nanograms
ORF	open reading frame
Rif	rifampicin
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
*S*I	*scyllo*-inosamine
Sp	spectinomycin
sp.	species
Sr	streptomycin sulfate

SSC	salt and sodium citrate solution
Tc	tetracycline hydrochloride
Tris-HCl	tris[hydroxymethyl]aminomethane
µg	microgram
µL	microliter
µM	micromolar
µm	micrometer
U	units
UV	ultra-violet
V	volts
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

Literature Review

1.1 General Introduction

Nitrogen is an essential element for healthy plant growth. Even though dinitrogen gas comprises 78% of the atmosphere, nitrogen in this form is not biologically available to plants. Plants require it in the form of nitrate or ammonium ions, or in simple organic compounds. Lack of accessible nitrogen is a common agricultural problem, perhaps second only to lack of water in factors limiting agricultural production (Maier and Triplett, 1996).

Nitrogen deficiency in many soils means that the application of inorganic fertilizers is often necessary. The disadvantages of using these fertilizers include the high energy input required for production, the pollution of waterways that results from leaching and surface run-off, as well as the volatilization of nitrogen oxides into the environment (Bohlool *et al.*, 1992).

The largest source of organic nitrogen in the global nitrogen cycle comes from a range of free-living and symbiotic bacteria. These micro-organisms are termed diazotrophs, and have the ability to convert gaseous dinitrogen to ammonium via a series of enzymatic reactions. This process is termed nitrogen fixation. The amount of nitrogen fixed by biological means is three times that produced by industry (Ishizuka, 1992).

Approximately 40% of biological nitrogen fixation is achieved by free-living micro-organisms (Gibson, 1990). These include anaerobes such as *Clostridium*, and aerobes such as *Azotobacter*, *Rhodospirillum* and *Azospirillum*. Many of these are found in association with plants.

Other nitrogen-fixing bacteria form a symbiotic relationship with higher plants. These associations include that of *Frankia* with *Alnus* (alder), the cyanobacterium *Anabaena* with the water fern *Azolla*, and members of the family Rhizobiaceae with legumes. The genera of nitrogen-fixing bacteria in the Rhizobiaceae are *Rhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Sinorhizobium.* These are the most extensively studied of the symbiotic diazotrophs (Mylona *et al.*, 1995). The terms *Rhizobium* or rhizobia are used throughout this thesis to collectively refer to the nitrogen-fixing members of the Rhizobiaceae. However, a distinction is made between the genera when necessary.

The symbiotic association of rhizobia with members of the plant family Fabaceae, or the legumes, is specific, and most species of rhizobia have a limited host range. The bacteria recognize appropriate plant hosts and enter the roots. They elicit the production of specialized structures, called nodules, on the roots. Within the nodules, the bacteria differentiate to form bacteroids, which are able to reduce molecular nitrogen to ammonium. The ammonium can then be assimilated by the plants. Although the bacteroids and undifferentiated bacteria are unable to use the ammonium themselves, they benefit from the association because the plant provides them with a low oxygen environment, and supplies photosynthetically-derived carbohydrates to support their metabolism.

Leguminous root nodules constitute by far the largest single source of organic nitrogen (Nap and Bisseling, 1990). The amount of nitrogen fixed by this process is thought to at least equal that produced by the fertilizer industry (Dénarié and Cullimore, 1993). The *Rhizobium*-legume symbiosis is therefore of great agricultural importance.

The importance of using legumes in crop rotations has long been recognized. The Greeks, Romans and Chinese used legumes in crop rotations more than 2000 years ago in order to increase agricultural productivity. By the 18th century, the use of legumes in crop rotations was well-established in most countries (Boussingalt, 1838, cited in Fred *et al.*, 1932), but it was not until 1888 that it was demonstrated that atmospheric nitrogen is

assimilated within the root nodules, and that this process is dependent upon bacteria (Hellriegel and Willfarth, 1888, cited in Nutman, 1987).

Today, rhizobial inoculants that enhance plant productivity by increasing the available nitrogen are often used in soils which contain low numbers of rhizobia, or which contain rhizobia that are inefficient at fixing nitrogen. Rhizobial inoculation also contributes to sustainable agriculture, by reducing the dependence on chemical fertilizers (Goss and Goorahoo, 1995).

1.2 Taxonomy of rhizobia

1.2.1 General characteristics

Rhizobia are aerobic, gram negative rod-shaped bacteria with multiple flagella. They form a symbiotic association with legumes in which nitrogen fixing nodules are formed on the roots. Classification of rhizobia at the genus level is currently under revision. Genera were originally designated on the basis of growth on yeast extract-mannitol medium. Fast-growing, acid-producing strains were assigned to *Rhizobium*, and slower-growing, non-acid-producing strains were assigned to *Bradyrhizobium* (Jordan, 1982). Two additional genera of fast-growing rhizobia, *Azorhizobium* and *Sinorhizobium*, have since been described (Dreyfus *et al.*, 1988; Chen *et al.*, 1988). The rhizobia are members of the family Rhizobiaceae, which also includes the genera *Agrobacterium* and *Phyllobacterium.*

Rhizobia have no cultural or biochemical characteristics which enable them to be easily distinguished from other soil micro-organisms, other than their ability to form nodules on legume roots. Indeed, even this is not diagnostic, as a number of non-symbiotic strains have been isolated (Sóberon-Chávez and Nájera, 1989; Segovia *et al.*, 1991; Laguerre *et al.*, 1993a; Sullivan *et al.*, 1996). These strains can regain the ability to infect legumes when an appropriate plasmid is added (Segovia *et al.*, 1991; Laguerre *et al.*, 1993a).

Rhizobia have traditionally been isolated indirectly from the soil, by retrieving free-living bacteria from within surface-sterilized nodules (Vincent, 1970). However, a number of methods for isolating rhizobia directly from soil have now been described (Jarvis *et al.*, 1989; Segovia *et al.*, 1991; Bromfield *et al.*, 1994).

1.2.2 Plasmids

Many rhizobia carry high molecular weight plasmids. Plasmids of up to 1500kb have been reported. Plasmid number and size is highly variable between strains, but can contain up to 25% of the total bacterial DNA (Martínez *et al.*, 1990). In *Rhizobium* and *Sinorhizobium*, the genes necessary for establishing symbiosis and nitrogen fixation are located on one or more of the megaplasmids, known as the Sym, or Symbiotic plasmids (see review by Mercado-Blanco and Toro, 1996).

Non-Sym, or cryptic plasmids, are often not required for the establishment of symbiosis, although some cases have been reported where they are essential. A megaplasmid from *R. leguminosarum* bv. *phaseoli*, for example, which carries lipopolysaccharide synthesis genes, is indispensible for symbiosis (Hynes and MacGregor, 1990; Brom *et al.*, 1992). Non-Sym plasmids more commonly influence traits such as growth and nodulation competitiveness (Brom *et al.*, 1992; see also Mercado-Blanco and Toro, 1996).

Evidence of Sym plasmid transfer between strains has been found, as single Sym plasmid types appear in diverse chromosomal backgrounds. However it is thought that such transfer events occur relatively rarely (Schofield *et al.*, 1987; Young and Wexler, 1988; Laguerre *et al.*, 1993c). Sym plasmids can also be transferred to other genera, such as *Agrobacterium*, which then develop the ability to infect legumes (Martínez *et al.*, 1987). The Sym and other megaplasmids generally transfer between strains by mobilization, although some are self-transmissable (Mercado-Blanco and Toro, 1996).

1.2.3 Classification

Rhizobia were originally classified on the basis of the plant from which they were isolated. This led to the identification of "cross-inoculation" groups; that is, groups of plants whose rhizobia are "mutually interchangeable" (Fred *et al.*, 1932). This classification system has a number of shortcomings, most importantly, it overlooks the huge genetic diversity within these genera (Flores *et al.*, 1988; Piñero *et al.*, 1988). Piñero *et al.* (1988) suggested that chromosomal genotype, and not phenotype, would be a much more reliable basis for classification. Many rhizobia will nodulate plants outside their cross-inoculation group, while others will nodulate only a very few species within their group (Jordan, 1984). Within the *Medicago* genus, for example, there are enormously differing degrees of specificity of symbiotic organisms (see Rome *et al.*, 1996). Nevertheless, this method, while simplistic, remains useful as a broad classification system.

Table 1.1 lists the species of *Rhizobium* described to date. The genus *Rhizobium* is more closely related to *Agrobacterium* than to the other members of the Rhizobiaceae, and contains a number of species and biovars. *Bradyrhizobium* is most closely related to *Rhodopseudomonas* and contains at least three species and probably more (Young and Johnston, 1989). A third genus, containing the single species *Azorhizobium caulinodans*, was created for the stem-nodulating bacteria isolated from *Sesbania rostrata*. *Azorhizobium* is distantly related to the other rhizobia and is genetically much more homogeneous (Dreyfus *et al.*, 1988). The most recently described genus is *Sinorhizobium* (Delajudie *et al.*, 1994), which contains five species. There has been much debate about whether *S. meliloti* and *S. fredii* belong to *Rhizobium* or *Sinorhizobium* (see Jarvis *et al.*, 1992), however it is now generally accepted that they are sufficiently different from other *Rhizobium* species to warrant their inclusion in a separate genus. A fifth genus, *Mesorhizobium*, has been proposed for *R. ciceri*, *R. huakuii*, *R. loti*, and *R. mediterraneum*, which form a distinct monophyletic taxonomic grouping (Lindström *et al.*, 1995).

Genus	Species	Plant hosts
Azorhizobium (Dreyfus *et al.*, 1988)	*A. caulinodans* (Dreyfus *et al.*, 1988)	*Sesbania rostrata*
Bradyrhizobium (Jordan, 1982)	*B. japonicum* (Jordan, 1982)	*Glycine max, Mimosa, Macroptilium, Parasponia, Lupinus*
	Bradyrhizobium sp. (Jordan, 1982)	*Leucaena, Lablab, Cicer, Ornithopus, Sesbania, Acacia, Vigna, Lotus, Aeschynomene, Glycine*
	B. elkanii (Kuykendall and Saxena, 1992)	*Glycine max*
Rhizobium (Jordan, 1984)	*R. ciceri* (Nour *et al.*, 1994)	*Cicer arietinum*
	R. etli (Segovia *et al.*, 1993)	*Phaseolus vulgaris*
	R. galegae (Lindström, 1989)	*Galega officinalis, G. orientalis*
	R. gallicum bv. *gallicum* (Amarger *et al.*, 1997)	*Phaseolus* spp., *Leucaena leucocephala, Macroptilium atropurpureum, Onobrychis viciifolia*
	R. gallicum bv. *phaseoli* (Amarger *et al.*, 1997)	*Phaseolus* spp.
	R. giardinii bv. *giardinii* (Amarger *et al.*, 1997)	*Phaseolus* spp., *L. leucocephala, M. atropurpureum*
	R. giardinii bv. *phaseoli* (Amarger *et al.*, 1997)	*Phaseolus* spp.
	R. huakuii (Chen *et al.*, 1991)	*Astragalus sinicus*

Table 1.1 The recognized species of *Rhizobium*, *Azorhizobium*, *Bradyrhizobium* and *Sinorhizobium* and their host plants

Genus	Species	Plant hosts
Rhizobium (Jordan, 1984)	*R. leguminosarum* bv. *viciae* (Jordan, 1984)	*Lathyrus, Lens, Pisum, Vicia*
	R. leguminosarum bv. *phaseoli* (Jordan, 1984)	*Phaseolus*
	R. leguminosarum bv. *trifolii* (Jordan, 1984)	*Trifolium*
	R. loti (Jordan, 1984)	*Anthyllis, Caragana, Cicer, Leucaena, Lotus, Lupinus, Mimosa, Ornithopus*
	R. mediterraneum (Nour *et al.*, 1995)	*Cicer arietinum*
	R. tropici (Martínez-Romero *et al.*, 1991)	*Leucaena* spp., *Phaseolus vulgaris*
	Rhizobium sp. NGR234 (Trinick, 1980)	more than 80 tropical legumes
Sinorhizobium (de Lajudie *et al.*, 1994)	*S. fredii* (Chen *et al.*, 1988)	*Glycine soja, G. max, Vigna unguiculata, Cajanus cajan*
	S. medicae (Rome *et al.*, 1996)	*Medicago truncatula, M. orbicularis, M. polymorpha, M. rugosa*
	S. meliloti (Jordan, 1984; Chen *et al.*, 1988)	*Medicago* spp., *Melilotus* spp., *Trigonella* spp.
	S. saheli (Delajudie *et al.*, 1994)	*Acacia seyal, Sesbania* spp., *L. leucocephala, N. oleracea*
	S. teranga (Delajudie *et al.*, 1994)	*Acacia* spp., *Sesbania* spp., *L. leucocephala, N. oleracea*

Table 1.1 cont. The recognized species of *Rhizobium*, *Azorhizobium*, *Bradyrhizobium* and *Sinorhizobium* and their host plants.

Two additional species of soybean-nodulating rhizobia have been described, *B. liaoningense* (Xu *et al.*, 1995) and *R. tianshanense* (Chen *et al.*, 1995), but these are not yet recognized as legitimate species (Lindström *et al.*, 1995). Further unnamed species have also been discovered (for example, Zhang *et al.*, 1991; Laguerre *et al.*, 1993b; Sullivan *et al.*, 1996). It is certain that as the symbiotic bacteria are isolated from new legume species, and the occurrence of non-symbiotic rhizobia is investigated more thoroughly, the species diversity of these genera will increase further.

1.3 Nodulation

1.3.1 Introduction

The nodules produced on the roots of legumes are highly organized and specialized structures. The establishment of an effective symbiosis uses an extensive network of plant and bacterial signals. Nodulation is increased under nitrogen limiting conditions, but is inhibited in the presence of large amounts of combined nitrogen. Ammonia represses the expression of nodulation genes (Dusha *et al.*, 1989; Wang and Stacey, 1990; Dusha and Kondorosi, 1993), whereas inhibition by nitrate is primarily controlled by the host plant (Carroll and Mathews, 1990). The production of flavonoid signal molecules is increased under nitrogen-limiting conditions, leading to a concomitant increase in expression of the nodulation genes (Coronado *et al.*, 1995).

There are two different types of nodules, indeterminate and determinate. Indeterminate nodules are cylindrial or coralloid in shape and are found in *Medicago* (lucerne), *Trifolium* (clover), *Pisum* (pea) and *Vicia* (bean). They are elongated and have protracted meristematic development. Determinate nodules are spherical in shape and are found in *Phaseolus* (bean), *Glycine* (soybean) and *Lotus*. These nodules have only transient internal meristematic development (Brewin, 1991).

1.3.2 Attraction to the root surface

Rhizobia are chemotactic towards plant root exudates, including nutrients such as sugars and amino acids (Bergman *et al.*, 1988; Triplett, 1990), and flavonoid compounds (Caetano-Anollés *et al.*, 1988; Kape *et al.*, 1991). After non-specific attraction of rhizobia to the legume root, there is an interchange of molecular signals between the plant and the *Rhizobium*. Each species of legume exudes a characteristic range of flavonoids. Certain flavonoids, such as luteolin and naringenin, as well as some non-flavonoids, such as trigonelline and stachydrine (Phillips *et al.*, 1994), induce the transcription of a series of rhizobial nodulation (*nod*) genes. Expression of these genes is essential for nodulation to take place. Although flavonoids are relatively widespread throughout the plant kingdom, it is only legume flavonoids which are able to induce nodulation (Johnston, 1989). Some flavonoids and isoflavonoids, such as umbelliferone, which are also chemoattractants, are *nod* gene inhibitors (Firmin *et al.*, 1986; Djordjevic *et al.*, 1987).

1.3.3 Induction of *nodD* gene expression

Induction of nodulation is mediated by an interaction between an appropriate flavonoid and the *nodD* gene product. *NodD* is the only *nod* gene to be constitutively expressed, and is an essential prerequisite for symbiosis (Recourt *et al.*, 1989; Schlaman *et al.*, 1990). NodD proteins from different species of rhizobia recognize particular flavonoids preferentially (Brewin, 1991). This interaction is one determinant of host specificity, as only in the presence of an appropriate flavonoid will NodD activate the transcription of the other *nod* genes. NodD is a positive regulator which binds to a conserved 60 base pair promoter sequence (the *nod* box) approximately 100-250 base pairs upstream from each inducible *nod* gene (Rostas *et al.*, 1986; Fisher and Long, 1989; Fisher and Long, 1992). The NodD protein binds to the *nod* box, usually independently of the flavonoid (Hong *et al.*, 1987; Fisher *et al.*, 1988; Fisher and Long, 1989; Kondorosi *et al.*, 1989; Fisher and Long, 1993). However, the flavonoid is required for NodD-dependent gene induction, and is thought to bind to NodD on the *nod* box (Goethals *et al.*, 1992), causing

a perturbation in the DNA conformation which permits the transcription of the downstream genes (Pueppke, 1996).

NodD is a member of the LysR family of transcriptional activators (Schell, 1993), which are cytoplasmic proteins. However, NodD is associated exclusively with the inner membrane of *R. leguminosarum* bv. *viciae* cells, despite lacking obvious membrane-spanning segments (Schlaman *et al.*, 1989). Flavonoids accumulate in the cytoplasmic membrane where they are thought to activate the NodD protein (Recourt *et al.*, 1989). The absorption of flavonoids is a complex process involving NodD as well as several other proteins (Hubac *et al.*, 1993).

While most rhizobia have one functional copy of *nodD*, several species contain multiple copies. In *S. meliloti*, there are three functional *nodD* genes (Honma and Ausubel, 1987), each with a different flavonoid specificity (Györgypal *et al.*, 1988). The presence of three *nodD* genes allows a greater diversity of host nodulation responses (Györgypal *et al.*, 1988; Demont *et al.*, 1994). The $nodD_1$ and $nodD_3$ genes play equivalent roles in the nodulation of sweet clover, but $nodD_1$ is more important than $nodD_3$ in the nodulation of lucerne. $NodD_2$ has an effect on lucerne nodulation but not on sweet clover nodulation (Homna and Ausubel, 1987). In *Rhizobium* sp. NGR234, which has a host range of more than 70 legume genera, there are two *nodD* genes (Perret *et al.*, 1991), with one more important than the other. Mutations to $nodD_1$ prevent nodulation entirely, while $nodD_2$ does not seem to play an active role (Broughton *et al.*, 1990).

1.3.4 Attachment to host

The first step in the establishment of symbiosis is the recognition and invasion of a legume root by rhizobia. The *Rhizobium* produces cellulose fibrils which help it become entangled in mucigel on the surface of the root hairs. The point of attachment is just behind the apical meristem, where the root hair cells are rapidly expanding (Bauer, 1981; Bhuvaneswari *et al.*, 1981; Sargent *et al.*, 1987). Binding may be reinforced by

ricadhesin, a calcium-dependent protein of rhizobial origin (Smit *et al.*, 1987; Swart *et al.*, 1994). Plant lectins have also been implicated in this process (Bohlool and Schmidt, 1974; Stacey *et al.*, 1980; Dazzo *et al.*, 1983), and a recent report has indicated that lectins play a role throughout nodule ontogeny (Bauchrowitz *et al.*, 1996).

Attachment is not host-specific, but does depend upon the presence, on the *Rhizobium* cell membrane, of exopolysaccharides (EPS), lipopolysaccharides (LPS), and ß-1,2-glucans (Brewin, 1991). As lectins are carbohydrate binding proteins, any of these cell-surface polysaccharides may serve as recognition molecules for lectin binding (Dowling and Broughton, 1986). Legumes from different cross-inoculation groups produce lectins with different binding specificities, and so host range is in part determined by the bacteria-lectin interaction (Kijne *et al.*, 1986; Díaz *et al.*, 1989).

After attachment, the bacteria induce the root hairs to become curled and twisted ("root hair curling"), by hydrolysis of the cell wall (Callaham and Torrey, 1981; van Spronsen *et al.*, 1994), and the bacterial cells become entrapped within these deformations. The deformations are elicited by very low concentrations of a signal molecule, known as the Nod factor, which is produced by the rhizobia. As little as five to ten minutes incubation with low concentrations of a Nod factor is sufficient to induce root hair deformations which are visible within one hour (Heidstra *et al.*, 1994). Nod factors will be discussed in more detail later in this chapter.

1.3.5 Infection

The rhizobia enter the plant at the site of root hair curling. They penetrate the wall of the root hair cell and a tubular infection thread forms which carries them, usually in single file, from the root surface to a new apical meristem which develops in the cortex. During the development of the infection thread, normal apical cell wall growth of the root hair is altered such that a normally outwardly growing cell wall cylinder is converted into an inwardly growing tunnel. This is probably brought about by a restructuring of the

cytoskeleton (Brewin, 1991).

An EPS is essential for infection thread initiation and development. The major EPS in *S. meliloti* is succinoglycan (Leigh and Walker, 1994), and this compound acts as a specific signal that promotes the growth of the infection thread (Battisti *et al.*, 1992; Leigh and Walker, 1994). EPS-deficient mutants (*exo*) often have defective nodule invasion and development (Leigh *et al.*, 1985), despite being able to produce signal molecules such as Nod factors (Lerouge *et al.*, 1990). Many genes involved in EPS synthesis have been described (e. g. Leigh *et al.*, 1985; Finan, 1988; Latchford *et al.*, 1991). For example, in *S. meliloti* there is a cluster of 13 EPS synthesis genes on one of the megaplasmids, with several more EPS genes located on the chromosome (Finan *et al.*, 1986; Long *et al.*, 1988).

LPS is required for the growth of the narrow infection threads typical of determinate nodules (Kijne, 1982). In rhizobia which give rise to determinate nodules, LPS-deficient mutants produce abortive infection threads (Cava *et al.*, 1989; Stacey *et al.*, 1991; Noel, 1992).

1.3.6 Nodule development

In plants which produce indeterminate nodules, the cortical cells begin to undergo multiple cell divisions within 12 - 24 hours of infection to form an incipient nodule (Bauer, 1981; Brewin, 1991). The addition of Nod factors to legume roots is sufficient to stimulate this mitotic activity (Spaink *et al.*, 1991; Truchet *et al.*, 1991; Relic *et al.*, 1993).

As the nodule grows, the infection threads branch and spread from cell to cell in the cortex. A cylindrical infection structure similar to a normal infection thread develops, but the end of it becomes enlarged, forming a vesicular infection droplet. The droplet is generally 10-25 μm in diameter and contains 10-100 bacteria which are enclosed by a

peribacteroid membrane (PBM). The PBM is derived from the plasma membrane of the root cells (Perotto *et al.*, 1991) and the fusion of vesicles from the golgi and endoplasmic reticulum (Brewin, 1993). As the infection droplet is released into the cytoplasm of the cortical cells by phagocytosis or endocytosis, the root cells become invaded by the bacteria released from the ends of the infection threads. Inside the cells, the PBM and bacteria within form organelle-like structures referred to as symbiosomes (Roth and Stacey, 1989a; Roth and Stacey, 1989b). The vesicles continue to divide in conjunction with division of the PBM. In pea and clover nodule cells, each bacterium eventually becomes individually enclosed, while in soybean, more than ten may remain in one membrane sac (Brewin, 1991).

The sequence of *Rhizobium*-induced cell division and cell invasion varies between legume species, with important consequences for nodule morphogenesis. In those legumes which give rise to determinate nodules, the cortical cell divisions occur just beneath the epidermis (Mathews *et al.*, 1989), and these cells are invaded by rhizobia before they become meristematic (Rolfe and Gresshoff, 1988). In this situation, the rhizobia are spread simply by dividing within the cytoplasm of cortical cells which are themselves dividing. Thus the nodule consists of a mixture of infected and uninfected cells derived from the root cortex, together with uninfected cells derived from the pericycle (Brewin, 1991).

1.3.7 Bacteroids

The bacteria released into the plant cytoplasm are termed bacteroids. They differentiate into larger, morphologically-altered endosymbionts of irregular shape (Werner, 1992). They remain enclosed by the PBM, and therefore are separated from the plant cell cytoplasm. In this state, they can synthesize the enzyme nitrogenase, which converts atmospheric nitrogen to ammonium. A number of developmentally-regulated genes in both the plant and the *Rhizobium* code for different steps of this reaction. The reaction takes place in the central tissues of the nodule, where the oxygen concentration is very

low (Brewin, 1991).

1.3.8 Nodule senescence

The senescence of bacteroids is a natural part of the developmental sequence in indeterminate nodules. This stage results in lysis of the bacteroids and host cells. A few undifferentiated bacteria are present within the infection thread, which persist in the soil after nodule disintegration (Vasse *et al.*, 1990). As stress proteins have been observed in nodules, it is thought that the reason for senescence is because the symbiosome is under physiological stress (Brewin, 1991).

1.4 Nodulins

Nodulins are plant proteins which are produced before the onset of nitrogen fixation and accumulate specifically in nodules (Sánchez *et al.*, 1991). Synthesis of early nodulins (ENODs) begins within hours of infection. ENODs are thought to be involved in root hair curling, infection and formation of the nodule. It has been shown that Nod factors activate the expression of two early nodulin genes, ENOD5 and ENOD12, which have been correlated with the formation of the infection thread wall in peas (Scheres *et al.*, 1990a; Scheres *et al.*, 1990b; Horvath *et al.*, 1993; Journet *et al.*, 1994). However, the precise functions of many ENODs remain unknown (Long, 1996).

Late nodulins are produced by a large group of genes that are expressed shortly before nitrogen fixation begins. By far the most abundant late nodulin is leghemoglobin. The protein component of leghemoglobin is synthesized by the plant, while the heme group was traditionally thought to be of bacterial origin (Nap and Bisseling, 1990). However, recent evidence suggests that the heme group may also be synthesized by the plant (O'Brian, 1996). Other late nodulins include proteins present in the PBM, enzymes involved in nitrogen assimilation, carbon metabolism and amide and ureide biogenesis (Sánchez *et al.*, 1991). Late nodulins aid in the establishment of the physiological

conditions required within the nodule for nitrogen fixation and ammonium assimilation and transport (Nap and Bisseling, 1990).

1.5 Nitrogen fixation

The reduction of atmospheric nitrogen to ammonium is catalyzed by the microbial enzyme nitrogenase. Chemically, the reaction is written as :

$$N_2 + 16ATP + 8e^- + 10H^+ \text{---> } 2NH_4^+ + H_2 + 16ADP + 16Pi.$$

The nitrogenase enzyme is irreversibly inactivated by oxygen (Shaw and Brill, 1977). However, the reaction has a large ATP requirement, derived from oxidative respiration. This conflict for oxygen requirements is overcome by leghemoglobin, which can reversibly bind oxygen, providing a flow of oxygen to the bacteroid, while at the same time protecting the enzyme from damage.

Nitrogenase is a complex metalloenzyme with two separable components, designated Fe protein and MoFe protein. Component 1, the MoFe protein, is a dinitrogenase, and component 2, the Fe protein, is a dinitrogen reductase (Hageman and Burris, 1978; Hageman and Burris, 1979). The *nifH* gene codes for the Fe protein, while the two subunits of the MoFe protein are encoded by *nifD* and *nifK*. The products of a number of other genes are required for processing of the two components before the enzyme becomes active. For example, an iron-molybdenum cofactor (FeMoco) must be inserted into the MoFe protein (Dean and Jacobson, 1992).

The fixed ammonium is excreted from the bacteroid to the cytosol of the infected plant cells. It is used to produce glutamine and glutamate, which are in turn used to synthesize various other nitrogenous compounds. These compounds are then translocated around the plant (Schubert, 1986; Cullimore and Bennett, 1992).

1.6 Genetic control of nodulation and nitrogen fixation

1.6.1 Introduction

In the genera *Rhizobium, Sinorhizobium* and *Azorhizobium*, the *nod* genes are located on the Sym plasmid. Many of the other genes involved in symbiosis, such as the nitrogen fixation (*nif* and *fix*) genes, are also located on the Sym plasmid. In *Bradyrhizobium*, the *nod*, *nif* and *fix* genes are chromosomally-borne (Long, 1989a; Hirsch, 1992).

1.6.2 The common nodulation genes

The genes involved in the initial steps of infection and nodulation are *nodA*, *nodB*, and *nodC*. These genes are responsible for the synthesis of the core of the Nod factor (Carlson *et al.*, 1994). *NodA*, *B* and *C* are present in all *Rhizobium* species and are called the common *nod* genes. They are located adjacent to *nodD*, but are transcribed divergently. Genes from one species can be used to complement mutations in another (Egelhoff and Long, 1985). Strains carrying mutations in any one of these genes cannot elicit any plant reaction and no nodules are formed (Long, 1989b).

NodC shows homology with chitin synthase genes and is thought to catalyze the synthesis of the chitin oligomer backbone of the Nod factor (Geremia *et al.*, 1994). This oligomer is modified by *nodB*, which de-*N*-acetylates the terminal non-reducing end (John *et al.*, 1993). At this position, NodA transfers a fatty acid from an acyl carrier protein (Atkinson *et al.*, 1994; Röhrig et al., 1994). This core structure is then modified by other Nod proteins, which either synthesize or add other substituents that restrict or extend host-range (Lerouge *et al.*, 1990; Roche *et al.*, 1991a; Spaink *et al.*, 1991; Krishnan and Pueppke, 1992; Firmin *et al.*, 1993; Mergaert *et al.*, 1993). It has recently been reported that allelic variation in the *nodABC* genes also influences host specificity (Roche *et al.*, 1996; Kamst *et al.*, 1997).

NodI and *nodJ* are also considered to be common *nod* genes, and are present in all rhizobial species. Their gene products are located on the cytoplasmic membrane (Schlaman *et al.*, 1990), and are involved in Nod factor export (McKay and Djordjevic, 1993). Several more genes are presumed to also be involved in Nod factor export, as *nodI*$^-$ and *nodJ*$^-$ mutants exhibit delayed, but not completely defective nodulation (Cárdenas *et al.*, 1996).

1.6.3 The specific nodulation genes

Other *nod* genes, such as *nodFE, nodG, nodH, nodPQ* and *nodL* cannot complement between species, and are known as the specific *nod* genes. Many play a role in defining host specifity (Brewin, 1991). These genes are located several kilobases from the common *nod* genes. Other specific nodulation genes include the *noe* and *nol* genes, and the *syrM* gene. A list of rhizobial nodulation genes is given in Table 1.2. It should be noted that only some of these genes function in any given organism.

Mutations in the specific *nod* genes cause a variety of phenotypes. Mutations in some genes can lead, for example, to infection of a normally unresponsive host, or abnormal root-hair curling (Goethals *et al.*, 1990; Göttfert *et al.*, 1990; Lerouge *et al.*, 1990; Schwedock and Long, 1990; Spaink *et al.*, 1991). Others, such as *nodT* in *R. leguminosarum*, show no observable abnormal phenotype (Surin *et al.*, 1990).

Three genes, *nodH, nodP* and *nodQ*, are responsible for the specificity of the *S. meliloti* Nod factor, NodRm-1 (Lerouge *et al.*, 1990; Roche *et al.*, 1991a). Mutations to these genes lead to the formation of NodRm-2, which is similar to NodRm-1 but lacks a sulfate moiety (Lerouge *et al.*, 1990). In the presence of the flavonoid luteolin, NodRm-2 elicits root hair deformations on vetch but not lucerne, indicating that host-specificity for lucerne is determined by sulfation of NodRm-2 (Schwedock and Long, 1990).

Gene	Proposed role of gene products
nodABC	synthesis of Nod factor backbone
nodD	transcriptional activator of *nod* genes
nodE	host range determinant
nodF	host range determinant
nodG	host range determinant
nodH	host range determinant
nodI	transport of Nod factor across cell membrane
nodJ	membrane protein, may act in conjunction with *nodI*
nodK	function unknown
nodL	host range determinant in *R. leguminosarum*
nodM	host range determinant in *S. meliloti*
nodN	biosynthesis of hair deformation factor
nodO	calcium binding protein
nodP	host range determinant in *S. meliloti*
nodQ	host range determinant in *S. meliloti*
nodR	host range determinant in *R. leguminosarum* bv. *trifolii*
nodS	possible host range determinant
nodT	outer membrane protein involved in Nod factor export
nodU	function unknown
nodVW	regulatory proteins in *B. japonicum*
nodX	host range determinant in some strains of *R. leguminosarum* bv. *viciae*
nodY	found in *B. japonicum*, function unknown
nodZ	host range determinant
noeAB	found in *S. meliloti*, function unknown
nolA	host range determinant in *B. japonicum*
nolBTUV	found in *S. fredii*, function unknown
nolBC	host range determinant in *B. japonicum*
nolFGHI	transport proteins
nolJ	unknown function
nolK	possible host range determinant
nolMNO	found in *B. japonicum*, may be a host range determinant
nolR	regulatory protein in *S. meliloti*
nolW	found in *S. fredii*, function unknown
nolX	found in *S. fredii*, function unknown
nolYZ	found in *B. japonicum*, function unknown
syrM	transcriptional regulator in *S. meliloti*

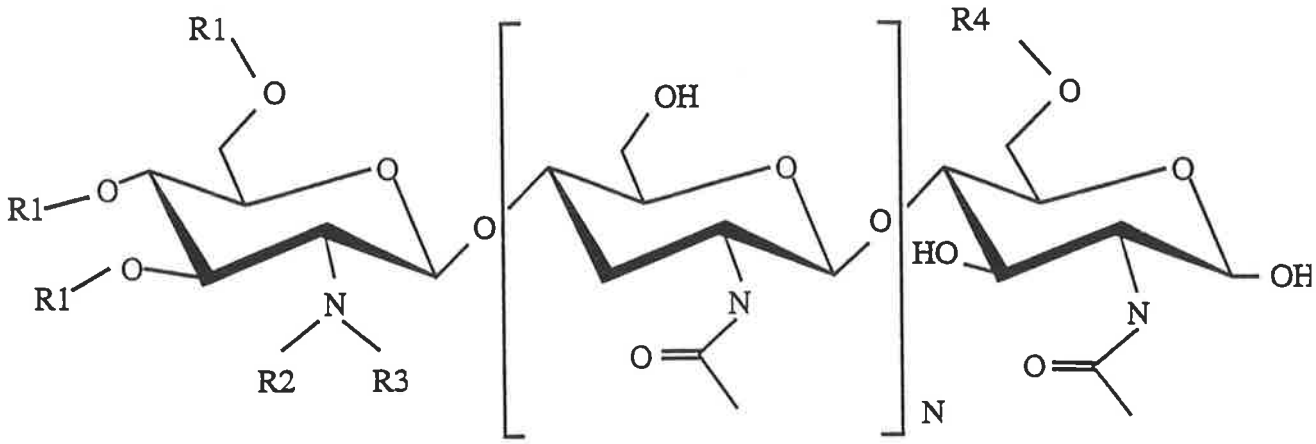
Table 1.2 Summary of nodulation genes and their function (adapted from Carlson *et al.*, 1994; Spaink, 1995; Dénarié *et al.*, 1996; Long, 1996; Pueppke, 1996).

Other *nod* genes have different functions. For example, *nodVW* in *B. japonicum* function as *nodD*-independent flavonoid-mediated regulators of *nod* gene expression (Sanjuan *et al.*, 1994; Stacey *et al.*, 1995). Another example is *NodO*, which has a role in binding calcium ions, and thus may mediate an early stage in the recognition between the *Rhizobium* and the host (Economou *et al.*, 1990).

1.6.4 Nod factors

Nod factors are chitin-like oligosaccharides of three to five ß-1,4-linked *N*-acetylglucosaminosyl residues which are *N*-acetylated on the non-reducing terminal residue (see Figure 1.1) (Carlsen *et al.*, 1994; López-Lara *et al.*, 1995). The specific *nod* genes are involved in modifying the Nod factor backbone, by adding further glycosyl residues, or adding sulfate, carbamyl, methyl, acetyl, or glycerol groups (Carlsen *et al.*, 1994). These groups are determinants of host specificity. A number of different Nod factors have been isolated from various species: *S. meliloti* (Lerouge *et al.*, 1990; Schultze *et al.*, 1995), *R. leguminosarum* bv. *viciae* (Spaink *et al.*, 1991; Firmin *et al.*, 1993), *B. japonicum* (Sanjuan *et al.*, 1992; Carlson *et al.*, 1993), *Rhizobium* sp. NGR234 (Price *et al.*, 1992), *A. caulinodans* (Mergaert *et al.*, 1993), *S. fredii* (Bec-Ferté *et al.*, 1993), *R. tropici* (Poupot *et al.*, 1993), *B. elkanii* (Carlson *et al.*, 1993), *R. loti* (López-Lara *et al.*, 1995), *S. saheli* (Lorquin *et al.*, 1997) and *S. teranga* (Lorquin *et al.*, 1997). Most species produce more than one type of Nod factor (Carlson *et al.*, 1993). The variety of Nod factors produced by the broad host range strain, *Rhizobium* sp. NGR234, facilitates its ability to nodulate more than 70 different legume genera (Price *et al.*, 1992).

Nod factors are involved in many different aspects of nodule development, such as the initiation of root hair curling, infection thread development, and cortical cell divisions (Brewin, 1991; Relic *et al.*, 1993; Roche *et al.*, 1991b; Truchet *et al.*, 1991). They may serve as a signal to trigger developmental events in the plant which are important for rhizobial infection. A recent study showed that Nod factors are the signal which allows rhizobia to penetrate the roots via infection threads. NodABC$^-$ mutants, which ordinarily



Non-reducing end

Reducing end

N = 1, 2 or 3

R1 = H or carbamoyl or acetyl

R2 = H or methyl

R3 = Long chain fatty acid

R4 = H or acetyl or sulfuryl or additional sugar

(arabinosyl or fucosyl, sometimes substituted)

Figure 1.1 Generic structure for nodulation factors (adapted from Long, 1996, and Pueppke, 1996).

cannot nodulate, have this ability restored if Nod factors are added at the time of inoculation (Relic *et al.*, 1993; Relic *et al.*, 1994).

1.6.5 Genes involved in nitrogen fixation

The genes responsible for nitrogen fixation are known as the *nif* and *fix* genes. The *nif* genes have homologues in the free-living nitrogen fixing bacterium, *Klebsiella pneumoniae*, whereas the *fix* genes are unique to *Rhizobium*. The genes *nifH*, *nifD* and *nifK* code for the nitrogenase enzyme. Other genes such as *nifE*, *nifN* and *nifB* are required for maturation of the enzyme, and a number of *fix* genes are required for an electron transport chain to the enzyme (de Philip et al., 1992).

The *nif* and *fix* genes are transcriptionally regulated by the *nifA* and *ntrA* genes. NifA is required for the symbiotic expression of all other *nif* genes (Szeto *et al.*, 1984), and is regulated by oxygen concentration. The product of *ntrA* is also required. This gene encodes a sigma factor subunit of RNA polymerase, which interacts with specific *nif* and *fix* promoters. Separate regulatory genes have been identified for some *fix* genes (David *et al.*, 1987; see also review by de Philip *et al.*, 1992).

In addition to the *ntrA* and *nifA* genes, *S. meliloti* has a parallel regulatory system, in which FixL and FixJ activate the transcription of both *nifA* and another regulatory gene, known as *fixK* (Hertig *et al.*, 1989; de Philip *et al.*, 1990; Batut *et al.*, 1991; Reyrat *et al.*, 1993). FixL is a transmembrane protein which senses microaerobiosis and transduces this signal by covalent modification to FixJ (David *et al.*, 1988). FixJ interacts directly with upstream regulatory sequences of *fixK* and *nifA* (Hertig *et al.*, 1989). Some *nifA*-independent *fix* genes (the *fixN* gene cluster) are directly activated by FixK in conjunction with NtrA. FixL and FixJ are also necessary for the expression of the *nif* genes and the *nifA*-dependent *fix* genes, because they are partially responsible for the microaerobic activation of *nifA* itself. After activation of *nifA*, the NifA protein in turn activates the other *nif* and *fix* genes (David *et al.*, 1988; Batut *et al.*, 1989; Hertig *et al.*, 1989). In this

way, FixL and FixJ regulate all of the NifA-dependent *nif* and *fix* genes, as well as the *nifA*-independent *fixN* regulon (David *et al.*, 1988).

1.7 Ecology of Rhizobia

1.7.1 Nodulation competitiveness

Rhizobia vary widely in their response to biotic and abiotic factors. Differences also exist between rhizobia with respect to characteristics such as nodulation competitiveness, nodulation efficiency, nitrogen fixing efficiency, and long-term persistence in the soil.

Nodulation competitiveness is defined as the ability of certain strains of *Rhizobium* to dominate nodulation in an environment containing many different strains (McDermott and Graham, 1990). Although many factors have been identified which can influence nodulation competitiveness, the basis for competitiveness remains largely unknown. However, no correlation has been found between nitrogen fixing efficiency and nodulation competitiveness. For example, Amarger (1981) demonstrated that some ineffective mutants of *S. meliloti* were equally competitive with the wild-type.

1.7.2 Factors which affect nodulation competitiveness

1.7.2.1 Speed of nodulation

The speed with which rhizobia can initiate infection, known as "nodule forming efficiency", is crucial to its competitiveness. Strains which display delayed nodulation, or have relatively low nodule forming efficiency, are often at a competitive disadvantage compared with those which are able to initiate and sustain an infection rapidly (Dowling and Broughton, 1986; Sargent *et al.*, 1987; Hahn and Hennecke, 1988; McDermott and Graham, 1990; Onishchuk *et al.*, 1994). One explanation for this may be that early infection by one strain induces an autoregulatory response which can prevent subsequent nodulation by other strains (Bhuvaneswari *et al.*, 1981; Kosslak and Bohlool, 1984;

Stephens and Cooper, 1988).

1.7.2.2 Environmental factors

Many environmental factors influence rhizobial competition for nodulation. These include soil type, pH, temperature, moisture and salinity (see reviews by Dowling and Broughton, 1986 and Vlassak and Vanderleyden, 1997). Rhizobia vary widely in their tolerance to environmental extremes, with some strains being better adapted to particular conditions than others.

In general, high acidity inhibits both growth and nodulation. Changes in soil pH may alter the relative proportion of nodules formed by competing strains. Acidity is often correlated with phosphate deficiency and high concentrations of metals such as manganese and aluminium, factors which also reduce nodulation. Most rhizobia have an optimum temperature for growth of between 25 and 30°C. Extremes of high and low temperatures reduce survival and nodulation in the field, and influence competition dynamics. Low nutrients, particularly carbon sources, can also diminish nodulation. However, some strains are better adapted to adverse conditions than others (Howieson *et al.*, 1993; McKay and Djordjevic, 1993). For example, rhizobia isolated from arid areas tend to be much more tolerant of high temperatures and dessication than those originating from cooler, wetter climates (Hartel and Alexander, 1984). Extremes of soil moisture and high salinity affect rhizobial survival, but are not thought to be major factors in competition (Vlassak and Vanderleyden, 1997).

1.7.2.3 Biological factors

A number of biological factors also play a role in nodulation competitiveness. Bacteriophages have the ability to lyse large numbers of rhizobia. Epiphytic bacteria such as *Erwinia herbicola* may prevent nodulation by producing a toxin or obstructing rhizobial attachment. Other organisms which may influence competition include predatory protozoa, and the intracellular parasite *Bdellovibrio* (reviewed by Dowling and Broughton, 1986).

Antibiotic production may confer a competitive advantage in nodulation (Triplett, 1988; Triplett, 1990). One example of this is the production of trifolitoxin by *R. leguminosarum* bv. *trifolii* strain T24. Trifolitoxin inhibits the nodulation of sensitive strains (Triplett and Barta, 1987; Triplett *et al.*, 1994), thus enhancing the competitiveness of resistant strains. However, results obtained under aseptic conditions are yet to be repeated in the field (Maier and Triplett, 1996). Spontaneous antibiotic resistance mutations can, but do not always, provide a competitive advantage. For example, chloramphenicol resistant mutants of *R. leguminosarum* bv. *trifolii* had reduced competitive ability (Rynne *et al.*, 1991), while some neomycin resistant mutants, although ineffective, were equally competitive with the parent strains (Amarger, 1981).

Biased rhizospheres, in which special compounds can be synthesized by either the plant or the bacteria, may influence competition by favoring those bacteria which are able to utilize the compound. One example is the betaine trigonelline. Three trigonelline catabolism genes are expressed in *S. meliloti* during all stages of infection, suggesting that this compound is available as a carbon source (Boivin *et al.*, 1990). A more specific example is that of rhizopines, which are inositol-based molecules synthesized in the nodule by some strains of *Rhizobium*. They may serve as a selective growth substrate for catabolizing strains, and are known to influence competition for nodulation (Gordon *et al.*, 1996). Rhizopines will be discussed in detail in Section 1.8.

1.7.2.4 Other factors

Many rhizobia are intolerant to even very low concentrations of fungicides containing substituents like iprodione, thiram or thiobendazole, and rhizobial colonization and nodulation in areas treated with these chemicals is often poor (Evans *et al.*, 1989).

1.7.3 Genes involved in nodulation competitiveness

Loci containing a number of genes involved in nodulation efficiency (*nfe*; nodule

formation efficiency) have been found in *S. meliloti* (Toro and Olivares, 1986; Sanjuan and Olivares, 1989; Sanjuan and Olivares, 1991; Soto *et al.*, 1993; Soto *et al.*, 1994) and *B. japonicum* (Chun and Stacey, 1994). Transposon insertion mutants in the identified region exhibit a delay in nodulation and reduced nodulation competitiveness compared to the wild-type (Sanjuan and Olivares, 1989; Chun and Stacey, 1994), while nitrogen fixation was unaffected (Chun and Stacey, 1994). Some poorly competitive natural isolates of *S. meliloti* showed no homology to an *nfe* probe, and two of these displayed increased competitiveness when transformed with *nfe* DNA (Sanjuan and Olivares, 1989; Sanjuan and Olivares, 1991). Expression of these genes is under direct regulation by the *nifA-ntrA* system, but the precise mode of action of the *nfe* locus is unknown (Sanjuan and Olivares, 1991). These genes are located on a cryptic plasmid (Toro and Olivares, 1986).

Cryptic plasmids, but not *nfe* genes, have been implicated in the competitiveness of a number of other species, such as *R. leguminosarum* bv. *phaseoli* and *R. etli* (Martínez-Romero and Rosenblueth, 1990; Brom *et al.*, 1992).

Several other loci with demonstrated involvement in nodulation competitiveness have been found. Two examples are a locus in *R. leguminosarum* bv. *phaseoli* known as ORF3 (Michiels *et al.*, 1995), and an unnamed region of DNA in *S. fredii* (McLoughlin *et al.*, 1987). The latter has not been genetically characterized. Mutations in this region render the strain defective only in competitiveness, as its symbiotic properties are identical to the wild-type when inoculated alone (McLoughlin *et al.*, 1987).

1.7.4 Rhizobial inoculants

Many soils contain low populations of rhizobia, strains which fix nitrogen inefficiently, or strains which do not nodulate the agriculturally grown legume species of that region. In these situations, particularly if the available nitrogen is very low, rhizobial inoculation can potentially be of benefit. Inoculation is especially necessary when a legume is first

introduced to a particular soil, as it is unlikely that appropriate rhizobia will already be present. When considering natural isolates for use as inoculants, it is very important to identify factors which influence both nitrogen fixing efficiency and competitve ability.

The success of nitrogen-fixing inoculants in the field is influenced by many factors, including the amount of inoculum added, the abundance of native rhizobia that are capable of nodulating the host plant (Ikram and Broughton, 1981; see also review by Dowling and Broughton, 1986), and the environmental and biological factors identified in Section 1.7.2 which influence nodulation competitiveness.

A superior inoculant strain would need to display the following characteristics: good competitive ability with other soil micro-organisms, good competitive ability for nodulation against indigenous strains, and efficient nodulation and nitrogen fixation rates, leading to enhanced plant productivity under nitrogen limiting conditions. An inability to persist for more than one growing season is also desirable. Reasons cited for this include reduced biological hazard, commercial success, and ability to subsequently introduce new, more effective inocula (Triplett, 1990). Other researchers disagree, taking the view that the most effective bacteria are those which are able persist over several growing seasons (Howieson, 1995).

Attempts to use superior inoculants to increase legume productivity often fail, because nodulation tends to be dominated by indigenous strains. The native bacteria are much better adapted to the environment, and are often able to occupy nodules to the exclusion of inoculant strains. When inoculation does improve crop yield, the population of native rhizobia is generally very small (Triplett and Sadowsky, 1992). Even when the level of inoculation is far in excess of the native population, the indigenous strains can still predominate (Weaver and Frederick, 1974a; Weaver and Frederick, 1974b). The frequent inability of inoculants to compete effectively is known as the *Rhizobium* competition problem (Triplett, 1990). This failure is generally due to an inability to compete against the native strains for nodulation, rather than an inability to colonize the

rhizosphere (Ikram and Broughton, 1981). Patterns of nodule occupancy do not always correlate closely with the relative populations of the competitors in the rhizosphere, nor with their specific growth rates (Demezas and Bottomley, 1986; Frey and Blum, 1994).

The *Rhizobium* competition problem can sometimes be overcome by repeatedly inoculating with cell numbers hugely in excess of the native population, although the success of an inoculant is not assured simply by numerical dominance. Weaver and Frederick (1974b) estimated that in order for half of the nodules on soybean to be occupied by a *Bradyrhizobium* inoculant, the inoculant had to be added in numbers 1000 times greater than the population of bradyrhizobia in the soil. Similar findings were obtained by Meade *et al.* (1985) for a *R. leguminosarum* bv. *viciae* inoculant. They found that when the populations of inoculant and native strains were equal, nodulation was dominated by the native bacteria, even though it had been shown that the strains were equally competitive when grown under laboratory conditions.

Other examples can be cited where rhizobial inoculants have initially survived, but failed to persist after the first growing season. Also, the phenomenon of autoregulation, whereby infection by one strain can suppress subsequent nodulation by another (Kosslak and Bohlool, 1984; Sargent *et al.*, 1987; Stephens and Cooper, 1988), suggests that indigenous rhizobia may be able to prevent even very competitive inoculant strains from nodulating (Dowling and Broughton, 1986; Stephens and Cooper, 1988).

There are some examples where inoculants have successfully become established. Dunigan *et al.* (1984) inoculated soybean plants with large numbers of *B. japonicum* over three consective years, leading to its eventual establishment in the soil. However, the establishment of inoculant strains can introduce further problems. Earlier this century, a *Bradyrhizobium* sp. was introduced to soybean-growing regions of North and South America which had extremely low numbers of native rhizobia. The inoculant was extremely competitive in nodulation and successfully persisted in the environment, but had low nitrogen fixing efficiency and did not increase crop productivity. The problem

which arose is that this strain is so highly competitive for nodulation that it is now extremely difficult to displace it with the introduction of more effective nitrogen fixing strains (Maier and Triplett, 1996).

In recent years, genes have been found which increase the level of nitrogen fixation. Strains capable of recycling the hydrogen gas generated as a result of the nitrogen fixation reaction, by hydrogen oxidation, are more efficient at fixing nitrogen due to conservation of energy (Evans *et al.*, 1987; Maier and Triplett, 1996). Many factors which influence rhizobial competitive ability have been identified, and it will be necessary to more fully understand these if the *Rhizobium* competition problem is to be solved.

1.8 Rhizopines

1.8.1 Introduction

It has been known for many years that different species and strains of *Agrobacterium* can induce the synthesis of a range of compounds known as opines. Each opine can be catabolized and used as a growth substrate only by the strain which induces its synthesis. This led to the establishment of the "opine concept" which states that the biological reason for opine synthesis is to provide a chemical environment which is selective for the strains carrying the pathogenic plasmid, thus creating an ecological niche for the pathogen (Tempé and Petit, 1983). Tempé and Petit (1983) tried to extend the opine concept by investigating whether members of the closely related genus *Rhizobium* had the ability to synthesize opine-like compounds and obtain a similar benefit. If the strain of *Rhizobium* nodulating a plant was to receive a specific nutritional advantage from the association, it would have an advantage in the rhizosphere over other competing rhizobia (Murphy *et al.*, 1995). Extracts from nodules produced by various strains of *S. meliloti* were biologically purified to remove non-specific carbon and nitrogen sources. Compounds which could not be catabolized by *Agrobacterium* were analyzed for

catabolism by the *Rhizobium* strain which had produced the nodules (Tempé and Petit, 1983). Those which were specifically catabolized by *Rhizobium* were later named rhizopines (Murphy *et al.*, 1987).

Rhizopines are simple sugar-like compounds that can be synthesized by some strains of *Rhizobium*. They are catabolized exclusively by those strains which elicit their synthesis, and not by other rhizobia or by other soil bacteria, and are thought to enhance the growth and competitive ability of the catabolizing strains (Murphy *et al.*, 1987; Gordon *et al.*, 1996).

A number of other compounds have been isolated which also enhance rhizobial growth. Trigonelline is a common secondary metabolite of legumes, and various *S. meliloti* strains are able to catabolize this compound, which may be available as a specific carbon source (Boivin *et al.*, 1990; Boivin *et al.*, 1991). Similarly, some flavonoids, such as luteolin, are able to enhance the growth of a variety of rhizobia independently of their capacity to induce *nod* gene expression (Hartwig *et al.*, 1991), while homoserine, which is produced by pea plants and secreted in root exudates, can serve as the sole carbon source for *R. leguminosarum* bv. *viciae* (van Egeraat, 1975). However, the most specific example of such compounds is that of rhizopines, in which both the synthesis and catabolism genes are contained in the bacterium and are fully regulated by the bacterium (Murphy *et al.*, 1995).

Rhizopines were initially studied by analogy to the *Agrobacterium*-opine system. Even though opines and rhizopines have some structural similarities, appear to function as selective growth substrates, and the interactions both involve plasmids, it soon became clear that the two systems are markedly different. There is no transfer of DNA to the host in the *Rhizobium*-legume association (Murphy *et al.*, 1988; Murphy and Saint, 1992), and the rhizopine genes are fully regulated by the bacterium (Murphy *et al.*, 1988), unlike in *Agrobacterium*. In this respect rhizopines differ also from trigonelline, flavonoids and homoserine (Murphy *et al.*, 1995).

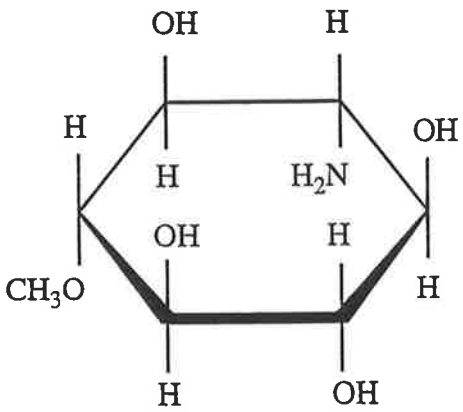
1.8.2 The occurrence of rhizopines

Two different rhizopines have been discovered so far, L-3-*O*-methyl-*scyllo*-inosamine (3-*O*-M*S*I) and *scyllo*-inosamine (*S*I). Both belong to the class of organic compounds known as inositols. 3-*O*-M*S*I is by far the most abundant and is produced by all known rhizopine strains except for *S. meliloti* strain Rm220-3, which produces *S*I. The two compounds differ only in the presence of a methoxy group in the C_3 position of 3-*O*-M*S*I (Figure 1.2).

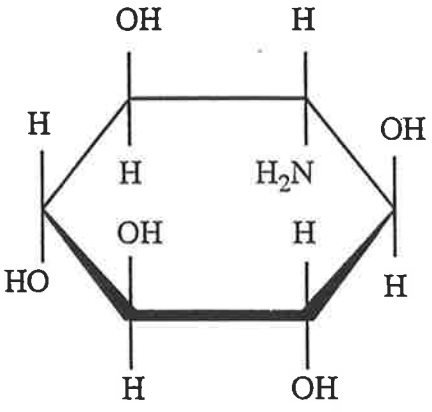
A related compound, rhizolotine, has been found in *Lotus*. Although rhizolotine can only be catabolized by the inducing strain, it does not seem to function as a growth substrate (Scott *et al.*, 1987), and so is not classed as a rhizopine.

A recent survey examining in excess of 300 strains of *Rhizobium* has revealed that 11% of *S. meliloti* and 12% of *R. leguminosarum* bv. *viciae* strains synthesize and catabolize 3-*O*-M*S*I. All of these strains were also able to catabolize *S*I. Rhizopine catabolism has always been found in association with rhizopine synthesis, supporting claims that the synthesis and catabolism genes have co-evolved as a functional unit (Murphy *et al.*, 1987). No *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *phaseoli*, *R. etli* or *R. tropici* strains were found with the ability to catabolize 3-*O*-M*S*I (Wexler *et al.*, 1995). Two other strains, from the former taxonomic grouping *Rhizobium* sp. (*Phaseolus*), were also found to catabolize 3-*O*-M*S*I, but these strains formed ineffective nodules and were not able to synthesize any rhizopine. This may have been due to the absence of an appropriate regulator, such as *nifA*, which is known to be necessary not only for symbiosis but also for rhizopine synthesis (Wexler *et al.*, 1995).

The fact that the rhizopine genes are located on the Sym plasmid in all species and strains examined so far suggests that they may also be involved in establishing symbiosis (Murphy and Saint, 1992). The coordinate regulation of *mos* and *nif* supports this assertion (Murphy *et al.*, 1988). However, non-rhizopine strains are still able to form an



(a) L-3-*O*-methyl-*scyllo*-inosamine (3-*O*-M*S*I)



(b) *scyllo*-inosamine (*S*I)

Figure 1.2 The structure of rhizopines (from Murphy and Saint, 1992).

effective symbiosis, and mutation studies have shown that rhizopines do not have any noticeable effect on nodulation or nitrogen fixation (e.g. Murphy *et al.*, 1988; Gordon *et al.*, 1996).

S. meliloti consists of two major divisions of strains, A and B (Eardly *et al.*, 1990). Division A is further divided into two subdivisions, A1 and A2. All of the rhizopine strains fall into subdivision A1. However, there is no evidence to suggest that the rhizopine strains constitute a single group of closely related strains within subdivision A1. Although rhizopine production is associated with particular Sym plasmid types, it is independent of the chromosomal genotype. In *R. leguminosarum* bv. *viciae*, there is some association between Sym plasmid type and rhizopine production, but rhizopine production is independent of chromosomal background. Rhizopine producing strains do not form a closely related subset of strains within this species (Wexler *et al.*, 1996).

Rhizopine strains have been isolated from geographically widespread locations and from both annual and perennial plants (Wexler *et al.*, 1995); however it is not known whether the strains examined were indigenous to the region from which they were obtained, or had been introduced.

The fact that the proportion of rhizopine strains is not larger may reflect recent evolution and on-going dispersal of the rhizopine genes in natural populations (Rao *et al.*, 1995). The location of the rhizopine genes on the Sym plasmid allows ample scope for their transfer and spread between rhizobial strains. This may explain their presence in diverse groups of rhizobia (Murphy *et al.*, 1995), although it is surprising that rhizopine strains have not been found in biovars of *R. leguminosarum* other than bv. *viciae*, considering that Sym plasmid transfer between the three biovars occurs naturally. It may be that they lack a necessary precursor for rhizopine synthesis, or can function only in strains with particular chromosomal backgrounds (Wexler *et al.*, 1995). It is possible that novel undiscovered rhizopines may be produced by other strains which are not inositols and cannot be detected by the methods currently used.

1.8.3 Genetics of rhizopines

1.8.3.1 Introduction

There are two loci involved in rhizopine synthesis and catabolism. The *mos* locus is responsible for rhizopine synthesis and the *moc* locus for rhizopine catabolism. Both loci consist of several genes. They are of bacterial origin and are located on the Sym plasmid in all strains examined so far. To date, the rhizopine genes have always been found together in strains which produce effective nodules: in a given strain of *Rhizobium* they are either both present or both absent. They are referred to as the rhizopine cassette and have probably evolved as a self-contained unit (Murphy and Saint, 1992).

Evidence confirming the bacterial location of the rhizopine genes was provided by *S.meliloti* strain L5-30. On the same species of plant, nodules produced by *S. meliloti* L5-30 contained the rhizopine 3-*O*-MSI, while nodules formed by some other strains of *S. meliloti* did not. Further studies revealed that the genes are on the Sym plasmid. A derivative of L5-30 which had a large deletion of the Sym plasmid, but with restored nodulation functions, was unable to produce either rhizopine (Murphy and Saint, 1992). The catabolism genes are also present on the Sym plasmid, and are closely linked to the synthesis genes, at least in *S. meliloti* (Murphy *et al.*, 1987; Saint *et al.*, 1993).

1.8.3.2 The *moc* locus

The rhizopine catabolism (*moc*) genes are expressed in free-living bacteria, which use rhizopine as a carbon growth substrate.

The *moc* locus was cloned by complementation of a Moc$^-$ strain with cosmid clones from strain L5-30. The region containing *moc* was narrowed down to 15kb, and two distinct functional regions were defined by Tn*5* mutagenesis and deletion analysis (Murphy *et al.*, 1987). Early reports suggested that in *S. meliloti*, four genes were involved in 3-*O*-MSI catabolism, *mocABC* and *mocR* (Rossbach *et al.*, 1994), although it was later reported

that only *mocCAB* were required (McSpadden *et al.*, 1996). Seven genes involved in catabolism of 3-*O*-M*S*I were identified in *R. leguminosarum* bv. *viciae*: *mocCABRDEF,* although *mocF* does not appear to be essential (Bahar *et al.*, 1999). The *mocD, E* and *F* genes have now also been found in *S. meliloti*, and these are in fact necessary for 3-*O*-M*S*I catabolism. Only *mocA* and *mocC* are required for the catabolism of *S*I in this species (Bahar, 1997).

Analysis of the DNA and protein sequences has led to putative functions being ascribed for some of these genes. *MocA* resembles an NADH-dependent dehydrogenase, and is thought to catalyze an NADH-dependent dehydrogenase reaction involved in the early stages of rhizopine degradation. The function of MocC is unknown and the protein shares little homology with any known protein (Rossbach *et al.*, 1994). *MocB* is likely to be a high affinity transporter which helps in the uptake of 3-*O*-M*S*I. It is thought to do this by interacting with some chromosomal gene products to sense and bind to the rhizopine, and subsequently import it into the bacterial cytoplasm. *MocD* is likely to be involved in the oxidation of the methyl group of 3-*O*-M*S*I, while *mocE* is probably a ferredoxin which supplies the electrons required for this oxidation. *MocF* shows homology to ferredoxin reductases. The product of this oxidation is *S*I, which would then be further broken down by *mocA* and *mocC* (Bahar, 1997). MocR is thought to play a regulatory role. It shows homology to the GntR family of regulatory proteins, which generally act as repressors of gene expression. However, *mocR* is unlikely to be a repressor, because when this gene is mutated, the phenotype is Moc$^-$. One member of the GntR family which shares homology with MocR, the FadR protein of *E. coli*, acts as a repressor of some genes but is an activator of others (Magnuson *et al.*, 1993). This supports the proposal that MocR may activate transcription of the other *moc* genes (Rossbach *et al.*, 1994). As *mocR* is required for 3-*O*-M*S*I catabolism but not *S*I catabolism, it might be a regulator of the oxidation process of 3-*O*-M*S*I to *S*I (Bahar, 1997).

1.8.3.3 The *mos* locus

The *mos* locus of *S. meliloti* was isolated from a clone bank by complementation of a Mos$^-$ strain, and the location was further delineated by deletion analysis (Murphy *et al.*, 1987). The *mos* genes of *R. leguminosarum* bv. *viciae* have not yet been found. The *S. meliloti mos* genes are organized as an operon with four open reading frames (ORFs). These are known as *mosA*, *mosB*, *mosC* and ORF1. The operon is transcriptionally activated by the common symbiotic regulator NifA. Direct regulation is thought to be achieved by binding to the promoter, which shows extremely high homology to the promoters of other NtrA-NifA-regulated genes, such as *nifH* (Murphy *et al.*, 1988). The coordinate regulation of *mos* and *nif* suggests that *mos* plays a symbiotic role (Murphy *et al.*, 1988). The proposed regulatory mechanisms of the *mos* gene would indicate that it is only expressed by the bacteroids in nodules, where oxygen concentration is low. *S. meliloti* Rm220-3 also has conserved *nifA* and *ntrA* recognition sequences in the 5' region of the *mos* genes, providing further evidence that the *mos* genes are directly regulated by a symbiotic promoter (Saint *et al.*, 1993). The *mos* genes are highly conserved in different rhizobial strains. A comparison between the L5-30 and Rm220-3 *mosB* and *mosC* genes has shown 97.5% homology at the DNA level (Saint *et al.*, 1993).

All of the *mos* genes except for ORF1 are expressed in nodules (Murphy *et al.*, 1993). The evidence indicates that the function of the MosA protein is to add a methyl group to *S*I, resulting in the formation of 3-*O*-M*S*I (Rao *et al.*, 1995). MosB is thought to have a regulatory function, while *mosC* has no significant homology to any known gene. The protein is very hydrophobic, and may play a role in membrane transport, either in the transport of precursors into the bacteroid or transport of rhizopine out of the bacteroid (Murphy *et al.*, 1993). Immunogold labelled antibodies have shown that the gene products of the L5-30 *mosA*, *B* and *C* genes are localized in the cytoplasm or membranes of bacteroids (Grzemski, 1994). The genetics of rhizopine synthesis will be discussed in more detail in Chapter 5.

1.8.4 The function of rhizopines

Rhizopines are synthesized by bacteroids in nodules, and are catabolized by the free-living bacteria of the same strain living in the infection thread and rhizosphere. In order to play a significant role in intra-specific competition, rhizopines must be reasonably widespread in nature, with the proviso that one particular rhizopine must not be too abundant, as this would result in no particular advantage for any strain (Murphy *et al.*, 1995). It was originally thought that the primary role of rhizopines was to give the bacteria a competitive advantage by providing a selective nutrient source in both the rhizosphere and the infection thread. They are a good carbon source but poor nitrogen source (Murphy and Saint, 1992; Murphy *et al.*, 1995), and are known to influence rhizobial competition for nodulation (Gordon *et al.*, 1996). This will be discussed in detail in Chapter 3. Other possible functions of rhizopines include improving the long term persistence under nutrient-limiting conditions, and providing an advantage for catabolizers in doubly infected nodules.

It was found that two rhizopine producing strains of *S. meliloti*, WSM922 and WSM826 (see Wexler *et al.*, 1994), were far superior at increasing the plant yield of various lucerne species compared to the commercial inoculant, CC169, which does not produce rhizopines (Evans and Howieson, 1993). The strains were not originally selected on the basis of rhizopine production. These experiments were carried out over a two year period and suggest both WSM922 and WSM826 persist and can efficiently nodulate plants over this time period. Another study in South Australia tested the persistence in the field of seven *S. meliloti* isolates which had been selected for high competitive ability (R. Ballard, unpublished). Subsequent studies of these strains revealed that four of the seven were able to synthesize and catabolize the rhizopine 3-*O*-MSI (A. Turner, unpublished data).

1.9 Scope of this thesis

Rhizopine-catabolizing rhizobia are known to have a competitive advantage in nodulating a compatible host plant. Although rhizopine was thought to be a selective growth substrate, competition studies have suggested that this is not their primary function (Gordon *et al.*, 1996). The first aim of the present study was to extend findings on the ability of rhizopines to influence intra-specific competition. Competition experiments were extended over a longer time frame, and under harsher conditions than experienced in the previous study. The objective was to investigate whether the dominance of the rhizopine-catabolizing strains would persist over several growing seasons, and secondly, to examine whether rhizopine would function as a specific growth substrate under harsh and nutrient-deficient conditions.

Studies were carried out examining the timing of rhizopine synthesis. The effects of competition observed in the Gordon *et al.* (1996) study occurred so rapidly that it was thought that rhizopine may be produced at an earlier developmental stage than was previously hypothesized. This was investigated by analyzing the roots of young lucerne seedlings to examine how soon after inoculation rhizopine could be detected.

The final aim was to isolate and characterize the rhizopine synthesis (*mos*) genes of *R. leguminosarum* bv. *viciae*. These genes have been located and sequenced in two strains of *S. meliloti* (Murphy *et al.*, 1987; Saint *et al.*, 1993), but previous attempts to locate the *R. leguminosarum* bv. *viciae mos* genes have not been successful (e. g. Wexler, 1994; Bahar, 1997). Transconjugant strains were used to show that the *mos* genes were present on the Sym plasmid. Two different approaches, R-primes and cosmid clones, were used in order to more closely define the genes.

CHAPTER 2

General materials and methods

2.1 Bacterial strains, plasmids and cosmids.

The bacterial strains and plasmids used in this study are listed in Tables 2.1 and 2.2.

2.2 Growth media and conditions

Rhizobium and *Agrobacterium* strains were grown at 28°C in TY complex media (Beringer, 1974) or GTS minimal media (Kondorosi *et al.*, 1984). *E. coli* strains were grown in LB media at 37°C (Miller, 1972), or M9 minimal media prior to transformation (Sambrook *et al.*, 1989). Solid media contained 1.5% agar (Oxoid, West Heidelberg, Vic.). Details of media are given in Appendix 1. Antibiotics were added where appropriate and were obtained from Sigma (Castle Hill, NSW). Details of antibiotic concentrations are given in Appendix 2.

Species	Strain description and reference/source
Agrobacterium radiobacter	K749; C58 pAt$^-$, pTi$^-$, Rif^r, Sr^r, Sp^r; (Donner *et al.*, 1993)
	K749(pSym1a::Tn*5-mob*); Km^r; (M. Wexler, unpublished)
Escherichia coli	HB101; *hsrR, hsrM, hsdS20, recA13;* Sr^r; (Boyer and Roulland-Dussoix, 1969).
	JM109; *recA1, hsrR17*; (Yanisch-Perron *et al.*, 1985).
	MC1061; F$^-$, araD139, *Δ(ara-leu)7696, galE15, galK16, Δ(lac)X74, rps*L, Sr^r; (Casadaban and Cohen, 1980).
Rhizobium leguminosarum bv. *viciae*	1a; Moc$^+$ Mos$^+$, produces 3-*O*-MSI; (Jensen, 1987)
	LS2090; 1a(pSym::Tn*5-mob*)(pJB3JI); Moc$^+$ Mos$^+$, Sr^r, Km^r, Tc^r; (L. Skot, unpublished)
	8401(pRL1); Moc$^-$ Mos$^-$, Sr^r, Rif^r; (Downie *et al.*, 1983)
	8401(pSym1a::Tn*5-mob*); Moc$^+$ Mos$^+$; Km^r; (M. Wexler, unpublished)

Table 2.1 List of bacterial strains used throughout this study. Strains constructed during this study are listed in Table 5.1.

Species	Strain description and reference/source
Rhizobium leguminosarum bv. *viciae*	P121; Moc$^-$ Mos$^-$; (G. Laguerre)
	P135; Moc$^-$ Mos$^-$; (G. Laguerre)
	P153; Moc$^-$ Mos$^-$; (G. Laguerre)
	P233; Moc$^-$ Mos$^-$; (G. Laguerre)
	P342; Moc$^-$ Mos$^-$; (G. Laguerre)
	Sp59; Moc$^-$ Mos$^-$; (J. P. W. Young)
Sinorhizobium meliloti	PM2338; L5-30; Moc$^+$ Mos$^+$; Gm^r, Sr^r; produces 3-*O*-M*S*I; (Kowalski, 1967)
	PM2126; pSym::Tn*5-mob*, derivative of L5-30; Moc$^+$ Mos$^+$; Gm^r, Sr^r, Km^r; (Murphy *et al.*, 1987)
	PM2129; *moc*::Tn*5-mob*, derivative of L5-30; Moc$^-$ Mos$^+$; Gm^r, Sr^r, Km^r; (Murphy *et al.*, 1987)

Table 2.1 cont. List of bacterial strains used throughout this study. Strains constructed during this study are listed in Table 5.1.

Species	Strain description and reference/source
Sinorhizobium meliloti	PM2168; *mos*::Tn*5*-*mob*, derivative of L5-30; Moc^{+} Mos^{-}; Gm^{r}, Sr^{r}, Km^{r}; (Murphy *et al.*, 1987) Rm1021; Moc^{-} Mos^{-}; (Meade *et al.*, 1982)

Table 2.1 cont. List of bacterial strains used throughout this study. Strains constructed during this study are listed in Table 5.1.

Plasmid	Size (kb)	Description and Reference
pJB3JI	58	Tra^+, Cb^r, Tc^r, Km^s derivative of R68.45; (Brewin *et al.*, 1980)
pLAFR3	22	Tc^r, broad host range Inc P-1 cosmid; (Staskawicz *et al.*, 1977)
pPH1JI	58	Gm^r, Inc P-1 plasmid of *Rhizobium* species; (Beringer *et al.*, 1978)
pRK2013	48	Km^r helper plasmid, contains RP4 *tra* genes cloned into a ColE1 replicon; (Figurski and Helinski, 1979)
R68.45	58	Ap^r, Tc^r, Inc P-1 conjugative plasmid of various gram negative bacteria; (Haas and Holloway, 1976)

Table 2.2 List of plasmids and cosmids used throughout this study.

2.3 Seed sterilization, plant growth conditions and inoculation

2.3.1 Seed and nodule sterilization

Pea (*Pisum sativum* L. cv. Greenfeast) and lucerne (*Medicago sativum* L. cv. Hunter River) seeds were surface sterilized by immersion for 2 minutes in 100% ethanol followed by 6 minutes in 2% sodium hypochlorite. They were then washed at least 6 times with sterile distilled water. Root nodules were surface sterilized for 5 minutes in 2% hypochlorite, followed by at least 6 washes with sterile distilled water. Rhizobia were recovered from nodules by plating squashed nodule extracts onto TY media containing appropriate antibiotics. Nodule squashes were incubated for 3 days at 28°C.

2.3.2 Plant growth conditions

2.3.2.1 Lucerne

Lucerne seedlings were grown in soil. The soil used was John Innes Mix (equal parts Gawler loam and coarse sand), and this was supplemented with superphosphate (1.2 kg/m^3), potassium sulfate (600 g/m^3) and calcium carbonate (600 g/m^3). The pH was adjusted to 7.4. The soil was steam pasteurized at 65°C for 30 minutes. Pots or test-tubes were used as appropriate. After surface sterilization, lucerne seeds were pre-germinated on TY agar and incubated at 28°C for 2 days prior to planting. In pot studies, several seedlings were sown in each pot and thinned as required after two weeks. In test-tube studies, one seedling was transferred to each tube. Plants were watered as required with filtered water, except for those grown in test-tubes which were watered with half-strength Hoaglands low nitrogen nutrient solution (Appendix 1.3). All plants were maintained in a glasshouse under natural light conditions, with a temperature of 22 - 26°C during the day and 16 - 18°C at night.

In studies on early rhizopine production, lucerne seeds were not pre-germinated and were sown at high density in 30 cm x 40 cm trays. Soil in this case was a 1:1 mix of steam pasteurized John Innes mix and vermiculite.

2.3.2.2 Peas

Peas were grown in 500 mL tissue culture flasks containing sterile vermiculite. They were grown in a growth cabinet with a photoperiod of 12 hours and a temperature range of 15 to 22°C. Plants were watered when required with sterile distilled water or sterile half-strength low-nitrogen Hoaglands solution. Pea seeds were not pre-germinated.

2.3.3 Inoculation with rhizobia

Natural isolates were grown in a 28°C shaking incubater (70 rpm, orbital mixer incubator, Ratek Instruments, Australia) for 2 days in 10 mL of TY broth supplemented with antibiotics where appropriate. Transconjugant strains were incubated for 3 days at 28°C, or until cell density was approximately 10^9 cells/mL. Immediately before inoculation, cells were centrifuged and resuspended in an equal volume of TY so that the culture was free of antibiotics. Seeds were inoculated at the time of sowing. The inoculum was applied by pipette to the soil surface close to the seeds. 500 mL flasks and 15 cm pots were inoculated with 1 mL of culture, 15 cm test-tubes were inoculated with 100 µL, and trays were inoculated with 10 mL per tray.

2.4 Gas Chromatography-Mass Spectrometry (GC-MS)

2.4.1 Sample preparation for GC-MS

A modification of the procedure of Anumula and Taylor (1992) was used for the acetylation of rhizopines for detection by GC-MS. Acetylation improves the volatility and thermal stability of the samples for the gas chromatography column. Samples were completely

dried in a vacuum and placed in glass test-tubes. 150 µL of acetic anhydride containing 5 mg/mL 4-*N*, *N'*-dimethylaminopyridine, 50 µL pyridine and 150 µL acetic anhydride were added separately with mixing after each addition. The reaction was allowed to proceed at ambient temperature for 4 hours and was terminated by the addition of 2.5 mL of water. After 10 minutes, acetylated derivatives were extracted from the acetic anhydride with an equal volume of dichloromethane. Samples were centrifuged for 10 minutes at 5000 rpm to aid phase separation, and the upper phase containing water and acetic anhydride was discarded. Samples were washed with water to remove any remaining acetic anhydride, and the water was removed with a pasteur pipette. Finally, the derivatives were dried in a stream of nitrogen.

The acetylated derivatives were resuspended in 20 µL dichloromethane immediately prior to injection into the gas chromatograph.

2.4.2 GC-MS conditions

The GC-MS analysis was performed using a Varian model 3400 gas chromatograph equipped with a Finnigan Mat TSQ 70 mass spectrometer (MS) (Hewlett Packard, Australia). The analytes were separated using a 30 m x 0.25 mm DB-1701 capillary column with a film thickness of 0.25 µm (J & W Scientific, Folsom, CA, USA). Samples were injected using splitless mode, and vented after 0.5 mins. The GC column was maintained at 50°C for 1 minute, ramped at 20°C/min to 150°C, then further ramped at 4°C/min to 250°C and held for 20 minutes. The injector and transfer-line temperatures were held at 84°C and 250°C respectively. Electron impact mass spectra at 70 eV were recorded by the MS over the range of m/z 35 to 350 with a scan time of 0.5 seconds.

Compounds were identified according to their mass spectrum, and by comparison of retention time with that of the internal standard, *myo*-inositol.

2.5 Triparental matings

Triparental matings were carried out in order to transfer plasmids from *E. coli* to *Rhizobium*. The helper plasmid pRK2013, in *E. coli* HB101, was used to facilitate this, as described by Ditta *et al.* (1980). Matings were incubated overnight at 28°C on solid TY media. Bacteria were resuspended in 0.95% saline and the transconjugants were selected either on GTS media or on TY media supplemented with appropriate antibiotics. The agar plates were incubated for 3-5 days at 28°C. Plasmid transfer was confirmed by standard minipreps using the small scale alkaline lysis procedure (Section 2.6.1.1), followed by electrophoresis of digested samples.

2.6 Recombinant DNA Techniques

2.6.1 Isolation of plasmid DNA

2.6.1.1 Small scale alkaline lysis

A standard alkaline lysis method was used (Sambrook *et al.*, 1989) to isolate cosmid DNA from *E. coli.* Isolation of cosmids from *Rhizobium* was also carried out using this procedure, with the slight modification that at the alkaline lysis stage, lysozyme was added to a final concentration of 10 mg/mL. This DNA was used for restriction analysis, and for confirmation of the presence of particular plasmids or cosmids in bacteria after conjugation or transformation.

2.6.1.2 Cesium chloride-ethidium bromide purification of plasmid DNA

Large scale preparations of plasmid DNA were performed by using a modified alkaline lysis procedure, followed by centrifugation on a cesium chloride-ethidium bromide gradient (Sambrook *et al.,* 1989). The modification used was that Brij-58 (polyoxyethylene 20 cetyl ether, Sigma) and sodium deoxycholate (Sigma) were used in the preparation of the cleared

lysates, as described by Clewell and Helinski (1970). Prepared DNA was used for restriction analysis and cloning.

2.6.2 Restriction digests, fragment isolation and ligations

Restriction enzymes and buffers were obtained from Boehringer-Mannheim (Castle Hill, NSW). Digests were carried out in the supplied buffer for 2-3 hours at 37°C.

DNA fragments were purified using a procedure based on the Progenius Bandpure DNA Purification Kit (Progen, Ipswich, Qld.). Fragments were excised from the gel and placed at 50°C in 3 volumes of 6 M NaI for 10 minutes or until the agarose slice had dissolved. An appropriate volume of silica matrix glass milk was added and centrifuged to pellet the silica matrix with the bound DNA. After washing three times in wash buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.5 in 50% ethanol), the pellet was resuspended in water, incubated 2.5 mins 50°C and re-centrifuged to elute the DNA. DNA was recovered by ethanol precipitation.

Dephosphorylation of fragments was achieved using 1.5 units of calf-intestinal alkaline phosphatase (Boehringer-Mannheim) in the recommended buffer at 37°C for 1 hour. Reactions were stopped with 2 µL of 0.5 M EDTA. This was incubated for 10 mins at 68°C and cooled rapidly on ice. The reaction volume was increased to 400 µL with water, and one phenol-chloroform extraction followed by a chloroform extraction were carried out to remove the inactivated enzyme (Sambrook *et al.*, 1989).

DNA ligations were carried out using T4 DNA ligase (Boehringer-Mannheim) at 15°C overnight, using the buffer supplied by the manufacturer.

2.6.3 Electrophoresis of DNA

DNA fragments were separated by electrophoresis in 0.8% agarose (Seakem ME, FMC Bioproducts, Rockland, ME, USA). The running buffer was either Tris-acetate-EDTA (40 mM Tris-HCl, 40 mM glacial acetic acid, 1 mM EDTA pH 8.0) (TAE) or Tris-borate-EDTA (89 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA pH 8.0) (TBE). 1 part gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll [type 400]) was added to 5 parts DNA sample prior to loading the gel. After electrophoresis, gels were stained with 10 μg/mL ethidium bromide and visualized under UV light.

2.6.4 Eckhardt gels

Large plasmids (greater than 60 kb in size) were separated by agarose gel electrophoresis, using the procedure of Eckhardt (1978) with minor modifications. In this procedure, the bacterial cells release their plasmids following lysis within the gel. Two combs were used in the gel, a 14 well comb, and a thick (5 mm) single-well comb. The thick comb was placed immediately behind, and in direct contact with, the 14 well comb. 100 mL of molten 0.65% agarose in TBE was allowed to set, and the large single-well comb was removed. Into this slot, a molten solution containing 0.5% agarose, 1% SDS and 0.05% bromophenol blue in TBE was added. The volume of cells to be used in the Eckhardt gel was based on the optical density A_{580} of the cell culture, and was determined by the calculations of Wexler (1994). Optical density was measured using a Turner Model 330 Spectrophotometer (Palo Alto, CA, USA). The cells were precipitated in a microfuge and the supernatant was removed. They were then frozen at -20°C for at least 10 minutes and up to 24 hours before use. Cells were resuspended in 20 μL of lysis buffer (50 μg/mL lysozyme, 125 μg/mL RNase A and 25% sucrose) immediately prior to loading onto the gel. Gels were run in TBE buffer for 30 minutes at 7 mA until the cells had lysed, then at 90 mA for 2.5 hours.

2.6.5 Competent cells and transformation

Competent cells were prepared using a calcium chloride method. 500 µL of overnight culture of *E. coli* strain JM109, HB101 or MC1061 was added to 10 mL LB and shaken at 37°C until the A_{580} was approximately 0.6. The cultures were chilled for 15 minutes and then precipitated by centrifugation. The cells were resuspended in 10 mL of ice cold filter-sterilized trituration buffer (100 mM $CaCl_2$, 70 mM $MgCl_2$, 40 mM NaAc, pH 5.5). The cells were then incubated for 10 mins on ice, then collected by centrifugation and resuspended in 160 µL of fresh trituration buffer.

The ligation mixture was mixed with the competent cells in a 1:4 ratio and incubated on ice for one hour. The mixture was heated for 2 mins at 42°C and then placed on ice for 2 mins. 500 µL of pre-heated SOC buffer (Appendix 1.6) was added to the cells, which were then incubated for 5 mins at 37°C without shaking, followed by 50 minutes at 37°C with shaking. Following this, 200 µl aliquots of the cells were plated onto LB agar plates containing the appropriate antibiotics.

2.6.6 Southern analysis

2.6.6.1 Southern Blotting

Southern blotting was carried out with minor modifications to the procedure of Southern (1975). Restricted DNA samples were separated on agarose gels, stained with ethidium bromide, and photographed. DNA was denatured in denaturation/transfer solution (0.4 M NaOH, 1 M NaCl) for 35 mins. The DNA molecules were transferred to a nylon membrane (Hybond-N+, Amersham, North Ryde, NSW) using transfer buffer (10x SSC) by capillary blotting overnight (Southern, 1975; Sambrook *et al.*, 1989). The membrane was washed in neutralization solution for 15 mins (500 mM Tris-HCl pH 7.2, 1 M NaCl). The transferred DNA was cross-linked to the membrane by exposure to UV light for 2 minutes.

2.6.6.2 Colony blots

Colony blots were carried out following the Genius System User's Guide supplied by Boehringer Mannheim, and all reagents were purchased from Boehringer Mannheim unless specified. Bacterial colonies were grown until the diameter of the colonies was 1-2 mm. The plates were chilled at 4°C for 1 hour. Membrane discs (Amersham) were placed on the colony-containing plates for 5 minutes. Membranes were placed cell side up on filter paper saturated with denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 minutes, followed by 15 minutes on filter paper saturated with neutralization solution (1 M Tris-HCl pH 8, 1.5 M NaCl). Finally, the membranes were placed on filter paper soaked in 2x SSC. The DNA was crosslinked to the membrane by exposure to UV light for 2 minutes. Membrane discs were then placed on a clean piece of aluminium foil, and 500 µL of freshly prepared 2 mg/mL proteinase K was pipetted onto each membrane disc. The solution was distributed evenly over the membrane, and this was incubated for 1 hour at 37°C. Cellular and agar debris was removed by blotting the membranes between filter paper.

2.6.6.3 Radioactive labelling of DNA

Radiolabelled probes were prepared by nick-translation incorporating [α–32P]dCTP. 1 µL (50-100 ng) of DNA and 1 µL of primer was used per labelling reaction. This was boiled for 5 mins to denature the DNA and immediately placed on ice for a further 5 minutes. 10x oligo buffer (60 µM each of dATP, dTTP and dGTP in 150 mM Tris-HCl, 150 mM NaCl, 30 mM $MgCl_2$, 300 µg/mL BSA), 3 µL of 32P dCTP and 2 µL (1-5 U) Klenow was then added. The labelling mix was incubated at 37°C for 2 hours. The labelled DNA was purified by spinning in a Sephadex G-50 column and used immediately.

2.6.6.4 Digoxigenin labelling of DNA

Probes were prepared by incorporation of digoxigenin-labelled dUTP (a thymidine analog) using the random primed method, following the manufacturer's instructions (Boehringer-Mannheim).

2.6.6.5 Hybridization and washing conditions

Digoxigenin labelled hybridizations were performed at 68°C in a shaking water bath (The Belly Dancer/hybridization water bath, Stovall Life Science Inc. Greensboro, NC, USA). Prehybridization and hybridization buffer was the standard solution described in the Genius System User's Guide (Boehringer Mannheim).

The prehybridization and hybridization buffer used for radiolabelled hybridizations was 6x SSC, 10% Denhardts solution, 0.5x SDS and 500 µg/mL herring sperm DNA. Radiolabelled hybridizations were performed in a bottle in a rotating hybridization oven (Hybaid Mini Hybridization Oven) at a temperature of 65°C. Membranes were washed in 2x SSC, 0.1% SDS at room temperature for 5 minutes and then twice in 0.1x SSC, 0.1% SDS at 65°C for 20 minutes.

2.6.6.6 Colorimetric detection of Digoxigenin labelled DNA

Digoxigenin labelled DNA was detected using the chemiluminescent substrate NBT solution (75 mg/mL nitroblue tetrazolium salt in 70% (v/v) dimethylformamide) and X-phosphate solution (50 mg/mL 5-bromo-4-chloro-3-indoyl phosphate, toluidinium salt in 100% dimethylformamide) according to the manufacturers (Boehringer Mannheim) instructions.

2.6.6.7 Autoradiography

Autoradiography was performed by exposure of radiolabelled membranes to X-ray film (Fuji RX) between two intensifying screens. X-ray cassettes were stored overnight at -80°C prior to development.

2.7 High voltage paper electophoresis (HVPE)

2.7.1 Electrophoresis conditions

HVPE was performed using the standard procedures described by Tate (1969). Whatman 1 Chr Chromatography paper (Dahl *et al.*, 1983) was cut into 15 cm x 45 cm rectangles. Samples were spotted, 0.5 µL at a time, along a pencil line ruled across the center of the paper, and air-dried. HVPEs were run at 3000 V for 15 minutes, however, longer runs of 45 minutes were occasionally performed to separate closely migrating compounds. The running buffer was formic/acetic acid pH 1.7 (28.4 mL 98% formic acid and 58.2 mL glacial acetic acid/2L). The inert buffer was perchloroethylene, which was purified by distillation.

2.7.2 Staining of paper electrophoretograms

Paper electrophoretograms were stained with silver nitrate (2 g/L in acetone) and air-dried. They were then transferred to 2% NaOH (w/v in ethanol) and left for 10 minutes to drip dry, then transferred to fixative (sodium thiosulfate 100 g/L and sodium metabisulfite 15 g/L). Finally they were washed in tap water for at least 5 hours.

2.8 Preparation and purification of 3-*O*-MSI

The rhizopine 3-*O*-MSI was extracted from pea nodules for use as a standard in GC-MS and HVPE. Pea plants were inoculated with the rhizopine producing strain, *R. leguminosarum* bv. *viciae* 1a (Section 2.3.3) and were grown for 4-6 weeks as described in Section 2.3.2.2. Batches of pea nodules (24 g) were ground in sterile distilled water with a sterile mortar and pestle. The extracts were centrifuged and the supernatant was filtered through 0.45 µm millipore filters to remove fine matter. In biological purification, nodule extracts were added to 200 mL flasks of Bergersen's medium (Appendix 1.1). Cells (1

mL) from an overnight culture of *Agrobacterium radiobacter* K749 were then added to the flasks. The flasks were incubated for 5 days in a 28°C shaking incubator. The cells were then pelleted by centrifugation and the supernatant was again filtered through 0.45 μm millipore filters and finally run through a Dowex 50W-X8 (H) cation exchange column (described in Section 2.11). The eluate was concentrated to a volume of 2 mL by rotary evaporation, and the pH was decreased to approximately 7 by adding several drops of dilute hydrochloric acid. Before use, catabolism tests were performed to ensure that the purified rhizopines could be catabolized by rhizobia.

2.9 Rhizopine synthesis tests

To test for rhizopine synthesis, *Rhizobium* strains were inoculated onto freshly sown pea seeds. Plants were grown for 5-6 weeks and the nodules were then removed from the roots. 100 mg of nodules were placed in a 1.5 mL eppendorf tube, and crushed in 300 μL of sterile distilled water with a wooden applicator stick. This was spun in a microfuge for 5 mins to remove particulate material. The supernatant was removed and extracted with 70% ethanol. After 1 hour at -20°C, it was re-centrifuged for 5 mins. The supernatant was collected, frozen in liquid nitrogen, and dried in a vacuum. The partially purified nodule extracts were resuspended in sterile distilled water at a concentration of 2.5 mg/mL. Detection of rhizopines in nodule extracts was achieved by HVPE (Section 2.7) and GC-MS (Section 2.4) where appropriate.

2.10 Rhizopine catabolism tests

Rhizobial cultures were grown in TY for 48 hours. 500 μL of the culture was harvested by centrifugation. The cells were washed in 0.95% saline and resuspended in Bergersen's minimal medium (Bergersen, 1961; Appendix 1). The A_{580} was adjusted to 0.5. 200 μL of the prepared cells, and 5 μL of 2 mg/mL 3-*O*-M*S*I, were added to sterile 10 mL polypropylene tubes. The tubes were incubated with shaking at 28°C for 5 days. To detect

rhizopine catabolism, 5 µL of samples were analyzed by HVPE. Known Moc^+ and Moc^- strains were used as controls.

2.11 Column Purification of 3-*O*-MSI

Root and nodule extracts were partially purified by passing through Dowex 50W-X8 (H) (BDH, Poole, England) cation ion exchange columns. The size of the columns depended on the volume to be purified, and varied from 6 mm x 5 cm for small-scale purification of extracts from 100 mg root nodules, up to 2.5 cm x 15 cm for large-scale purification of 3-*O*-MSI. Columns were left overnight in 0.1 M HCl. Prior to loading the samples, the pH was increased to approximately 2.5 by running water through the column. Samples were loaded at 1 ml per minute and left on the column overnight. The column was washed with 5 bed volumes of water which was also added at 1 mL per minute. The negatively charged compounds were eluted slowly (15 mL/hour) with 5 bed volumes of 2 M ammonia solution. The eluate was collected once the pH had reached 9. Higher yields were obtained when columns were left overnight in ammonia. Columns were discarded after one use to prevent cross-contamination of samples.

CHAPTER 3

Rhizopines and rhizobial competition

3.1 Introduction

According to the rhizopine concept (Murphy and Saint, 1992), rhizopines were proposed to function primarily as a selective growth substrate. A number of experimental tests of the rhizopine concept have been carried out, showing that strains able to catabolize rhizopine have a competitive advantage in nodulating a compatible host plant compared to those which do not have this ability (e. g. J. Nicol, P. Murphy and M. Ryder, unpublished; Gordon *et al.*, 1996). The experiments described in this chapter contribute to and extend findings on the effects of rhizopines on rhizobial competition for nodulation.

The competition experiments were carried out using the rhizopine strain, *S. meliloti* L5-30, and some Tn*5* transposon insertion mutants of L5-30. Three mutants were created (Gordon *et al.*, 1996): a mutant defective for rhizopine catabolism (Moc^-); one defective for rhizopine synthesis (Mos^-); and a neutral mutant, in which the transposon was inserted near the rhizopine gene cluster, but whose phenotype was Moc^+ Mos^+.

In the earlier competition study by Gordon *et al.* (1996), strain L5-30 was competed in a 1:1 ratio against each of the three mutants, and the nodule occupancy of the competitors was examined. When L5-30 was competed against the neutral mutant, approximately half of the nodules were occupied by each strain, showing that the insertion of the transposon did not adversely affect nodulation competitiveness. Similarly, when L5-30 was competed against the Mos^- mutant, each strain initially occupied about half of the nodules. This demonstrated that rhizopine synthesis *per se* did not affect nodulation competitiveness. However, the significant benefit of rhizopine catabolism was observed in competition

experiments between L5-30 and the Moc$^-$ mutant. In this situation, more than 70% of the nodules were occupied by L5-30, and less than 30% by the Moc$^-$ strain. It was evident that the Moc$^-$ mutant was at a considerable disadvantage in nodulating a host in a competition situation (Gordon *et al.*, 1996).

Although it was originally thought that rhizopine would influence intraspecific competition by providing a selective growth substrate, two observations of the competition dynamics suggested that this was not happening. The first is the speed with which L5-30 dominated nodulation. The dominance of this strain in nodules was already apparent when the first harvest was carried out, two weeks after inoculation. This was the earliest point at which nodules could be harvested, and would not have allowed time for sufficient rhizopine production to enable the catabolizing strain to multiply in the rhizosphere and elicit new nodules. Secondly, the degree of dominance of L5-30 over the Moc$^-$ mutant did not increase over time. It was expected that the proportion of rhizopine-catabolizing bacteria in the rhizosphere population would increase over time, leading to a concomitant increase in the proportion of nodules occupied by the rhizopine strain. Eventually 100% of nodules should be occupied by the rhizopine strain (Gordon *et al.*, 1996). The results were also inconsistent with the proposed mechanism for the regulation of rhizopine synthesis. There is evidence that the *mos* genes are under symbiotic regulation, and therefore rhizopines should only be produced by bacteroids in nodules (Murphy *et al.*, 1988). This will be discussed further in Chapter 4.

According to the model proposed for the synthesis and catabolism of rhizopine, it was expected that there would be a delay after inoculation before either strain would obtain an advantage, because initially no rhizopine would be present in the growth medium. Rhizopine synthesis would only begin once the first nodules were formed by the rhizopine strain. The synthesis of rhizopine and its subsequent release into the rhizosphere would then give the catabolizers a competitive advantage in initiating new infections. The

proportion of nodules occupied by the rhizopine strain would increase, as more rhizopine becomes available to free-living catabolizers in the infection thread and rhizosphere.

Competition between L5-30 and the Moc$^-$ mutant can be partially explained in terms of a frequency-dependent relationship. When L5-30 comprised 5% of the inoculum, nodule occupancy of the two strains was approximately equal, but when 85% of the inoculum was L5-30, nodule occupancy by L5-30 approached 100% (Gordon *et al.*, 1996).

Each of the mutants was able to induce an effective symbiosis, and a number of growth studies were carried out in both solid and liquid media, which showed no significant difference in the rate of growth between L5-30 and the mutants (Gordon *et al.*, 1996). However, it was also necessary to establish that these mutants did not have a reduced nodulation ability. Accordingly, the first aim of this chapter was to examine the individual nodulation characteristics of the Tn*5* mutants. Although the inserted transposons did not affect growth, they may have reduced the strains capability to nodulate a compatible host plant. In order to investigate this, the nodulation dynamics of each of the four strains was examined individually.

The second aim was to continue the competition experiments. The objective was to investigate whether the nodulation dominance of L5-30 over the Moc$^-$ mutant would persist over a much longer period of time. As rhizopine did not seem to be acting as a selective growth substrate under favorable conditions for the bacteria in a shorter term (10 month) experiment (Gordon *et al.*, 1996), the conditions in this extended study were altered. In this experiment, the plants and bacteria encountered various physiological stresses. It was reasoned that under nutrient-limiting conditions, and when the bacteria and plant were under some degree of physiological stress, rhizopine might be more likely to be a specific growth substrate. This could potentially lead to rhizopine providing a benefit in the long-term persistence of certain rhizobia in harsh or dessicating environments.

3.2 Materials and methods

3.2.1 Strains and strain construction

The four strains used in these experiments were *S. meliloti* L5-30 (PM2338), and the Moc$^-$ (PM2129), Mos$^-$ (PM2168) and neutral (PM2126) mutants. The mutants were derived from strain L5-30, and were created by insertional mutagenesis with the Tn*5* transposon. Their construction is described by Murphy *et al.* (1987) and Gordon *et al.* (1996). PM2338 is the wild-type L5-30 strain, except that it contains the plasmid pPH1JI. This plasmid was used in the construction of the Tn*5* mutants and is also present in the strains bearing the Tn*5* insertions. All strains are listed in Table 2.1.

3.2.2 Single strain nodulation experiments

3.2.2.1 Plant culture

Germination, surface sterilization of seeds, and soil, were described in Section 2.3. Two day old lucerne seedlings were sown in 15 cm x 2 cm test-tubes containing sterile soil. One seedling was sown in each test-tube. Plants were grown in a glasshouse under natural light conditions. They were watered when required with sterile half-strength Hoagland's low nitrogen nutrient solution (Appendix 1).

3.2.2.2 Nodulation experiments

The test-tubes were randomly assigned to one of four groups. Immediately after transplanting, each seedling was inoculated at the base with 100 µL (10^8 cells) of stationary-phase culture of either L5-30, Moc$^-$, Mos$^-$, or the neutral mutant. At regular intervals after inoculation, 15 plants were randomly selected from each of the four groups and the number of nodules on each plant was recorded. To confirm that nodules were formed by the inoculant strain and not by a contaminant, bacteria were re-isolated from nodules and tested for correct antibiotic resistance (Section 2.3.1).

3.2.2.3 Statistical analysis

The statistical analyses involved a standard one way analysis of variance, and least-squares linear regression.

3.2.3 Competition experiments

3.2.3.1 Plant culture

Germination, seed surface-sterilization and soil are described in Section 2.3. The original lucerne plants were sown in November, 1993 (Gordon *et al.*, 1996). Pre-germinated lucerne seedlings were sown in soil in 1 liter pots, with no added fertilizer. Several seedlings were sown in each pot, and later thinned to 1 per pot at the time of inoculation. Plants were periodically watered with filtered water, and were grown in a glasshouse under natural light conditions.

3.2.3.2 Competition experiments

The design of competition experiments was as described by Gordon *et al.* (1996). Lucerne seedlings were inoculated at the base two weeks after sowing with a mixture of the two competing strains. The mutant strains and L5-30 were grown separately in TY broth for 48 hours, and were mixed in equal numbers (5 x 10^8 cells of each) immediately prior to inoculation. The following three strain combinations were tested: Moc$^-$ and L5-30 (strain PM2129 versus strain PM2338), Mos$^-$ and L5-30 (strain PM2168 versus strain PM2338) and neutral mutant and L5-30 (strain PM2126 versus strain PM2338).

3.2.3.3 Nodule and bacterial sampling

Results for plants harvested prior to September 1994 were published by Gordon *et al.* (1996). The experiment was continued and the remaining plants were harvested in November, 1997. The frequency of both cell types in the nodules was monitored after inoculation by re-isolating free-living rhizobia from the nodules on the lucerne host plant.

The method for sampling nodules and determining bacterial occupancy was similar to that described by Gordon *et al.* (1996). The nodules were removed from the roots such that approximately 1 mm of root remained on either side of the nodule. They were surface sterilized (Section 2.3.1) and then placed individually in the wells of a microtiter plate. 40 µL of sterile distilled water was added to each well. The nodules were then crushed with a sterile wooden applicator stick, and the mixture was streaked out onto two TY agar plates, one containing 250 µg/mL streptomycin and the other with no antibiotics added. The plates were incubated for 3 days at 28°C. From each streptomycin plate, ten well-separated colonies were transferred with a toothpick to a fresh TY agar plate containing 250 µg/mL streptomycin, as well as one containing 250 µg/mL kanamycin and 250 µg/mL streptomycin. After incubation at 28°C for 3 days, the number of kanamycin resistant (mutant) and kanamycin sensitive (L5-30) colonies was counted. Strain L5-30 is resistant to streptomycin, and this antibiotic was used to prevent the growth of contaminant rhizobia. Less than 2% of natural isolates of *S. meliloti* have resistance to streptomycin (D. Gordon, unpublished data). The mutants could be distinguished from L5-30 due to the presence of a kanamycin resistance gene on the Tn*5* transposon.

3.2.3.4 Statistical analysis

For the purpose of analysis, a plant was considered the experimental unit. Six plants were analyzed from each treatment. The total number of L5-30 and mutant colonies was determined for each plant, and expressed as the ratio of L5-30 to total colonies. The total number of colonies per plant was equal to the number of colonies tested per nodule (10) multiplied by the number of nodules recovered from the plant. It was not possible to examine the same number of nodules on every plant. The statistical analysis was a Kruskal-Wallis one way analysis of variance. This is a non-parametric test which makes no assumptions about the distribution of the data (Kruskal and Wallis, 1952). A standard parametric analysis of variance, in which a normal distribution of data is assumed, was also carried out.

3.3 Results

3.3.1 Single strain nodulation studies

Lucerne seedlings were inoculated individually with one of the four strains used in competition experiments. Plants were inoculated with either strain L5-30, or with one of the mutants of L5-30: Moc$^-$, Mos$^-$, or the neutral mutant. At regular intervals after inoculation, plants were harvested and the number of nodules on each plant was counted.

The number of nodules per plant increased in a linear manner and at a similar rate for all four strains ($p < 0.001$) (Figure 3.1). No differences between the strains in the number of nodules recovered per plant were seen ($p > 0.65$), nor was any interaction between strain and time detected ($p > 0.32$).

Nodules were excised from the roots, surface sterilized, and plated onto selective media to retrieve the free-living bacteria. This was done to ensure that the occupants of the nodules were the strain which had been inoculated. All nodules were found to contain the correct bacteria based on antibiotic resistance and colony morphology. Control plants, which were not inoculated, were not nodulated.

3.3.2 Competition Studies

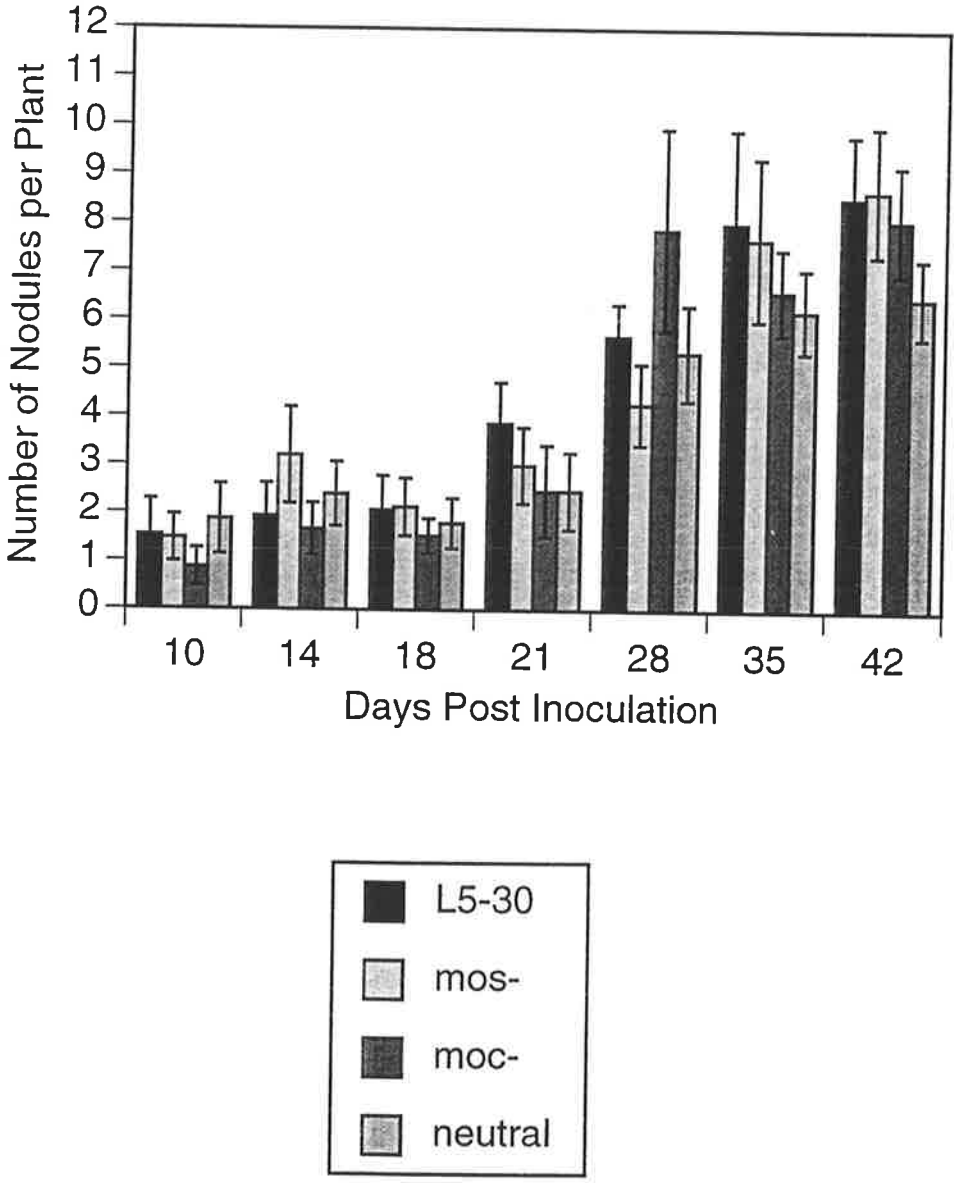
The rhizopine producing strain *S. meliloti* L5-30 was competed in a 1:1 ratio against the Moc$^-$ mutant, the Mos$^-$ mutant, and the neutral mutant. Nodule occupancy by each strain was examined after four years. The plants were grown under harsh conditions, which included confined space within pots restricting root growth, nutrient deficiency (no fertilizer was added), occasional water-stress, and periodic harvesting of shoots. These conditions reflect, in part, the type of conditions found in dryland lucerne production in southern Australia.

The growth of the plants after four years was poor, and the number of nodules was low. The number of nodules on each plant varied widely, several plants carried no effective nodules and were discarded, while others had more than 50 effective nodules. For this reason it was not possible to sample the same number of nodules from each plant. Many nodules were observed to be senescing or senescent, these nodules were not sampled due to the low probability of retrieving viable bacteria (data not shown).

The mean number of nodules sampled per plant for each treatment is given in Table 3.1. Very few nodules were found to be the result of mixed infections. The proportions of doubly infected nodules were 2%, 2.5% and 6% for the competition treatments involving the neutral mutant, the Mos$^-$ mutant, and the Moc$^-$ mutant respectively (see Table 3.1).

The Moc$^-$ mutant suffered a considerable disadvantage in competition for nodulation. L5-30 occupied almost 74% of the nodules when in competition with the Moc$^-$ mutant (Table 3.2). On the contrary, L5-30 occupied only 34% and 27% of the nodules when in competition with the Mos$^-$ and neutral mutants respectively. The proportion of nodules occupied by L5-30 in competition with Moc$^-$ was significantly greater than that occupied by L5-30 in the Mos$^-$ and the neutral mutant treatments ($p = 0.03$) (Kruskal-Wallis test). In addition, a standard parametric analysis of variance, in which a normal distribution of the data is assumed, was carried out. This test also showed a significant difference between the treatments: $F = 5.4$ ($p = 0.017$). The nodule occupancy of L5-30 in competition with the Mos$^-$ mutant was not significantly different from the nodule occupancy of L5-30 in competition with the neutral mutant.

Figure 3.1 Accumulation on lucerne plants of nodules produced by *S. meliloti* L5-30 and the Tn*5* mutants of L5-30. The number of nodules per plant increased in a linear manner (note that the x axis is not an interval scale). The vertical lines indicate 95% confidence limits of the means.


Number of Nodules per Plant
12
11
10
9
8
7
6
5
4
3
2
1
0
10
14
18
21
28
35
42
Days Post Inoculation
L5-30
mos-
moc-
neutral

Inoculant strains	Number of plants sampled per treatment	Mean number of nodules sampled per plant	% of nodules containing both strains
L5-30 and Neutral mutant	6	10	2
L5-30 and Moc$^-$ mutant	6	11	6
L5-30 and Mos$^-$ mutant	6	7	2.5

Table 3.1 Sample sizes in the competition experiment, and the proportion of nodules occupied by mixed infections in each treatment.

Competing strain	Initial frequency of L5-30 in inoculum	Frequency of L5-30 in nodules at 10 months	Frequency of L5-30 in nodules after 4 years
Neutral mutant	51.8 ± 3.26	29.9 ± 3.91	26.9 ± 11.41
Moc⁻ mutant	46.8 ± 3.26	74.1 ± 4.55	73.5 ± 5.41
Mos⁻ mutant	57.0 ± 3.22	23.9 ± 4.48	33.9 ± 17.51

Table 3.2 Frequency of nodules occupied by L5-30 in the competition experiments (% of total nodules). Figures are given for frequency in initial inoculum, 10 months after inoculation, and four years after inoculation.

3.4 Discussion

3.4.1 Single strain nodulation studies

It has been shown that each of the Tn*5* mutants of strain L5-30 does not have a significantly different growth rate from L5-30, either in liquid broth, or on agar plates (Gordon *et al.*, 1996). It was, however, necessary to check the individual nodulation of these strains, as a control for the competition experiments. It is possible that the growth studies might not have been sensitive enough to pick up a subtle reduction in growth rate, which could conceivably have significantly altered nodulation capacity. Alternatively, the nodulation of the mutants may have been impaired by the presence of the transposon, without affecting specific growth characteristics.

The poor nodulation competitiveness of the Moc$^-$ mutant, which was detected very early on in the initial nodulation experiments when this strain was competing against L5-30, could have resulted from one of two possibilities. Either the poor nodulation may have been caused by a reduction in ability to nodulate a host plant, or alternatively, by a reduction in nodulation competitiveness in the presence of other rhizobia. A reduction in ability to nodulate could be a non-specific effect of carrying the transposon, caused for example by a decrease in the speed of nodulation. A reduction solely in nodulation competitiveness would only be observed in competition for nodulation against a rhizopine catabolizing-strain, and would specifically implicate the *moc* genes as an important factor in intra-specific competition for nodulation amongst rhizobia.

No differences between the strains could be detected in the rate of nodulating a compatible host plant (*Medicago sativum*) when inoculated individually onto the plant. The insertion of the Tn*5* transposon did not affect the demographic characteristics of the strains (other than rhizopine synthesis and catabolism in the case of the Mos$^-$ and Moc$^-$ mutants respectively). No differences were found in the rate of nodule formation of each of the

strains over a period of five weeks, and there was no indication of any decline in the rate of nodule formation at this point.

The reason for the poor competitive performance of the Moc$^-$ mutant was not because this mutant has a reduced capacity to nodulate a host, caused by the transposon insertion. This poor competitive ability is a specific effect, which is appearing only when the Moc$^-$ mutant is in direct competition with a rhizopine synthesizing and catabolizing strain. The presence of the transposon neither enhances nor impairs the rate of nodulation of any of the mutants on a compatible host plant. This result suggests that an important underlying assumption of the competition experiments is justified, that is, that the transposon itself does not exert an adverse effect on the nodulation of the Moc$^-$ mutant.

3.4.2 Competition Studies

The presence at four years of low numbers of effective nodules on many plants, and an abundance of senescent nodules, was probably due to the harsh growth conditions. The plants and bacteria suffered occasional water stress, nutrient deficiency as no fertilizer was added to pots, lack of root-space within pots, and annual harvesting of shoot material.

Nodules normally form on the zone of young roots lying immediately behind the apical meristem, where the root hair cells are rapidly expanding (Bhuvaneswari *et al.*, 1981). Root hairs form primarily on young roots, and only occasionally do new root hairs emerge on older roots. Older root hairs do not become infected (Dart, 1975). Probably due to the growth conditions (particularly the lack of root-space within pots) and the age of these plants, many plants had relatively few new roots. This would have resulted in a lack of availability of appropriate sites for infection, leading to the formation of fewer nodules than would be expected on younger plants with an actively growing and expanding root system.

Lucerne root nodules do not normally persist for longer than one growing season, or one year. Under suitable conditions, new nodules would normally form on the ephemeral roots (Gault *et al.*, 1995). In this experiment, the initiation of new infections appeared to be occurring at low frequency. In addition to the limited production of new nodules, another factor contributing to the low nodule numbers may have been premature senescence of nodules and bacteroids. It is known that in indeterminate nodules, such as those found on lucerne, the bacteroids senesce naturally (Vasse *et al.*, 1990). Under optimum growth conditions, an equilibrium would ordinarily be reached, whereby there is a turnover in the nodule population. As the older nodules senesce, they would be replaced by a new generation of nodules forming on fresh ephemeral roots (Munns, 1968; Gault *et al.*, 1995).

Perennial legumes such as lucerne often lose nodules when unfavorable conditions are encountered. For example, loss of nodules can occur after harvest or grazing, during conditions of drought or dessication, or when there is a lack of nutrients (Kijne, 1975). Furthermore, a lack of nutrients, particularly photosynthates, or an interruption or interference to the carbohydrate supply, results in rapid senescence of bacteroids (Dart, 1975; Vance *et al.*, 1980). Interruption to the supply of photosynthates is commonly caused by factors such as harvesting and grazing, and this can result in very rapid nodule loss. As the amount of leaf tissue is reduced, the photosynthetic capacity of the plant is also decreased, and it may then be unable to supply sufficient photosynthates to the nodules. The common response to this is senescence of bacteroids and shedding or disintegration of the nodules (Vincent, 1974). Attack by insects, nematodes and fungi can also result in nodule senescence (Vincent, 1974), although these agents were not a factor in these experiments.

In the 10 month study (Gordon *et al.*, 1996), it was shown that the Moc$^-$ mutant was at a competitive disadvantage in nodulation compared to L5-30. This indicated that in the presence of rhizopine, a strain which can catabolize the rhizopine is at a distinct advantage

in forming nodules compared to a competitor which can not. Maximal dominance of the wild-type occurred within two weeks of inoculation, and this did not change even over a period of 10 months (Gordon *et al.*, 1996). This experiment was described in detail in Section 3.1.

It was initially thought that rhizopines would influence intraspecific competition by providing a selective growth substrate in the rhizosphere and infection thread (Murphy *et al.*, 1995). Although both the present competition study and the previous one (Gordon *et al.*, 1996), as well as several unpublished experiments by J. Nicol, P. Murphy and M. Ryder, have all shown that a rhizopine-catabolizing strain does have a competitive advantage in nodulation over the Moc$^-$ mutant, the results are not consistent with rhizopine acting solely as a selective growth substrate. A steady state was reached very soon after inoculation, whereby the proportion of rhizopine-catabolizing bacteria in the rhizosphere did not continue to increase. If rhizopine was acting as a specific growth substrate, it would be expected that the proportion of the rhizopine strain in the rhizosphere population would increase, leading to an increase in the percentage of nodules occupied by this strain.

The Gordon *et al.* (1996) study did not preclude the possibility that under harsher conditions, in particular, when nutrient sources were limiting, that rhizopine may be a growth substrate. Perhaps, in a nutrient-deficient environment, the rhizopine-catabolizing strains would utilize rhizopine as a nutrient source. In such a situation they should grow and survive better than other bacteria which can not benefit from these compounds. If this was happening, the proportion of nodules occupied by the catabolizers should increase slowly over a much longer time frame, as other nutrient sources become depleted.

The findings of the current experiment extend the previous investigation by Gordon *et al.* (1996) from 10 months to four years, with the added feature that the plants were grown under harsh nutrient-deficient conditions with occasional water stress. Plants were also

severely cut back three times, and, as they were grown for the entire four years in the same pots, the root system was stunted and potbound. The results presented here are almost identical to those of the shorter study. The level of dominance of L5-30 over the Moc$^-$ strain after four years has essentially not changed from that observed at 10 months. The results of this experiment therefore indicate that even under adverse conditions, rhizopines are not acting as a growth substrate.

The fact that the frequency of L5-30 had not changed in any of the treatments suggests that in the period between 10 months and four years, a steady state of competitiveness was reached, whereby there was some turnover in nodule population, and a stabilization of bacterial frequency. These results might suggest that nothing had happened over the previous three years, however this is extremely unlikely. It is unlikely that the original nodules, formed soon after inoculation, would have survived for four years, and even more improbable for there to have been neither cell division nor cell death of rhizobia during this time. In fact, there is some evidence to the contrary. In lucerne, the nodules resulting from the initial infections normally appear on the tap root and other main roots. After the initial phase of rapid nodulation, subsequent nodulation is much slower, and the new nodules form in groups on the newer roots (Munns, 1968). The nodules recovered after four years were found exclusively on fine, pale, ephemeral roots, indicating that they had been more recently formed.

In the Gordon *et al.* (1996) study, it was found that in the first two months after inoculation, nodule occupancy of the neutral mutant was equal to that of the wild-type. The neutral mutant was included in the experiments to measure any non-specific effects the Tn*5* insertion may have on the growth or nodulation of the bacteria, and the results showed that nodulation by this strain was not disadvantaged by the presence of the transposon. The Mos$^-$ mutant also occupied approximately half of the nodules when competed against the wild-type. Despite an inability to synthesize rhizopine, this strain was still able to catabolize the rhizopine produced by L5-30.

However, in both treatments, after approximately two months, nodule occupancy by L5-30 declined slowly (estimated decreases of 0.6% and 0.7% per week) to approximately 30% after 10 months (Gordon *et al.*, 1996). In this extended study, a similarly low nodule occupancy of L5-30 was observed. The frequency of L5-30 had not continued to fall, but had stabilized levels similar to those reported at 10 months. If the decline had continued at the same rate, it is unlikely that any L5-30 cells would have been isolated from nodules at four years.

It is difficult to explain why the frequency of L5-30 has declined. Although unlikely, it is possible that in the neutral mutant, a gene negatively affecting competition was inactivated by the transposon. The Mos$^-$ strain does not produce rhizopine, but can catabolize the rhizopine produced by L5-30. It would therefore be predicted that neither strain would derive a competitive advantage. A possible explanation for the advantage that the Mos$^-$ mutant enjoys, is that rhizopine synthesis is normally a metabolic drain on the strain, reducing it competitiveness. However, in normal circumstances, this is more than countered by the advantage conferred by rhizopine catabolism. Another explanation is that there may have been kanamycin-producing micro-organisms present in the soil, which reduced the growth, and particularly the nodulation, of L5-30.

The competition results presented in this chapter further support the conclusion that in the presence of rhizopine, the strain which can catabolize it has a competitive advantage in nodulation. It was shown that a rhizopine synthesizing and catabolizing strain had a competitive advantage in nodulation (Gordon *et al.*, 1996), and the competition study described in this chapter extends these findings over a period of four years under suboptimal growth conditions. Even after a long period of stressed growth, where there is minimal nutrition, the advantage conferred by rhizopine does not appear to be that of a growth substrate. The single strain nodulation studies eliminated the possilibility that the reason for the competitive difference was due to a reduction in the capability of the Moc$^-$

mutant to nodulate compatible roots. The Moc$^-$ mutant is at a disadvantage because of a decrease in nodulation competitiveness in a situation where there is a rhizopine synthesizing and catabolizing competitor.

These experiments have not alluded to the mechanism of the competitive advantage, but have shown that it does not result in complete dominance of the rhizopine-catabolizing strain even after four years. Rather than acting as a growth substrate, it is possible that rhizopines may have a direct effect on nodulation. For example, they may interfere with, or inhibit, one of the early stages in the infection process. The rapid effects of competition indicate that this interaction is occurring at a very early developmental stage, and suggests that perhaps rhizopines are being synthesized by rhizobia earlier than has been previously thought. The timing of rhizopine synthesis is discussed in detail in Chapter 4.

CHAPTER 4

The timing of rhizopine synthesis

4.1 Introduction

4.1.1 Regulation of the *mos* operon

Rhizopines are thought to be produced only by bacteroids within root nodules. Rhizopine synthesis has not been detected in free-living bacteria (P. Murphy, pers. comm.). The promoters of both the *S. meliloti* L5-30 and Rm220-3 *mos* genes have recognition sequences for the common symbiotic regulators, NifA and NtrA, which are activators of transcription (Murphy *et al.*, 1988; Saint *et al.*, 1993). The promoters show extremely high homology to those of other NifA-NtrA-regulated genes, such as *nifH* (Murphy *et al.*, 1988), which encodes the Fe-S dinitrogen reductase component of the nitrogenase enzyme (Ruvkun *et al.*, 1982). Rhizopines should therefore be produced under the conditions in which nitrogen fixation occurs, that is, in bacteroids in nodules. There is further evidence to indicate the involvement of NifA and NtrA in the regulation of the *mos* operon, as the start site for transcription of *mos* is almost identical to that of *nifH* (Murphy *et al.*, 1988). NifA and NtrA are both required for rhizopine synthesis, and NifA$^-$ and NtrA$^-$ mutants are unable to produce any rhizopine. The free-living nitrogen fixation regulator NtrC has no effect on rhizopine synthesis (Murphy *et al.*, 1988). Either NifA is directly involved in *mos* regulation, or the *mos* genes have a requirement for NifA-regulated functions. The high homology with the *nifH* promoter suggests that the former is more likely (Murphy and Saint, 1992).

The co-ordinate regulation of *mos* and *nif* suggests that *mos* plays a symbiotic role, although the *mos* genes are not needed for the establishment of an effective symbiosis

(Murphy *et al.*, 1988). Furthermore, nitrogen fixation is not a requirement for rhizopine synthesis, as NifH$^-$ mutants of L5-30 and Rm-220-3 retain the ability to synthesize their respective rhizopine (Murphy *et al.*, 1988; Saint *et al.*, 1993).

4.1.2 Rhizobial competition

The catabolism of rhizopines by some strains of *Rhizobium* results in a competitive advantage in nodulation for these strains over non-catabolizing strains (Gordon *et al.*, 1996). In Chapter 3, competition studies were described which demonstrated this competitive advantage. Chapter 3 also outlined how the competition results published previously by Gordon *et al.* (1996), and those described in this thesis, contradicted the assumption that rhizopines influence intra-specific competition solely by serving as a proprietary growth substrate. Briefly, this was suggested by the extremely early effects of competition, and the failure of the wild-type isolate, L5-30, to increase its dominance over the Moc$^-$ strain over an extended period of time. This indicated that rhizopines may be produced at a very early stage of nodule development, or that there may be a low level of *mos* gene expression in free-living rhizobia prior to the establishment of symbiosis.

The aim in this chapter was to investigate the timing of rhizopine synthesis. The reason for undertaking this study was to determine whether rhizopines are synthesized at an earlier stage of development than was previously thought. Very early expression of the *mos* genes, or a low level of constitutive expression, might explain the early effects of rhizopine on competition for nodulation. Expression of the *mos* genes was examined by phenotype. Root extracts from very young plants were analyzed for the presence of the rhizopine 3-*O*-MSI. The method chosen for the detection of rhizopines was gas chromatography-mass spectrometry (GC-MS).

4.2 Materials and Methods

4.2.1 Plant growth conditions

Lucerne seeds were sown in trays containing a 1:1 mixture of vermiculite and John Innes soil mix. Soil was steam pasteurized at 65°C for 30 minutes. Surface sterilized lucerne seeds (Section 2.3.2.1) were sown at high density in trays with dimensions of 40 cm x 30 cm x 12 cm. Approximately 1000 seeds were sown per tray. Seeds were not germinated prior to planting. Half of the trays were inoculated with a 10 mL culture of *S. meliloti* strain L5-30 (10^9 cells/mL) at the time of sowing. The remaining trays were not inoculated. At intervals after germination (1-2 days), one tray of inoculated plants and one tray of uninoculated plants were harvested. Plants were harvested daily between 3 and 12 days after inoculation, and then every three days until 21 days.

4.2.2 Extraction, purification and analysis of plant material

60 g of plant tissue was collected from each sample. Compounds were extracted from plant material for 1 hour in 70% ethanol. This was followed by a second 70% ethanol extraction, also for 1 hour. Samples were then centrifuged (10,000 rpm, 15 mins) to remove heavy particulate matter, and the ethanol was removed by rotary evaporation. The extracts were resuspended in sterile distilled water and filtered through 0.45 μM filters to remove fine material. They were further purified on Dowex 50W-X8 (H) ion exchange columns (Section 2.11). Partially purified plant extracts were examined for the presence of the rhizopine 3-*O*-M*S*I by GC-MS analysis of the acetylated derivatives of the plant compounds (Section 2.4).

4.2.3 Preparation of purified 3-*O*-MSI

Large-scale preparations of 3-*O*-MSI were made from pea nodules. Pea plants were grown as described in Section 2.3.2.2, except that they were watered with tap water rather than sterile distilled water. The plants were harvested after 8 weeks, and the 3-*O*-MSI was prepared from the nodules as described in Section 2.8. The final concentration of the rhizopine was approximately 2.5 mg/mL in sterile water, and the pH was adjusted to 7.0 by addition of several drops of dilute (0.1 M) HCl. The purified sample was used in preliminary experiments to determine the mass spectrum and expected retention time for 3-*O*-MSI, and later also as a standard for high voltage paper electrophoresis (HVPE) (described in Chapter 5).

4.3 Results

4.3.1 Detection of rhizopine by GC-MS

In GC-MS analysis, 3-*O*-M*S*I was identified firstly by its expected retention time, and by comparison with the retention time of standards such as *myo*-inositol and ononitol. *Myo*-inositol (Streeter and Bosler, 1976), and several epimeric and isomeric methyl-inositols, including ononitol, bornesitol, D-pinitol and *O*-methyl-*scyllo*-inositol are among the cyclitols which have been found previously in legume roots and nodules (Wagner *et al.*, 1969; Streeter and Bosler, 1976; Skot and Egsgaard, 1984; Streeter, 1985).

A 25 µg sample of biologically purified 3-*O*-M*S*I was acetylated and run on GC-MS using the conditions described in Section 2.4.2. Due to the large amount of the compound run through the gas chromatography column, and the fact that the sample was reasonably pure, the peak corresponding to 3-*O*-M*S*I was easily located (Figure 4.1a). The mass spectrum corresponding to this peak proved to be the expected spectrum for this rhizopine (Figure 4.1b). The penta-acetate derivative of 3-*O*-M*S*I has a molecular weight of 403 but due to the fragmentation in the gas chromatography column, the molecular ion was not observed in the mass spectrum. This was not unexpected, as the molecular ions of acetyl inositols, such as *myo*-inositol hexa-acetate, are barely detectable at ordinary sample pressures (Sherman *et al.*, 1970). 3-*O*-M*S*I is characterized particularly by a highly abundant peak of m/z 181. As this is the most intense peak, it was used as the base from which to normalize the relative abundance of all other ions. The molecular weight of 3-*O*-M*S*I is 193 amu, but this ion was not observed in the spectrum. Such an ion is precluded by the fragmentation pattern, which involves three consecutive losses of acetic acid to give an ion of m/z 223. The loss of one ketene group from this ion gives the base ion of m/z 181, and the subsequent loss of a second ketene group results in an ion of m/z 139. Therefore, the characteristic ions of 3-*O*-M*S*I are m/z 139 and m/z 181, which are always highly abundant, and are used as a "fingerprint" to identify 3-*O*-M*S*I. These ions have a stable

aromatic structure, which is very characteristic of cyclitols, resulting from the series of eliminations (Sherman *et al.*, 1970; Skot and Egsgaard, 1984).

The two most abundant and characteristic fragmentation ions of 3-*O*-MSI are 29 amu below the characteristic *myo*-inositol ions of m/z 168 and 210 (Sherman *et al.*, 1970), and 1 amu below the characteristic ions of methyl inositols of m/z 140 and 182 respectively (Skot and Egsgaard, 1984). These differences result from hydroxyl groups at all six carbon positions of *myo*-inositol becoming acetylated during derivatization, to form *myo*-inositol hexa-acetate (R-O-$COCH_3$; mw = 59), whereas in 3-*O*-MSI and all methyl-inositols, the methoxy group which is present at one carbon position does not become acetylated (R-O-CH_3; mw = 31). The difference between these species is 28 mass units. 3-*O*-MSI differs further from *myo*-inositol, and also from the methyl-inositols, in that it contains an amine group at the C 3 position. In the acetylation procedure, the amine group becomes acetylated (R-NH-$COCH_3$; mw = 58). In *myo*-inositol and the methyl inositols, a hydroxyl group is present at the corresponding carbon position, which also becomes acetylated, to form R-O-$COCH_3$ (mw = 59). The difference between these species is 1 amu.

Apart from the characteristic ions of m/z 139 and 181, other ions present in the 3-*O*-MSI mass spectrum which aided positive identification included m/z 128, 167, 209 and 223. Due to the absence of any common and specific fragments greater than m/z 350, this region of the spectra was not routinely examined in detail. The presence of a relatively abundant ion of m/z 128, as well as the highly characteristic ion of m/z 139, suggests that there may be parallel fragmentation pathways for 3-*O*-MSI, as differences of 11 amu are not observed in nature (Watson, 1985).

It should be noted that retention times do sometimes vary slightly between samples, and this is due to frequent replacement of columns to prevent cross-contamination. If two rhizopine samples are run consecutively on the same column, the rhizopine will have an identical retention time in both samples. However, when they are run on two different

columns, they often have slightly different retention times due to packing differences between the columns. Nevertheless, if they are run on the same GC-MS program, the retention time of rhizopine in different samples will remain similar, and its position relative to other compounds, such as the internal standard, will be the same. The time at which 3-*O*-*MSI* was predicted to come off the column was scanned with single ion traces for m/z 181. This ion is highly characteristic of 3-*O*-*MSI*, and was found to be rare in other compounds encountered in lucerne plant and root samples. Compounds containing ions of m/z 181 were analyzed further for the presence of other ions that help in the identification of rhizopine, such as m/z 128, 139, 167 and 223, and single ion traces were also sometimes also performed to locate compounds containing these ions.

4.3.2 Early detection of the rhizopine 3-*O*-*MSI* in plant tissue

Inoculated and uninoculated plants were harvested at daily intervals after sowing. Compounds were extracted from 60 g of plant material by two 70% ethanol extractions, and partially purified on a Dowex 50W-X8 (H) ion exchange column. The partially purified extracts were examined for the presence of the rhizopine 3-*O*-*MSI* by GC-MS. The extracts were resuspended in 20 µL dichloromethane, and 4 µL of this (equivalent to approximately 12 g of fresh plant tissue assuming 100% extraction efficiency) was injected into the GC column. The GC columns were changed between each sample to prevent cross-contamination. When this was not practical, blanks containing dichloromethane alone were run through, to ensure there was no carry-over of compounds from the previous sample. In addition, samples with the least amount of rhizopine were always run first, and those expected to contain larger quantities of rhizopine were run later.

The earliest that the rhizopine 3-*O*-*MSI* could be detected in plant extracts was six days after inoculation. It should be noted that as the seeds were not pre-germinated, and took approximately two days to germinate, the seedlings were on average four days old at this point. It was impractical to pre-germinate seeds in this experiment, due to the large number

of seeds required. The amount of rhizopine in root extracts gradually increased with time after six days, but remained extremely low until approximately 18 days after inoculation, when there seemed to be a massive induction of rhizopine synthesis (see Table 4.1).

In four day old plants, 3-*O*-*MS*I could barely be detected above background. No rhizopine peak could be seen on the total ion trace (Figure 4.2a). When the scale was magnified, a very small peak could be distinguished on a scan for the single ion m/z 181, and a corresponding peak at the same position could then be identified on the multiple ion scan, but only when the scale was magnified (Figure 4.2b) (note that figures 4.2a and 4.2b are not to the same scale). Confirmation that the compound was 3-*O*-*MS*I, and not a trace amount of an unrelated compound, was achieved with the correct mass spectrum (Figure 4.2c). The quantity of 3-*O*-*MS*I was close to the limit of detection of this system, and was estimated to be approximately 1 ng. Some ion peaks which are not characteristic of 3-*O*-*MS*I were observed. These have appeared because the amount of rhizopine is barely above background, leading to interference by contaminant peaks from other compounds. However, the mass spectrum contained sufficient detail for a positive identification to be made (Y. Hayasaka, pers. comm.). The rhizopine peak was absent from the uninoculated sample, (Figures 4.2d and 4.2e) and rhizopine could not be detected even when the scale was magnified substantially. In this sample, the mass spectrum obtained for the expected retention time of 3-*O*-*MS*I contained none of the characteristic ions, and it was thus concluded that this sample does not contain rhizopine (Figure 4.2f).

At 21 days after inoculation (19 day old plants), a rhizopine peak could be clearly seen on the total ion trace (Figure 4.3a). Single ion traces for m/z 181 and m/z 223 showed the expected peaks for 3-*O*-*MS*I (Figure 4.3b). The amount of rhizopine present was estimated to be approximately 50 ng. The rhizopine peak was not present in the 21 day old uninoculated plant samples, and a scan for m/z of 181 revealed no peaks providing a mass spectrum similar to that of 3-*O*-*MS*I (Figures 4.3c and 4.3d).

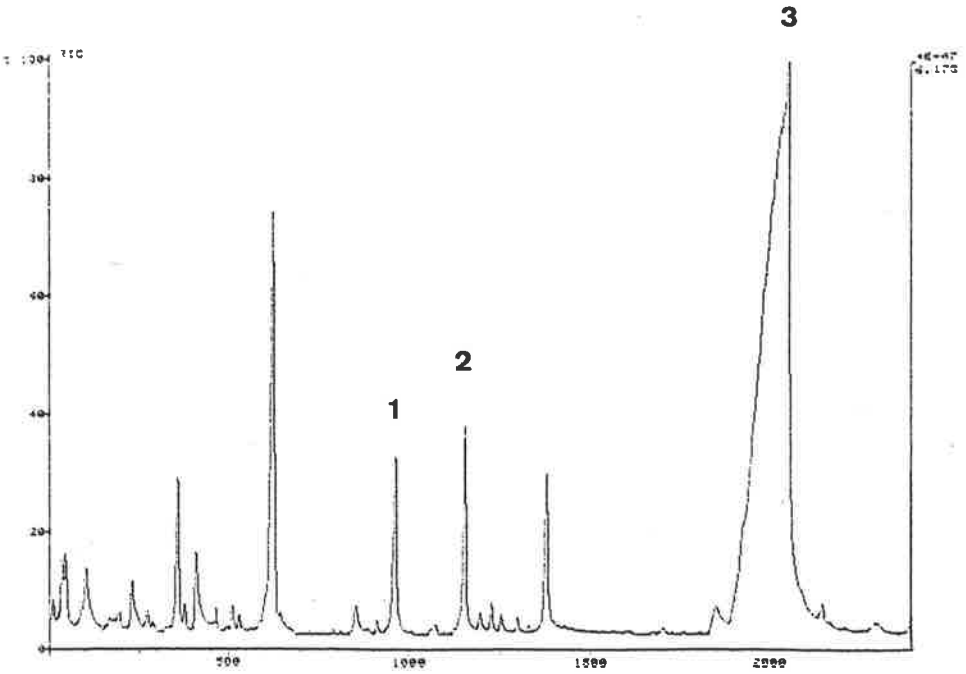
The first nodules became visible on the plants seven days after germination. By 19 days after germination, a number of nodules were visible on almost all plants. This correlates with the time of extensive rhizopine synthesis noted in the GC-MS results. Earlier than this time, nodule numbers were small, and many plants did not yet have visible nodules on their roots. At 21 days, small nodules (diameter less than 1 mm) were visible on the roots of most inoculated plants. All nodules were white in colour. The roots of uninoculated plants contained no nodules.

Quantification of rhizopines in samples is only approximate, due to difficulty in obtaining appropriate internal standards. The internal standard needed to be a compound of similar size and structure to 3-*O*-MSI, to enable it to be easily detected under the same GC conditions and the same MS analysis. *Myo*-inositol was used most frequently as an internal standard, but was used primarily as a guide to locate the rhizopine peak. It could not be used for precise quantification, because *myo*-inositol is a commonly occurring compound in legume root nodules (Streeter and Bosler, 1976), and use of this compound for quantification will therefore tend to over-estimate the amount of rhizopine present.

Figure 4.1 Detection of the rhizopine 3-*O*-M*S*I by GC-MS in a 25 μg sample of biologically purified rhizopine from pea nodules.

(a) Gas chromatogram showing large 3-*O*-M*S*I peak. The x axis indicates retention time on the GC column. The y axis indicates abundance. The scale on the left reflects relative abundance (relative ion current, RIC), the scale on the right provides absolute values.
peak 1, ononitol; peak 2, *myo*-inositol (internal standard); peak 3, 3-*O*-M*S*I.
(b) The mass spectrum corresponding to peak 3 identified in (a), note the characteristic highly abundant ions of m/z 139, 181 and 223, confirming this compound to be 3-*O*-M*S*I.

a


1
2
3

b

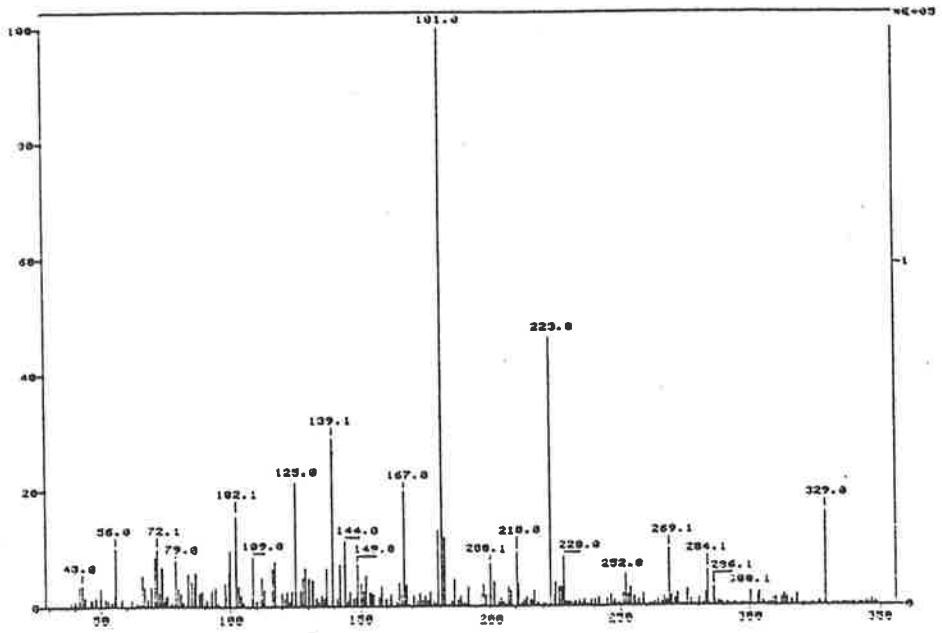
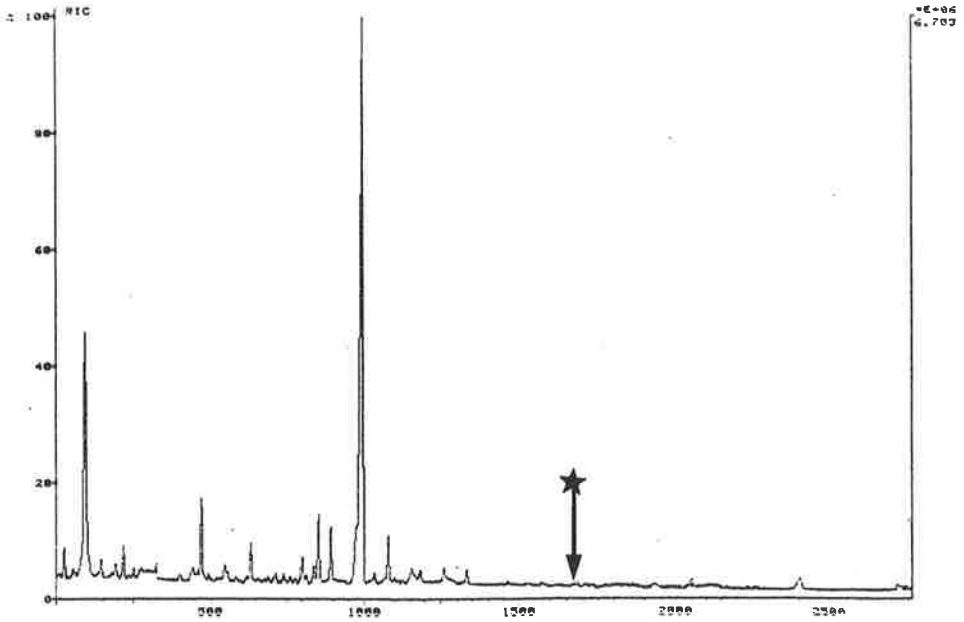

191.0
223.0
139.1
125.0
167.0
102.1
329.0
56.0
72.1
79.0
43.0
102.0
144.0
148.0
200.1
210.0
220.0
252.0
269.1
284.1
296.1
300.1

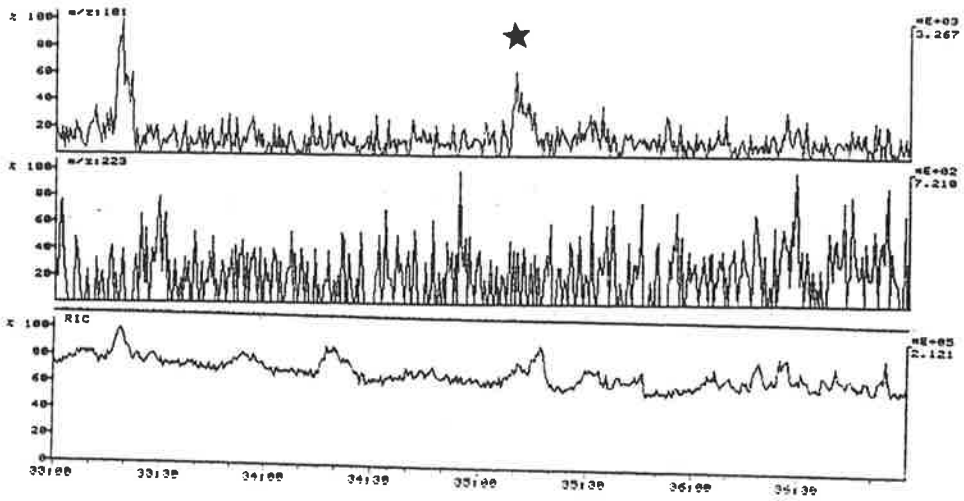
Figure 4.2 Detection by GC-MS of 3-*O*-*MSI* in four day old lucerne seedlings. Figures (a), (b) and (c) show results from inoculated plants, figures (d), (e) and (f) show results from uninoculated plants. In figures (a), (b), (d) and (e), the x axis indicates retention time on the GC column. The y axis indicates abundance. The scale on the left of the figures indicates relative abundance (relative ion current, RIC), the scale on the right indicates absolute values. In figures (c) and (f), the x axis shows molecular weight and the y axis represents ion abundance.

(a) The chromatogram shows no peak at the position where 3-*O*-*MSI* is expected to appear (indicated with a star and arrow). The asterisk denotes the internal standard, *myo*-inositol.
(b) In the single ion traces, using a magnified scale, a very small peak can be seen at the appropriate position in the m/z 181 scan (upper chromatogram) but not in the m/z 223 scan (middle chromatogram). On this magnified scale, an extremely small peak can also be seen on the total ion trace (lower chromatogram). The 3-*O*-*MSI* peak is shown by the star. The three chromatograms are on the same scale and are directly comparable.
(c) Confirmation of the presence of 3-*O*-*MSI* was achieved by obtaining the correct mass spectrum for the peak identified in (b). Note that several non-characteristic peaks are present, for example m/z 267. These come from a background of compounds of extremely low abundance, but appear more significant in these traces because of the magnified scale and the extremely low level of rhizopine present.

a


RIC

b


m/z:181
m/z:223
RIC

c

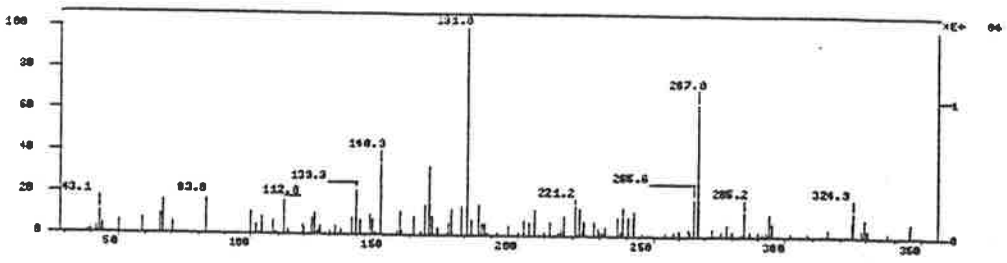

331.0
267.0
160.3
133.3
112.0
93.0
43.1
221.2
285.0
285.2
324.3

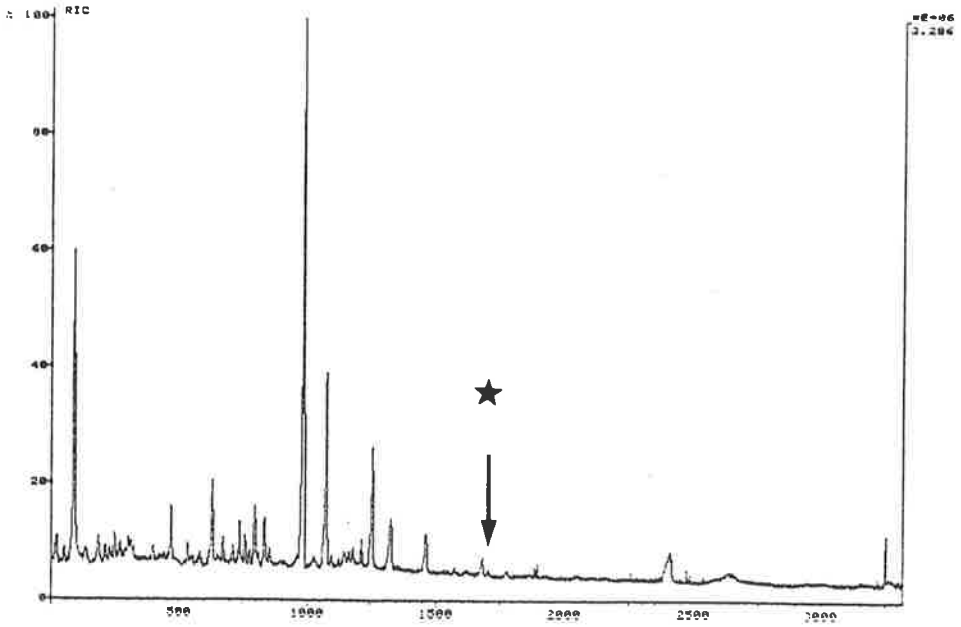
Figure 4.2 cont. Detection by GC-MS of 3-*O*-M*S*I in four day old lucerne seedlings.

(d) No 3-*O*-M*S*I peak can be seen on the multiple ion trace of four day old uninoculated plants. The position on the gas chromatogram where rhizopine is expected to appear is shown with a star and arrow.

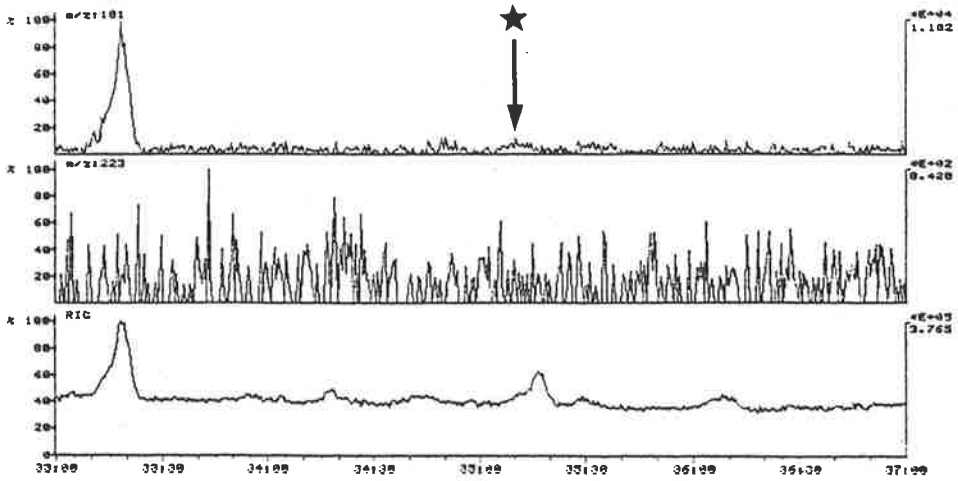
(e) No 3-*O*-M*S*I peak can be seen when the scale is magnified. At the expected position, indicated by the star and arrow, there are peaks neither in the total ion trace, nor in the single ion traces for m/z 181 and 223. The three chromatograms are on the same scale and are directly comparable.

(f) The mass spectrum taken for the retention time expected for 3-*O*-M*S*I reveals none of the characteristic ions, suggesting that this compound is not present in this sample.

d



e



f

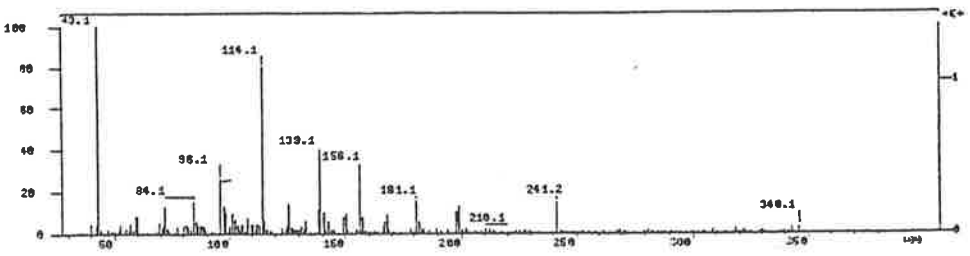
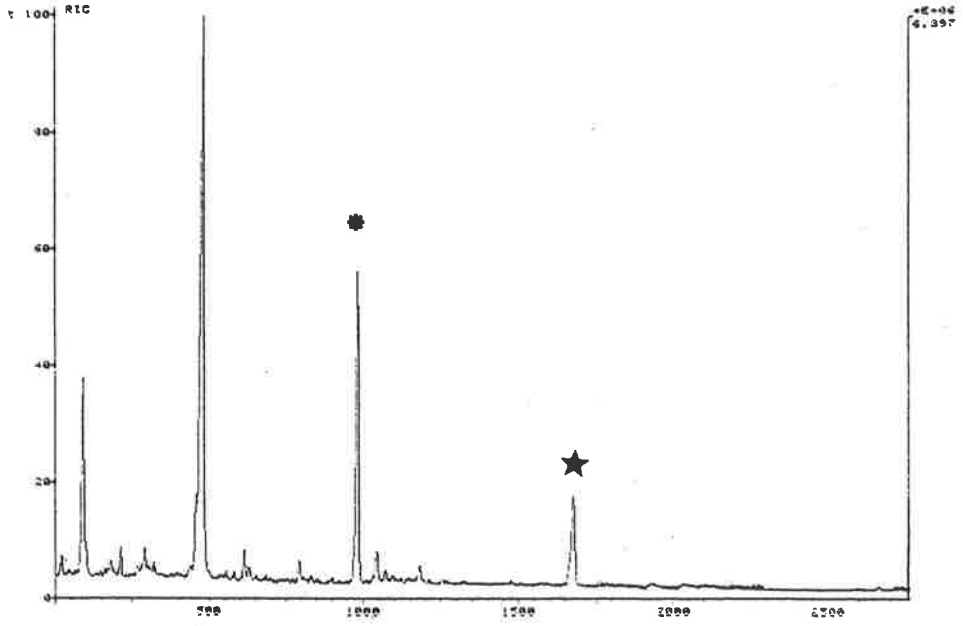


Figure 4.3 Detection by GC-MS of 3-*O*-MSI in 19 day old lucerne plants. Figures (a) and (b) show results for inoculated plants, figures (c) and (d) show results for uninoculated plants. In all diagrams, the x axis indicates retention time on the GC column. The y axis indicates abundance. The scale on the left of the figures indicates relative abundance (relative ion current, RIC), the scale on the right indicates absolute values.

(a) In the multiple ion trace, the 3-*O*-MSI peak is easily visible. This peak is marked with a star. The asterisk denotes the internal standard, *myo*-inositol.

(b) The peak can be further seen on single ion traces, where the common ions of m/z 181 and 223 show clear peaks corresponding to the peak identified on the multiple ion trace. The three chromatograms are on the same scale and are directly comparable.

a



b

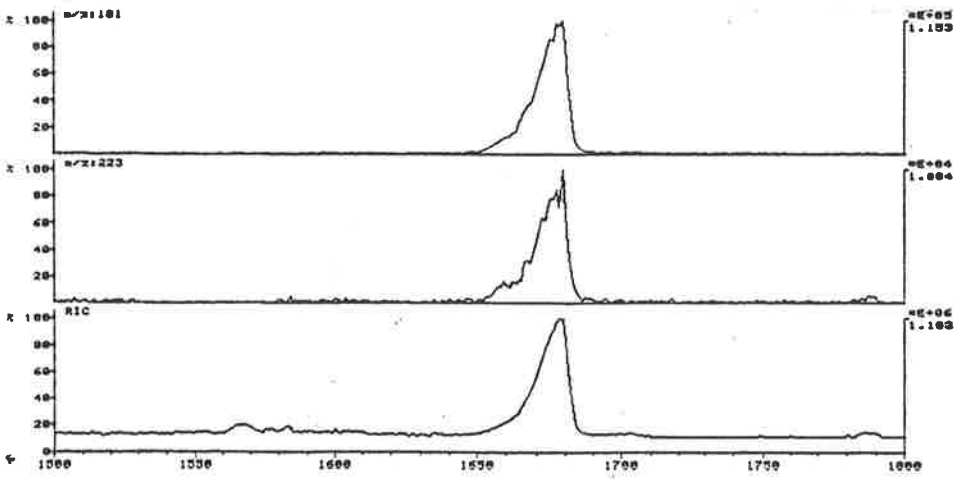
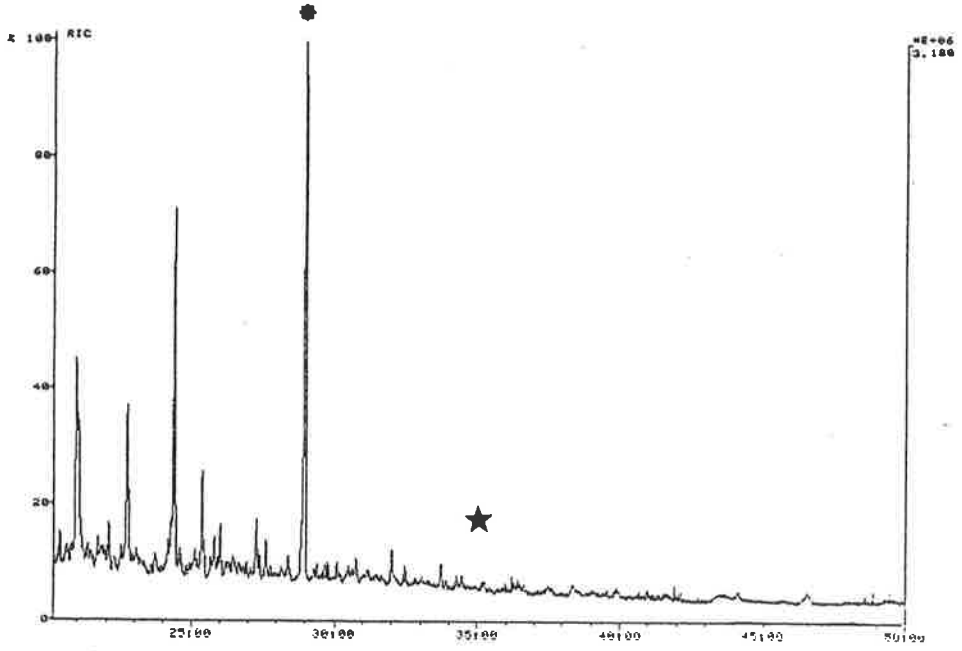


Figure 4.3 cont. Detection by GC-MS of 3-*O*-M*S*I in 19 day old lucerne plants.

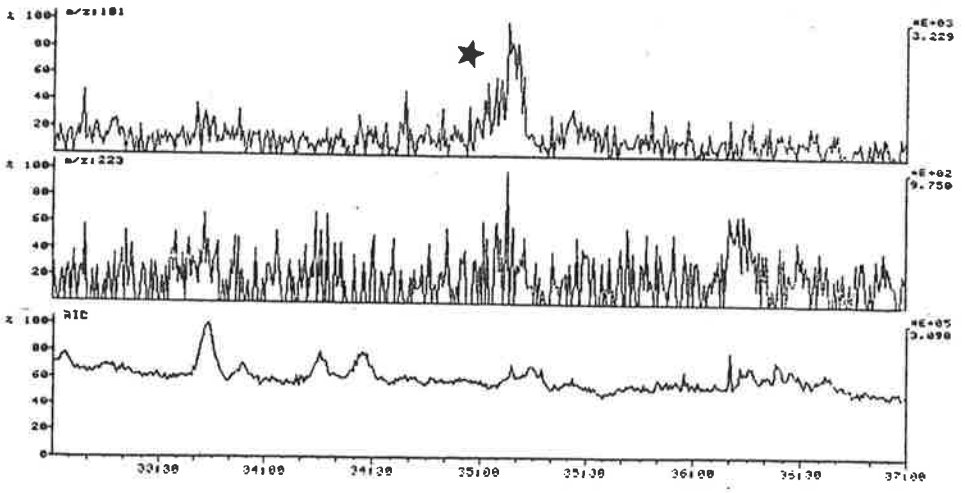
(c) No peak corresponding to the retention time of 3-*O*-M*S*I is present on the multiple ion trace of 19 day old uninoculated plants. The *myo*-inositol peak is indicated with an asterisk, the star indicates the expected position for 3-*O*-M*S*I.

(d) When the region of the gas chromatogram where 3-*O*-M*S*I is expected to appear is examined more closely on an expanded scale and using single ion traces, no peaks can be seen in this region, and no mass spectrum corresponding to 3-*O*-M*S*I was obtained. No 3-*O*-M*S*I can be detected in uninoculated plants after 19 days. The three chromatograms are on the same scale and are directly comparable.

c



d



Time after inoculation (days)	Approximate age of plants (days)	Relative amount of 3-*O*-MSI
3	1	-
4	2	-
5	3	-
6	4	+
7	5	+
8	6	+
9	7	+
10	8	+
11	9	+
12	10	++
15	13	++
18	16	+++
21	19	++++++

Table 4.1 The relative amount of the rhizopine 3-*O*-MSI present in plant samples at time intervals after inoculation.

4.4 Discussion

The rhizopine 3-*O*-MSI was first detected in inoculated lucerne seedlings four days after germination, well before the appearance of the first nodules at seven days. This is the earliest positive identification of 3-*O*-MSI to date, and indicates that there is a low level of *mos* gene expression in rhizobia prior to the induction of nitrogen fixation in the bacteroids. It is possible that rhizopine may be synthesized at an even earlier stage, at levels which were too low to detect. However, GC-MS is an extremely sensitive technique. For a compound the size of 3-*O*-MSI, GC-MS can detect nanogram amounts (Y. Hayasaka, pers. comm.), although when used for specific detection, GC-MS has been reported to pick up quantities as small as one picogram (McLafferty, 1980).

The GC-MS findings are consistent with the hypothesis that maximal rhizopine synthesis is achieved after the establishment of effective nitrogen-fixing nodules. Although these experiments showed the detection of 3-*O*-MSI in root extracts before nodules were visible, the appearance of rhizopine four days after germination was certainly after nodules had already been initiated, and therefore it is unlikely that this alone is sufficient to account for the observed competition results. Functional bacteroids are unlikely to be present at this early stage. However, the initiation of infection begins very soon after inoculation, and can occur within one hour of root hairs coming into contact with Nod factors (Heidstra *et al.*, 1994). For this reason, further studies should be carried out using other techniques, to investigate whether rhizopine can be produced by rhizobia at an earlier developmental stage, or by free-living rhizobia.

It is important to note that rhizopine synthesis was observed before fully functional nitrogen fixing nodules were present on the plant roots. This suggests that there may be a low level of rhizopine synthesis by rhizobia in pre-emergent nodules, when bacteroid development is minimal. Otherwise, if there is even a very low level of expression of the *mos* genes in free-living rhizobia, this could account for the competition phenomenon which is seen at

two weeks. This is a feasible hypothesis, as micro-aerobic conditions close to the root surface have been reported to result in the induction in free-living bacteria of some other NifA-NtrA-regulated genes. These include *melA* , which is involved in melanin synthesis (Hawkins *et al.*, 1988), *groESL3*, which encodes a chaperone (Fischer *et al.*, 1993), and *glnII*, which encodes a glutamine synthetase (Carlson *et al.*, 1993).

The massive increase in rhizopine found in plants between 18 and 21 days after inoculation is consistent with the *mos* genes being activated in bacteroids by the symbiotic regulators NifA and NtrA, as shown by Murphy *et al.* (1988). The low level of rhizopine recorded before this time is consistent with there being an early induction of these genes, perhaps in the free-living rhizobia close to the root surface.

Experiments examining the timing of rhizopine synthesis should be continued, so that the time of onset of rhizopine production can be more precisely determined and the levels of synthesis quantified. This could be done by looking at expression of the *mos* genes, rather than analyzing for the presence of the rhizopine compound in plant extracts. This would indicate exactly when the genes are being expressed, and would show, for example, when the genes are switched on, or if they are expressed constitutively. This could be done using a gene fusion in which the *mos* promoter is fused to a marker gene. Potential marker genes would be the *gus*, *lux* or *lac* genes. However, some problems have been encountered when using GUS in *Rhizobium,* because of read-through transcription from the *lacZ* transcript into the GUS coding region, followed by translational initiation at the GUS initiator (Jefferson, 1987). A better prospect may be to use the green fluorescent protein (GFP), which does not have the same problems associated with its use. In addition, the assay is easier to carry out because it does not require any exogenous substrates, and no fixing or staining of the organism is required in order to monitor GFP expression. GFP has already been used in *Rhizobium* to visualize colonization on the root surface, and growth and behavior of rhizobia within the infection thread (Gage *et al.*, 1996).

It was considered necessary to inoculate the plants at the time of sowing rather than after germination, due to the problem of contamination from extraneous rhizobia. It was not practical to pre-germinate the seeds on agar as was done in other experiments, because of the large number of seeds used. Although the soil had been steam pasteurized before use, the open glasshouse conditions meant that contamination from the air or water was possible. Some preliminary experiments had shown that even when all precautions were taken, lucerne, when grown in the glasshouse and inoculated after germination, sometimes contained a small proportion of contaminant nodules. However, in the experiments performed in this study, the lack of any nodules on uninoculated plants even after 21 days, and the inability to detect the rhizopine 3-*O*-*MSI* in uninoculated root samples by GC-MS, suggested that contamination was not occurring.

As discussed in Chapter 3, rhizopine did not function as a selective growth substrate for the catabolizing rhizobia in pot trials under glasshouse conditions. The experiments described in this chapter have shown that rhizopines are synthesized by rhizobia at a much earlier developmental stage than had been expected. Based on sequence data of the *mos* genes, it was anticipated that rhizopine would only be synthesized by bacteroids within nodules, under the conditions required for gene induction by the symbiotic regulators NifA and NtrA.

The synthesis of a small quantity of rhizopine at a very early stage of the symbiotic association probably facilitates the competitive advantage enjoyed by catabolizing strains. The small quantity also suggests that rhizopines act only in a localized environment, such as in the infection thread and in the rhizosphere immediately near the root surface. Despite a very low concentration in the rhizosphere, this would enable them to influence nodulation dynamics. The amount of 3-*O*-*MSI* at four days after germination was estimated to be approximately 100 picograms per gram of fresh weight of plant tissue, based on an extrapolation of the data presented here, and assuming 100% extraction efficiency. At this concentration, rhizopine may exert a direct effect on the nodulation process. Another

possibility is that rhizopine may increase the proportion of the infection threads initiated by non-catabolizing rhizobia which are aborted before nodule development. If this is the case, rhizopine would probably not need to be produced earlier than four days after inoculation in order to influence nodule occupancy, as the initial infection events would not be affected. Further competition experiments could be carried out to examine nodule occupancy within the first two weeks of inoculation, when the very first nodules are being formed. Also, microscopic studies could be performed to examine events on the root surface after spot inoculation of rhizobial cells onto the root.

To date, most of the progress towards understanding the *mos* genes, and rhizopine synthesis and its regulation, has been achieved in *S. meliloti.* Although some work has commenced in the search for the *mos* genes of *R. leguminosarum* bv. *viciae*, the genes have not yet been located in this species. Chapter 5 describes experiments carried out towards this aim.

CHAPTER 5

Isolation of the rhizopine synthesis genes of *R. leguminosarum* bv. *viciae*

5.1 Introduction

The *mos* genes of *S. meliloti* have been extensively studied and characterized, and have been sequenced in two strains, L5-30 and Rm220-3 (Murphy *et al,*. 1987; Saint *et al.*, 1993). They form an operon with four open reading frames, *mosA, mosB, mosC* and ORF1. The *mos* operon is approximately 5 kb in size and is located 4.5 kb from the *moc* gene cluster (Murphy *et al.*, 1987).

A comparison between the L5-30 and Rm220-3 *mosB* and *mosC* genes revealed 97.5% homology at the DNA level (Rao *et al.*, 1995). Restriction fragment length polymorphism (RFLP) analysis of the *mos* genes in other *S. meliloti* strains suggests that although there is a degree of genetic polymorphism, there seems to be a high level of sequence conservation throughout the species (Wexler, 1994; Wexler *et al.*, 1996).

The *mosA, B* and *C* genes are expressed in nodules. No protein product derived from ORF1 has been detected. This could be because this ORF is not expressed, or because the protein produced may be rapidly degraded. However, as a frameshift mutation in ORF1 still results in rhizopine production, it is assumed that no protein product is required for rhizopine synthesis (Murphy *et al.*, 1993). Although a protein product is not needed, the secondary structure of ORF1 may be important for mRNA stabilization (M. Wexler, pers. comm.).

As discussed in Chapter 4, the *mos* genes are thought to be under direct transcriptional regulation by the common symbiotic regulatory genes *nifA* and *ntrA*. The *mos* promoter contains putative binding sites for NifA and NtrA (Murphy *et al.*, 1988), and the absence of further putative promoters suggests that the entire operon is covered by one transcript (Murphy *et al.*, 1993). Based on the extensive homology with the *nifH* promoter, leader, and coding regions, it has been proposed that the *mos* locus acquired a symbiotic promoter by a duplication of the *nifH* gene and its regulatory region (Murphy *et al.*, 1988). Such rearrangements of symbiotic genes are relatively common in rhizobia (Petes and Hill, 1988; Romero *et al.*, 1991).

Immunogold antibody studies have shown that the gene products of the *mosA, B* and *C* genes in L5-30 are localized in the cytoplasm or membranes of bacteroids (Murphy *et al.*, 1993; Grzemski, 1994). Possible functions have been proposed for each of these genes. The function of the MosA protein appears to be to add a methyl group to *S*I, resulting in the formation of 3-*O*-M*S*I. *mosA* is completely absent from Rm220-3, and this strain can only synthesize *S*I. Further, deletion of *mosA* from the L5-30 *mos* locus leads to the production of *S*I instead of 3-*O*-M*S*I (Rao *et al.*, 1995). Curiously, MosA has extensive homology over its entire length to the DapA proteins of *E. coli*, *Corynebacterium glutamicum* and wheat. However, the MosA protein has approximately 30 additional amino acids at the *C*-terminal end (Murphy *et al.*, 1993). The *dapA* gene encodes the enzyme dihydrodipicolinate synthetase, which is the first enzyme in the lysine biosynthesis pathway. A recent study indicated that a *mosA*-hybridizing region in *R. leguminosarum* bv. *viciae* contains the conserved region corresponding with *dapA*, but not the additional amino acid residues. As a functional role in rhizopine biosynthesis has not yet been attributed to this region, it has been suggested that *mosA* may be a modified *dapA* gene (Bahar, 1997).

MosB was proposed to be a regulatory protein. It has two domains, one at the *N*-terminal end, and a central domain. The domain at the *N*-terminal end shows homology to the NifT

proteins from *Klebsiella pneumoniae* and *Azotobacter vinelandii*. The function of NifT is unknown, but in both species it lies immediately distal to the *nifHDK* operon and arises from the same transcript. MosB also has extensive homology with the FixU protein from *R. leguminosarum* bv. *trifolii* (Murphy *et al.*, 1993). The central domain of MosB has homology to several known bacterial proteins. Many of these, including DnrJ, EryCl, Prg-1 and StrS, are involved in carbohydrate metabolism. MosB has greatest homology with the *E. coli* DegT protein, which is a pleiotropic regulatory protein. DnrJ is also known to have a regulatory function. The similarity of MosB at the amino acid level to these two proteins suggested that MosB might also have a regulatory role (Murphy *et al.*, 1993), although this has not yet been shown experimentally (Murphy *et al.*, 1995). Interestingly, *stsC*, the gene responsible for the enzymatic conversion of *myo*-inositol to streptidine in the streptomycin biosynthesis pathway of *Streptomyces griseus,* was recently isolated (Ahlert *et al.*, 1997). The DNA sequence of this gene revealed considerable homology to *mosB*. It is possible, therefore, that the *mosB* gene may have both an enzymatic and a regulatory function.

The *mosC* gene shows no significant homology to any known gene, but the protein is very hydrophobic and has 12 putative membrane-spanning regions. This is characteristic of proteins involved in transporting sugars across membranes, and MosC has been shown to bind to bacteroid membranes (Grzemski, 1994). MosC may either be involved in transport of a precursor into the bacteroid, or, more likely, transport of rhizopine out of the bacteroid (Murphy *et al.*, 1993).

Several attempts have been undertaken to locate the *mos* genes in *R. leguminosarum* bv. *viciae* (e. g. P. Murphy, unpublished; Wexler, 1994; Bahar, 1997). This has proven to be a difficult task, and to date they have remained elusive. Previous studies have depended upon the use of *mosABC* probes from *S. meliloti* to isolate the equivalent genes from *R. leguminosarum* bv. *viciae*. These studies revealed a region of high homology to the L5-30 *mosA* gene, but limited homology to *mosB* and *mosC* (Wexler, 1994, C. Owuama,

unpublished data). The *mosB* and *mosC*-hybridizing regions are closely linked to each other, but are not linked to *mosA*. When separate clones containing these regions were transferred on compatible vectors to rhizobia, the recipients were unable to induce rhizopine production in nodules (Wexler, 1994; Bahar, 1997).

The objective of the work presented in this chapter was to isolate and characterize the *mos* genes of *R. leguminosarum* bv. *viciae* strain 1a. The aim was to initially show that in this strain, the *mos* genes are located on the Sym plasmid, and subsequently, to identify clones isolated from the Sym plasmid which have the ability to direct rhizopine synthesis after transfer to non-rhizopine strains.

5.2 Materials and Methods

5.2.1 Plasmid transfer

The Sym plasmid from *R. leguminosarum* bv. *viciae* strain 1a was mobilized into other rhizobia using LS2090. LS2090 is a derivative of strain 1a in which the Sym plasmid is marked with the Tn*5-mob* transposon (i. e. RP4 mobilizing genes cloned into Tn*5*). This strain also contains a broad host range helper plasmid, pJB3JI (see Table 2.1). Mobilization of the Sym plasmid into various rhizobial strains was facilitated using the transfer genes on this plasmid.

The recipient rhizobia were spontaneous chloramphenicol and/or rifampicin resistant derivatives of natural Moc$^-$ Mos$^-$ isolates of *R. leguminosarum* bv. *viciae*. These strains are listed in Table 5.1. Transconjugants were selected on TY medium containing kanamycin, chloramphenicol and rifampicin. To ensure that the rhizobia growing on the selective media were transconjugants and not spontaneous antibiotic resistant mutants of LS2090, Eckhardt gels were performed on cells (Section 2.6.4) to examine plasmid profiles.

Cosmid clones and R-primes were transferred to *Rhizobium* by triparental mating (Section 2.5). Transconjugant strains were selected on TY media containing tetracycline, and chloramphenicol and/or rifampicin. Confirmation of the presence of the plasmid of interest was achieved by Eckhardt gel electrophoresis of cells (for R-primes), or agarose gel electrophoresis of re-isolated plasmids after digestion with the restriction enzyme *Eco* RI (for cosmid clones).

5.2.2 General Recombinant DNA techniques

Small-scale isolation of plasmid DNA was described in Section 2.6.1.1. Large scale plasmid preparation and cesium chloride-ethidium bromide purification was described in Section 2.6.1.2. Restriction digests and ligations were performed as described in Section 2.6.2. The size of DNA fragments was determined by measuring their mobility in relation to a molecular weight standard, bacteriophage λ digested with *Hin*d III, following electrophoresis in 0.8% TBE agarose gels. Large plasmids were examined using Eckhardt gels (Section 2.6.4). Triparental matings were carried out as described in Section 2.5. Confirmation of the presence of appropriate fragments was achieved by digestion of plasmids with restriction enzymes, followed by visualization under UV light after separation on 0.8% TBE agarose gels (Section 2.6.3). Southern analysis, hybridizations and washing conditions were described in Section 2.6.6.

5.2.3 Selection of antibiotic resistant strains of *Rhizobium*

Spontaneous mutants were created by growing the natural isolates of *Rhizobium* or *Sinorhizobium* in flasks containing 50 mL TY broth for 1 week in the presence of low concentrations (10 μg/mL) of selected antibiotics (rifampicin and/or chloramphenicol). Resistant derivatives were transferred to media containing higher concentrations until the desired resistance was achieved (listed in Appendix 2).

5.2.4 Construction of R-primes

R-primes of the 1a Sym plasmid were constructed using R68.45. R68.45 is a conjugative plasmid which can mobilize large fragments of DNA between various Gram negative bacteria. This plasmid carries the insertion sequence IS21, which is able to transpose into the genome, thus generating a region of R68.45 homology. The recombination that follows can produce plasmids carrying large sections of inserted DNA.

Tn*5*-marked Sym plasmids from three bacterial strains were used as a basis for R-prime construction: LS2090 [1a(pSym1a::Tn*5*-*mob*)], 8401(pSym1a::Tn*5*-*mob*) (both strains of *R. leguminosarum* bv. *viciae*), and *Agrobacterium radiobacter* K749(pSym1a::Tn*5*-*mob*) (see Table 2.1). The latter two strains were constructed by transfer of the Tn*5*-marked Sym plasmid from LS2090, and were selected by kanamycin resistance, conferred by the Tn*5*-*mob* transposon (L. Skot, unpublished; M. Wexler, unpublished).

These bacteria were mated with R68.45. Matings were incubated overnight at 28°C on non-selective solid media. Bacteria were resuspended in 0.95% saline, and selection was then carried out on TY medium for matings in which the donor was a *Rhizobium* strain, and on GTS minimal medium where the donor was *Agrobacterium*. In both cases, media were supplemented with kanamycin and tetracycline, and bacteria were incubated at 28°C for 3 - 5 days. R68.45 containing Tn*5*-*mob* and a section of Sym plasmid DNA was then transferred to *E. coli* HB101 Nal^r. Matings were plated onto TY medium, and colonies containing the R-prime were selected by resistance to kanamycin, tetracycline, and naladixic acid, and were incubated at 37°C. R-primes were checked to be of sufficient size by Eckhardt gel electrophoresis (Section 2.6.4) and were then transferred to *Rhizobium* strains as described in Section 5.2.1.

5.2.5 Construction of a cosmid clone bank

A cosmid clone bank of the *R. leguminosarum* bv. *viciae* 1a Sym plasmid was constructed. The Sym plasmid was extracted from *Agrobacterium radiobacter* K749(pSym1a::Tn*5*-*mob*) in a large scale preparation and purified on a CsCl-EtBr gradient (Section 2.6.1.2). This strain was chosen for the extraction because it contains no plasmids besides the Tn*5*-marked Sym plasmid from LS2090. The megaplasmid is known to be the Sym plasmid, as this strain has the ability to catabolize rhizopine (Wexler, 1994). The Sym plasmid was partially digested with the restriction enzyme *Eco* R1 (Section 2.6.2) and ligated into

completely digested and dephosphorylated vector pLAFR3 (Section 2.6.2) (a map of pLAFR3 is provided in Appendix 3). pLAFR3 confers tetracycline resistance on its bacterial host. Ligated DNA was packaged and transfected into *E. coli* JM109 with lambda phage. This preferentially size selects for larger inserts of approximately 30 kb. This was done using the Stratagene Gigapack II XL kit following the manufacturers instructions (Stratagene, La Jolla, CA, USA).

The most important cosmids constructed during the course of this study are listed in Table 5.2.

5.2.6 Screening of transconjugant strains

Screening was achieved by analyzing for the phenotype of rhizopine synthesis. Once rhizobial transconjugants were confirmed to be carrying the desired plasmids, rhizopine synthesis tests were carried out. These were done essentially as described in Section 2.9. Pea seeds were inoculated with 10^9 cells of the strain to be tested. A positive control, the rhizopine producing strain *R. leguminosarum* bv. *viciae* 1a, was used in all experiments. Strain 8401(pRL1) was used as the negative control except where indicated otherwise in the text. The growth conditions and soil were described in Section 2.3. The plants were harvested after 5 - 6 weeks, and nodules were harvested and analyzed for the presence of the rhizopine 3-*O*-MSI. Crude nodule extracts were partially purified by ethanol extraction. Detection and confirmation of the presence or absence of 3-*O*-MSI was achieved by high voltage paper electrophoresis (HVPE) (Section 2.7) and gas chromatography-mass spectrometry (GC-MS) (Section 2.4) as appropriate.

Strain	Description
E. coli	
HB101	Nal^r
S. meliloti	
Rm1021	Moc^- Mos^-, Cm^r
R. leguminosarum* bv. *viciae	
8401(pRL1)	Moc^- Mos^-, Cm^r Rif^r
P121	Moc^- Mos^-, Cm^r Rif^r
P135	Moc^- Mos^-, Cm^r Rif^r
P153	Moc^- Mos^-, Cm^r Rif^r
P233	Moc^- Mos^-, Cm^r Rif^r
P342	Moc^- Mos^-, Cm^r Rif^r
Sp59	Moc^- Mos^-, Cm^r Rif^r
P121(pSym1a::Tn*5-mob*)	Cm^r Km^r Rif^r, contains pSym1a::Tn*5-mob* from LS2090, produces 3-*O*-M*S*I
P135(pSym1a::Tn*5-mob*)	Cm^r Km^r Rif^r, contains pSym1a::Tn*5-mob* from LS2090, produces 3-*O*-M*S*I
P233(pSym1a::Tn*5-mob*)	Cm^r Km^r Rif^r, contains pSym1a::Tn*5-mob* from LS2090, Nod^-, Mos^-

Table 5.1 List of some of the strains constructed during this study.

Strain	Description
R. leguminosarum* bv. *viciae	
8401(pSym1a::Tn*5-mob*)	Cm^r Km^r Rif^r, contains pSym1a::Tn*5-mob* from LS2090, produces 3-*O*-M*S*I
P342(pSym1a::Tn*5-mob*)	Cm^r Km^r Rif^r, contains pSym1a::Tn*5-mob* from LS2090, produces 3-*O*-M*S*I
8401(pRL1)(pKH104)	Cm^r Rif^r Tc^r, contains pKH104, produces 3-*O*-M*S*I
P135(pKH104)	Cm^r Rif^r Tc^r, contains pKH104, produces 3-*O*-M*S*I
P121(pKH104)	Cm^r Rif^r Tc^r, contains pKH104, Mos^-
P233(pKH104)	Cm^r Rif^r Tc^r, contains pKH104, Nod^-
P342(pKH104)	Cm^r Rif^r Tc^r, contains pKH104
8401(pRL1)(pKH085)	Rif^r Tc^r, contains pKH085
P121(pKH085)	Rif^r Tc^r, contains pKH085
P135(pKH085)	Rif^r Tc^r, contains pKH085
P342(pKH085)	Rif^r Tc^r, contains pKH085
8401(pRL1)(pKH227)	Rif^r Tc^r, contains pKH227
8401(pRL1)(pKH246)	Rif^r Tc^r, contains pKH246

Table 5.1 cont. List of some of the strains constructed during this study.

Cosmid	Description
pKH085	pSym1a cosmid clone in *Eco* RI site of pLAFR3, in *E. coli* JM109, Tc^r
pKH104	pSym1a cosmid clone in *Eco* RI site of pLAFR3, in *E. coli* JM109, Tc^r, produces 3-*O*-M*S*I
pKH227	pSym1a cosmid clone in *Eco* RI site of pLAFR3, in *E. coli* JM109, Tc^r
pKH246	pSym1a cosmid clone in *Eco* RI site of pLAFR3, in *E. coli* JM109, Tc^r

Table 5.2 List of the most important cosmids constructed during this study.

5.3 Results

Transconjugant strains were used to show that the *mos* genes of *R. leguminosarum* bv. *viciae* strain 1a are located on the Sym plasmid. These transconjugants, which are not normally rhizopine-producing rhizobia, had their native Sym plasmid displaced by the incoming Sym plasmid from strain 1a (pSym1a). After the introduction of pSym1a, these strains were able to produce rhizopine.

Two approaches were used to determine the regions of pSym1a which are required for rhizopine synthesis. R-primes and a cosmid clone bank of pSym1a were constructed. These approaches provide relatively large clones. If large clones are used, fewer would be required in order to have a reasonable chance of covering the entire Sym plasmid. Up to approximately 100 kb of DNA can be inserted into a single R-prime plasmid (Haas and Holloway, 1978), and up to 30 kb into the cosmid vector used. Clones were transferred to non-rhizopine producing strains of *R. leguminosarum* bv. *viciae* and screened for ability to synthesize rhizopine in root nodules.

5.3.1 The *mos* genes are located on the Sym plasmid in strain 1a.

The marked Sym plasmid from the rhizopine strain 1a was transferred to the non-rhizopine strain 8401(pRL1) Cm^r Rif^r, using LS2090 as the donor of the Sym plasmid. Strain 8401(pRL1) Cm^r Rif^r is an antibiotic-resistant derivative of strain 8401(pRL1) (see Tables 2.1 and 5.1). Transfer of pSym1a was confirmed by comparing the plasmid profiles of the donor and recipient with that of the transconjugant after Eckhardt gel electrophoresis (Figure 5.1). This was considered necessary, to ensure that the colonies obtained after plasmid transfer were real transconjugants, and not variants of LS2090 which had undergone mutations that could confer resistance to chloramphenicol and rifampicin. The Sym plasmid of strain 8401(pRL1) is smaller than pSym1a. The transconjugant,

8401(pSym1a::Tn*5-mob*), carries a Sym plasmid which is slightly larger than that of strain 8401(pRL1), but is identical in size to pSym1a.

Strain 8401(pSym1a::Tn*5-mob*) produced large, pink nodules which contained leghemoglobin. These were judged to be healthy nitrogen-fixing nodules (effective), as plant growth was good and plants did not suffer any symptoms typical of nitrogen deficiency. In comparison, six week old uninoculated plants suffered severe chlorosis of stems and leaves.

When bacteria were re-isolated from the nodules, only rhizobia resistant to the appropriate antibiotics (kanamycin, chloramphenicol and rifampicin) were recovered, indicating that the nodules were produced by the inoculated transconjugant, and not by a contaminant. In addition, the roots of control plants, which were not inoculated, contained no nodules.

The rhizopine 3-*O*-M*S*I was detected in nodules produced by strain 8401(pSym1a::Tn*5-mob*). A silver-staining compound, which migrated to the same electrophoretic position as 3-*O*-M*S*I, was observed after HVPE of the partially purified nodule extracts (Figure 5.2). The 3-*O*-M*S*I samples in different tracks vary slightly in electrophoretic mobility. This is due to retardation of movement of the rhizopine caused by impurities in the samples. The biologically purified sample of 3-*O*-M*S*I has the greatest electrophoretic mobility. Most of the nodule extracts contained various common nodule compounds, and the presence of these compounds hindered the electrophoretic movement of 3-*O*-M*S*I.

Although this is only a semi-quantitative test, the amount of the silver-staining compound identified in Figure 5.2 was estimated to be 10-20% less than the quantity of 3-*O*-M*S*I in nodules produced by the wild-type rhizopine-producing isolate, 1a. This finding was replicated several times. The identity of 3-*O*-M*S*I in the extracts of nodules produced by strain 8401(pSym1a::Tn*5-mob*) was confirmed by GC-MS. The gas chromatogram revealed a distinct peak corresponding to the retention time predicted for 3-*O*-M*S*I (peak 4,

Figure 5.3a). The single ion trace for m/z 181, an ion characteristic of 3-*O*-MSI, showed a large peak corresponding to the peak identified from the total ion trace shown in Figure 5.3a (Figure 5.3b). The mass spectrum of this compound is shown in Figure 5.3c, and displays all the characteristic ions expected for 3-*O*-MSI (described in Section 4.3.1). The gas chromatogram of strain 1a nodule extracts (Figure 5.3d) shows a larger rhizopine peak than was seen for strain 8401(pSym1a::Tn*5*-*mob*) nodule extracts. No equivalent compound was found in nodules produced by the non-rhizopine isolate, 8401(pRL1) (Figure 5.3e).

These results provide definitive evidence that the *R. leguminosarum* bv. *viciae mos* genes are present on the Sym plasmid in the strain 1a. They also demonstrate that *mos* genes introduced on a foreign Sym plasmid can be expressed in strain 8401(pRL1), although expression is at a slightly reduced level compared to the wild-type.

Figure 5.1 Eckhardt gel confirming Sym plasmid transfer from strain LS2090 to strain 8401(pRL1).

Lane 1, 8401(pRL1); lane 2, 1a; lane 3, 8401(pSym1a::Tn*5-mob*).

Chr: broken chromosomal DNA.

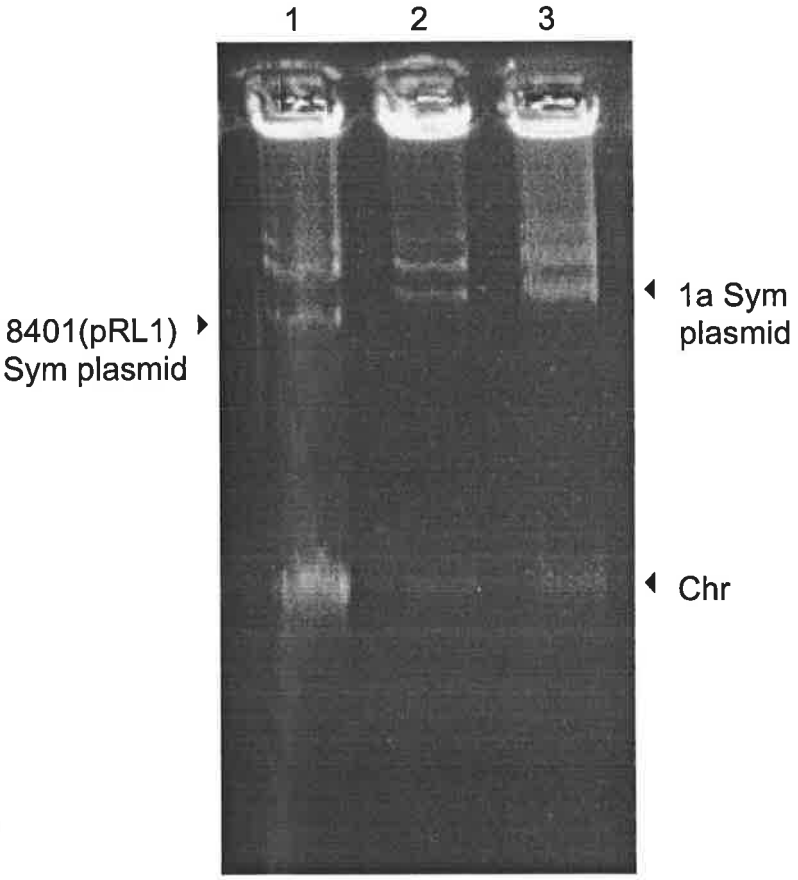

1
2
3
8401(pRL1)
Sym plasmid
1a Sym
plasmid
Chr

Figure 5.2 Synthesis of 3-*O*-M*S*I by a transconjugant of 8401(pRL1) which contains the Sym plasmid from strain 1a, 8401(pSym1a::Tn*5-mob*). Results of HVPE in formic/acetic buffer pH 1.7 of crushed nodules. 0.5 µL (1.25 µg) of nodule extract was loaded in each lane.

Lane 1, 8401(pRL1); lane 2, 1a; lane 3, 8401(pSym1a::Tn*5-mob*); lane 4, purified 3-*O*-M*S*I; lane 5, standard (mannitol).

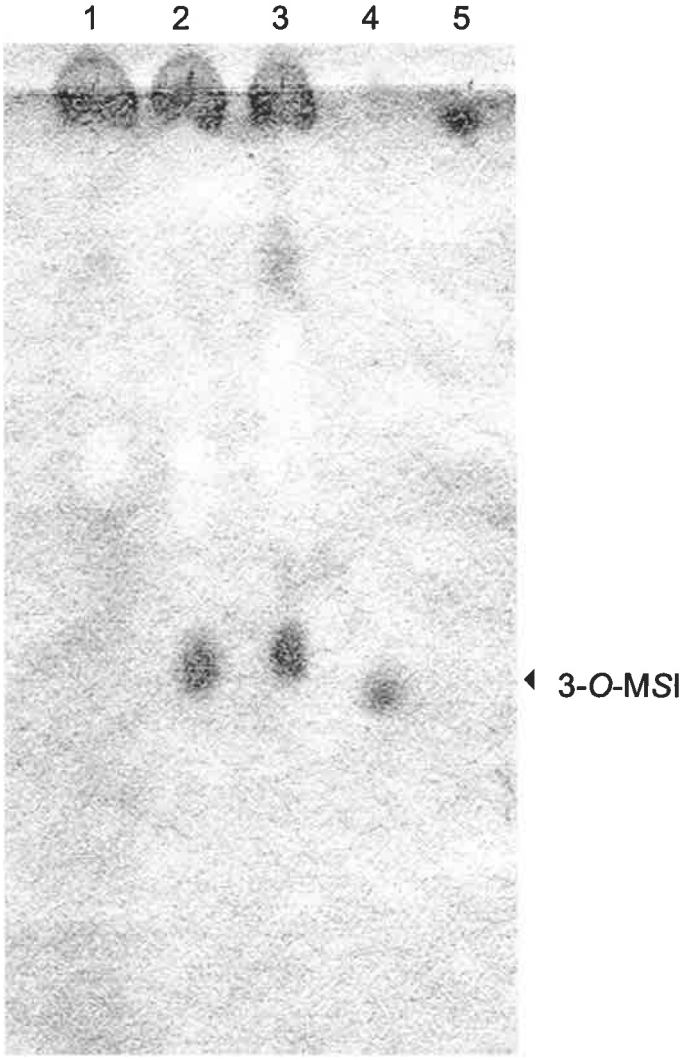

1
2
3
4
5
3-O-MSI

Figure 5.3 Confirmation of the identity of the rhizopine 3-*O*-M*S*I in nodules produced by 8401(pSym1a::Tn*5*-*mob*) by GC-MS. In figures (a), (b), (d) and (e), the x axis indicates retention time (minutes) on the GC column. The y axis indicates abundance. The scale on the left reflects relative abundance (relative ion current, RIC), the scale on the right provides absolute values. In figure (c), the x axis represents mass/charge ratio (m/z) and the y axis represents ion abundance.

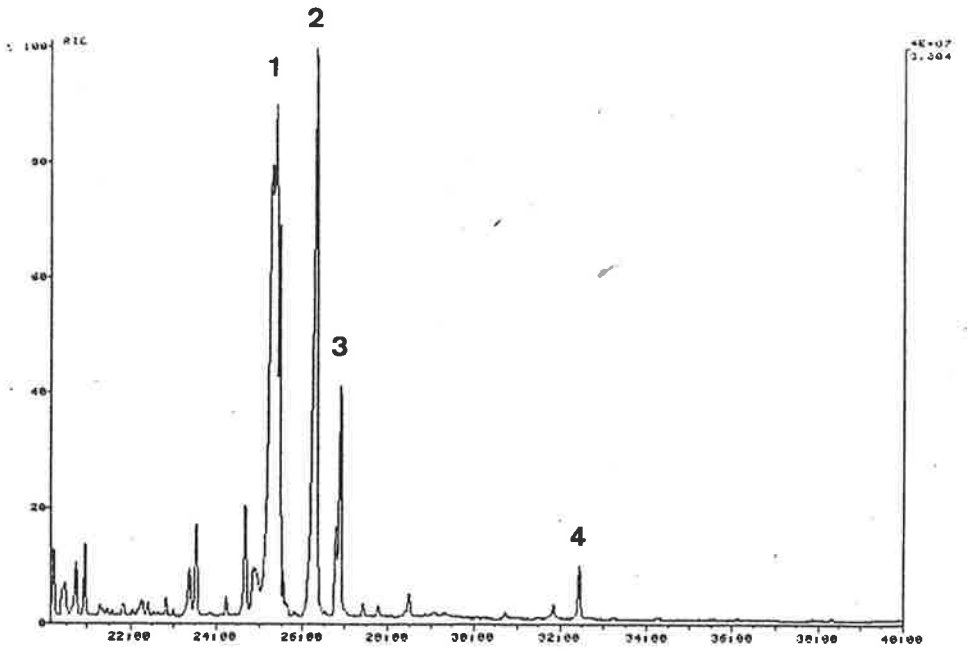
(a) Gas chromatogram showing the presence of rhizopine in the nodule extracts from strain (8401pSym1a::Tn*5*-*mob*).

Peak 1, an unidentified mono-methyl inositol; peak 2, ononitol; peak 3, *myo*-inositol; peak 4, 3-*O*-M*S*I.

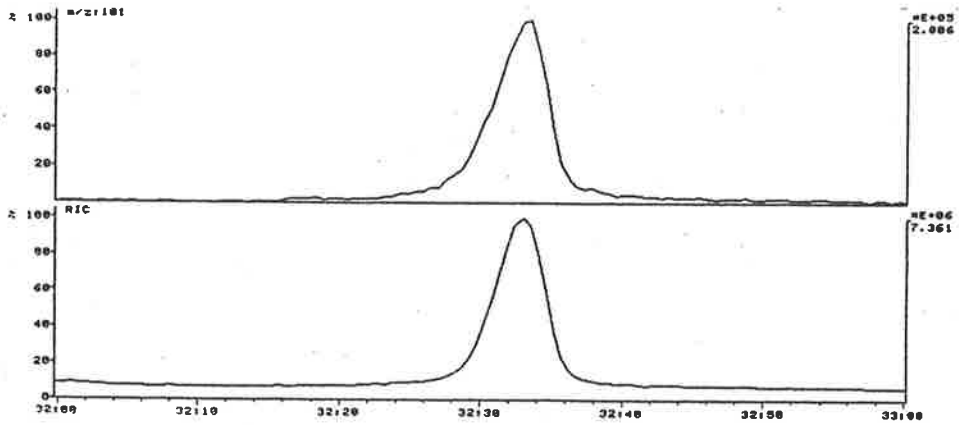
(b) Total ion scan, and single ion scan for m/z 181, which is highly characteristic of 3-*O*-M*S*I, using a magnified scale showing only the distinct peak representing 3-*O*-M*S*I. The two chromatograms are on the same scale and are directly comparable.

(c) The mass spectrum for the peak identified in (b), confirming the identity of this compound to be 3-*O*-M*S*I. Note the highly abundant peaks at m/z 139, 181 and 223 which are diagnostic for 3-*O*-M*S*I.

a


RIC
1
2
3
4

b


RIC

c

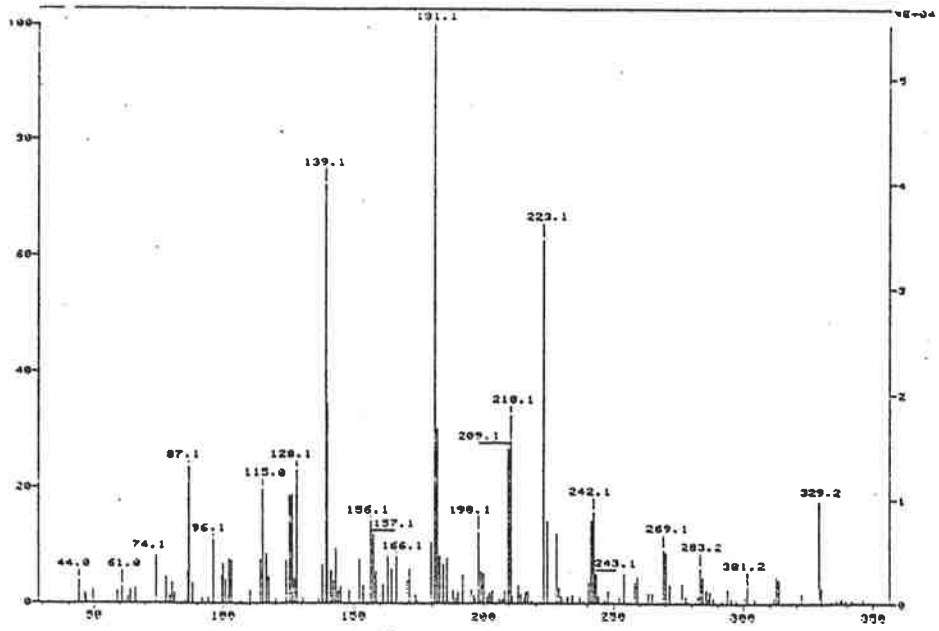

191.1
139.1
223.1
218.1
209.1
87.1
128.1
115.0
242.1
329.2
156.1
157.1
198.1
96.1
269.1
74.1
166.1
283.2
44.0
61.0
243.1
301.2

Figure 5.3 cont. Confirmation of the identity of the rhizopine 3-*O*-M*S*I in nodules produced by 8401(pSym1a::Tn*5-mob*) by GC-MS.

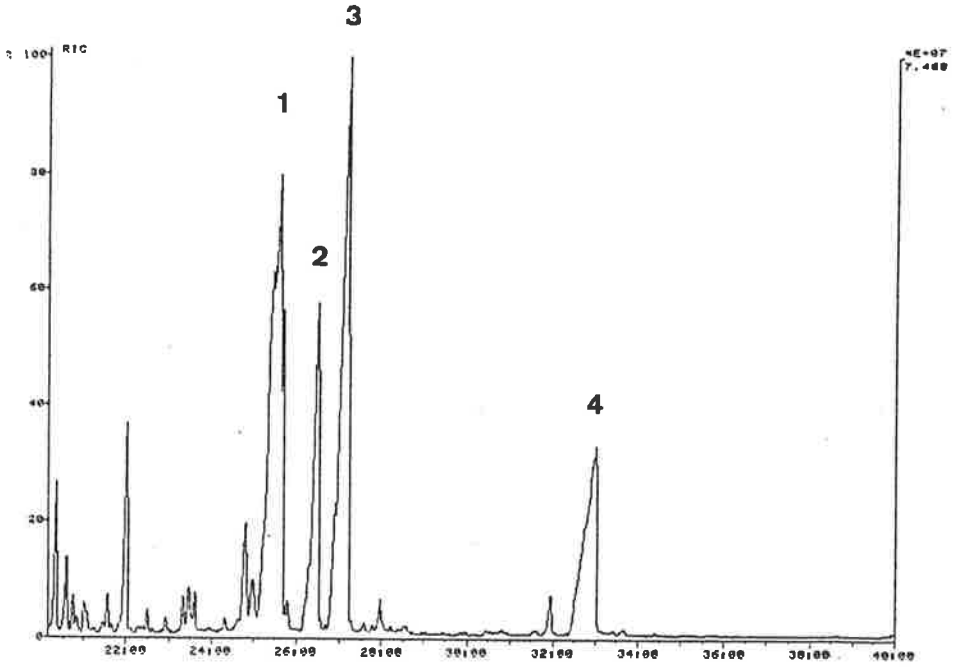
(d) Gas chromatogram for nodule extracts from the rhizopine-producing strain, 1a. The rhizopine peak (peak 4) in this sample is much larger than the one observed in (a) for extracts from nodules produced by 8401(pSym1a::Tn*5-mob*).

Peak 1, an unidentified mono-methyl inositol; peak 2, ononitol; peak 3, *myo*-inositol; peak 4, 3-*O*-M*S*I.

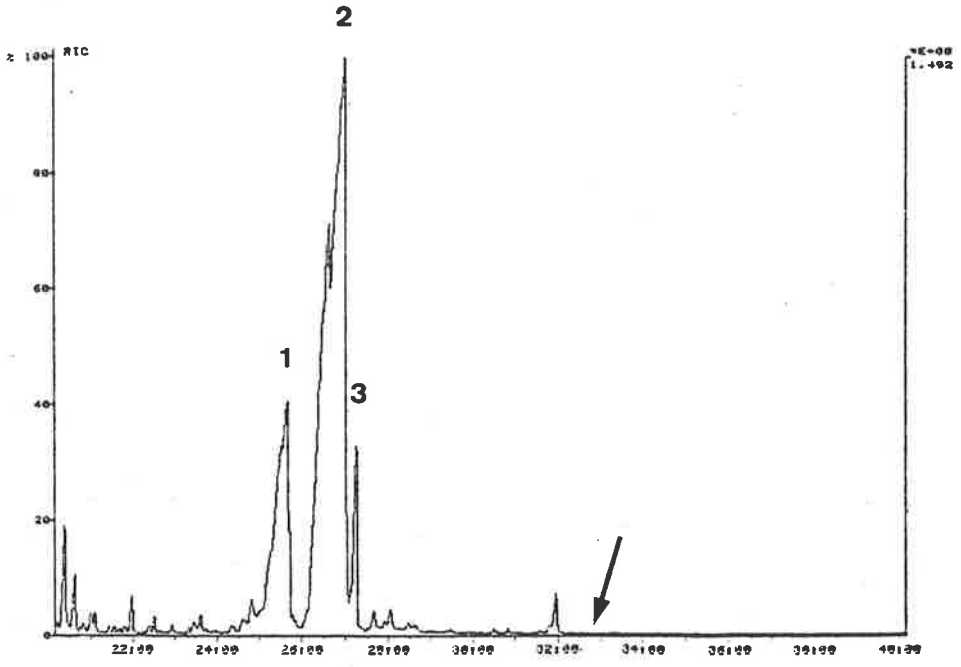
(e) Gas chromatogram for nodule extracts from the non-rhizopine-producing strain, 8401(pRL1), showing the absence of a peak corresponding to 3-*O*-M*S*I.

Peak 1, an unidentified mono-methyl inositol; peak 2, ononitol; peak 3, *myo*-inositol. The arrow shows the expected position for 3-*O*-M*S*I as deduced by relative mobility of this compound compared to the other compounds.

d



e



5.3.2 pSym1a R-primes

5.3.2.1 Construction of pSym1a R-primes and transfer to strain 8401(pRL1)

Ten R-primes, all of which were constructed from a single Tn*5*-marked Sym plasmid from LS2090, were selected for detailed analysis. Due to their common origin (from the same Tn*5* insertion), the R-primes were expected to share a large amount of Sym plasmid DNA, but some differences were expected between them in size and region incorporated. The Sym plasmid in the strain 1a is 230 kb in size (Wexler, 1994), and if the R-prime inserts were 100 kb in size as expected, a number of different R-primes should together cover over half of the Sym plasmid. The R-prime technique was selected as it would increase the probability of obtaining all the relevant genes on one clone in the event that they are not closely linked.

Ten colonies of *E. coli* HB101 Nal^r containing the different R-primes were blotted and hybridized with digoxigenin-labelled *mosA* and *mosB* probes from *S. meliloti*. The probes were a 770 bp *Bam* HI internal fragment from the *mosA* gene, and a 1170 bp *Nsi* I - *Hin*d III internal fragment from the *mosB* gene (see Saint *et al.*, 1993). Both probes hybridized to the rhizopine-producing strains 1a and *S. meliloti* L5-30, as well as to *A. radiobacter* K749(pSym1a::Tn*5-mob*). They did not hybridize to any of the R-prime strains, to the non-rhizopine strains 8401(pRL1) and Rm1021, or to *Agrobacterium radiobacter* K749 (data not shown).

Large scale preparations of R-prime plasmids from HB101 Nal^r were purified on a CsCl-EtBr gradient. The R-prime DNA was digested with the restriction enzyme *Not* I to obtain size information and to highlight differences between the R-primes. The digests showed that most of the R-prime plasmids were approximately 100 kb or less in size, although one, pKHRP4, was much larger (estimated to be 180kb) (data not presented). The inserted Sym plasmid DNA in most of the R-prime plasmids was therefore somewhat smaller than

expected, ranging between 30 and 50 kb in size, although pKHRP4 had an insert of approximately 120kb. The one large and nine smaller R-primes were retained for further analysis. They were transferred into *R. leguminosarum* bv. *viciae* 8401(pRL1) by triparental mating. Confirmation of plasmid transfer was shown by Eckhardt gel electrophoresis (Figure 5.4).

Transconjugant rhizobia containing R-primes were inoculated onto pea plants and after 5 - 6 weeks growth, the roots were examined for nodules. Many of these strains did not form an effective symbiosis and the pea plants suffered typical symptoms of nitrogen deficiency. Some of these plants had not been nodulated, while on others, the roots contained large numbers of small (less than 1 mm in diameter), white nodules. These were thought to be ineffective. Ineffective as well as effective nodules were harvested for analysis. Tables describing the nodulation of each of the R-primes in strain 8401(pRL1), and the results of rhizopine synthesis tests, are presented in Appendix 4.

No rhizopine could be detected in nodules induced by rhizobia containing any of the R-primes. Some nodule extracts were purified further on a Dowex 50W-X8 (H) ion exchange column (Section 2.11) to remove impurities, whilst others were partially purified by ethanol extraction (Section 2.9). No evidence was found in HVPE or GC-MS analysis to suggest that rhizopine was present in any nodule samples. Nodules produced by the wild-type strain, 1a, which were inoculated in the same experiment, contained sufficient 3-*O*-MSI for detection by HVPE.

5.3.2.2 Transfer of pSym1a to other non-rhizopine rhizobia

Once it had been ascertained that none of the R-prime plasmids were able to direct rhizopine synthesis after transfer to strain 8401(pRL1), they were transferred to other strains of *R. leguminosarum* bv. *viciae* to examine the possibility that more effective nodulation may be obtained when the R-primes were introduced to different chromosomal backgrounds. In

addition, the *mos* genes might not be expressed in all strains even when present, particularly if the chromosomal genotype has some influence on *mos* gene expression. The strains P121, P135, P153, P233, P342 and Sp59 were chosen as recipients, as these are non-rhizopine-producing rhizobia which are most closely related to strain 1a in terms of chromosomal genotype (Figure 5.5, see also Wexler *et al.*, 1996).

Before these rhizobia were used as recipients for R-primes, their ability to accept foreign DNA, and their ability to express foreign Sym plasmid genes, were verified. Spontaneous rifampicin and chloramphenicol resistant derivatives of these strains were used as the recipients for pSym1a.

pSym1a was introduced to each of the selected rhizobia by triparental mating. LS2090 was again used as the Sym plasmid donor. Bacteria containing pSym1a were selected by antibiotic resistance, and plasmid transfer was confirmed by Eckhardt gel electrophoresis. LS2090 also contains the 60kb helper plasmid, pJB3JI. Co-transfer of pJB3JI to the recipient strains suggested that transfer of pSym1a had been successful. It is unlikely that pJB3JI transferred alone, as this plasmid cannot confer kanamycin resistance upon its host. It is also unlikely that the transconjugant strain is actually a spontaneous antibiotic resistant derivative of LS2090, as it is improbable that a *Rhizobium* strain would develop resistance to two different antibiotics (chloramphenicol and rifampicin) at such high frequency.

Transconjugants created by the introduction of pSym1a are listed in Table 5.1. These strains were inoculated onto pea plants and when suitable nodules were formed, the nodule extracts were analyzed for the presence of rhizopine. P233(pSym1a::Tn*5-mob*) did not nodulate. The other strains tested, with the exception of P342(pSym1a::Tn*5-mob*), were able to induce an effective symbiosis (see Table 5.3). P342(pSym1a::Tn*5-mob*) produced large numbers of white, ineffective nodules. These nodules had a maximum diameter of 1 mm, plants were yellow in color and growth was poor. Other strains produced fewer nodules, but the nodules were larger in size (up to 5 mm x 2 mm). The nodules varied

from pale pink to red in color, and plant growth was healthy, suggesting that the nodules were effective nitrogen-fixing nodules.

All of the transconjugant strains which produced nodules were able to direct the synthesis of 3-*O*-M*S*I, although some strains produced a greater quantity of rhizopine than others (Table 5.3). The strain used in the initial experiments, 8401(pSym1a::Tn*5*-*mob*), produced the most 3-*O*-M*S*I. Of the other strains tested, P135(pSym1a::Tn*5*-*mob*), which produced the largest number of effective nodules, was estimated to produce less than one quarter as much rhizopine as strain 1a (Figure 5.6). Interestingly, P342(pSym1a::Tn*5*-*mob*), despite forming ineffective nodules, was able to synthesize a small amount of 3-*O*-M*S*I (Figure 5.7), which was estimated to be one hundred times less than that produced by strain 1a.

5.3.2.3 Transfer of R-primes to other non-rhizopine rhizobia

Once it had been established that various other natural isolates of *Rhizobium* (described in Section 5.3.2.2) were good recipients for foreign Sym plasmid DNA, and that these strains were able to express the *mos* genes, the R-primes were then transferred to these rhizobia. Some R-primes were also transferred to *S. meliloti* strain Rm1021, an unrelated Moc$^-$ Mos$^-$ strain. Most strains were able to accept the R-prime plasmids. This was demonstrated by growth of bacterial colonies with correct antibiotic resistance, and confirmed by Eckhardt gel electrophoresis of these cells. However, in some strains, the R-prime was not stably maintained in the absence of selection.

The nodulation characteristics of rhizobia containing various R-primes, and results of rhizopine synthesis tests, are summarized in Appendix 4. Many of these strains were able to form a successful and effective symbiosis with their host plant, although there were some exceptions. Most of the transconjugants in which strain P233 was the recipient for the R-prime plasmids were unable to nodulate. Those which did nodulate produced very few nodules, although these nodules appeared to be effective. Also, transconjugants constructed using strain P342 either produced ineffective nodules, or in some cases failed

to produce any nodules. Those constructed with strains P121 and P135 usually produced effective nitrogen-fixing nodules.

Rhizopine was not found in nodules produced by any of the R-prime-containing strains. P135 strains containing several different R-primes appeared to produce a silver-staining compound which corresponded to 3-*O*-*MSI*, giving faint and diffuse spots in paper electrophoresis. However, when larger quantities (2 μL) were loaded on HVPE, a faint spot also appeared in the corresponding position in the track containing P135 (without R-prime plasmid) nodule extract (Figure 5.8). This strain is known to be unable to produce rhizopine, casting some doubt on the result with the P135 R-prime strains. The nodule extracts of the strains producing this silver-staining compound were analyzed by GC-MS. Some unpurified nodule extracts, and some column-purified nodule extracts, were analyzed, but the presence of 3-*O*-*MSI* could not be confirmed in any sample.

Figure 5.4 Example of an Eckhardt gel confirming the presence in rhizobia of some R-prime plasmids after transfer to *R. leguminosarum* bv. *viciae*. Agarose gels were run in TBE buffer.

Lane 1, R68.45; lane 2, 8401(pRL1); lane 3, 8401(PRL1)(pKHRP1); lane 4, 8401(pRL1)(pKHRP2); lane 5, 8401(pRL1)(pKHRP3).

Chr: broken chromosomal DNA.

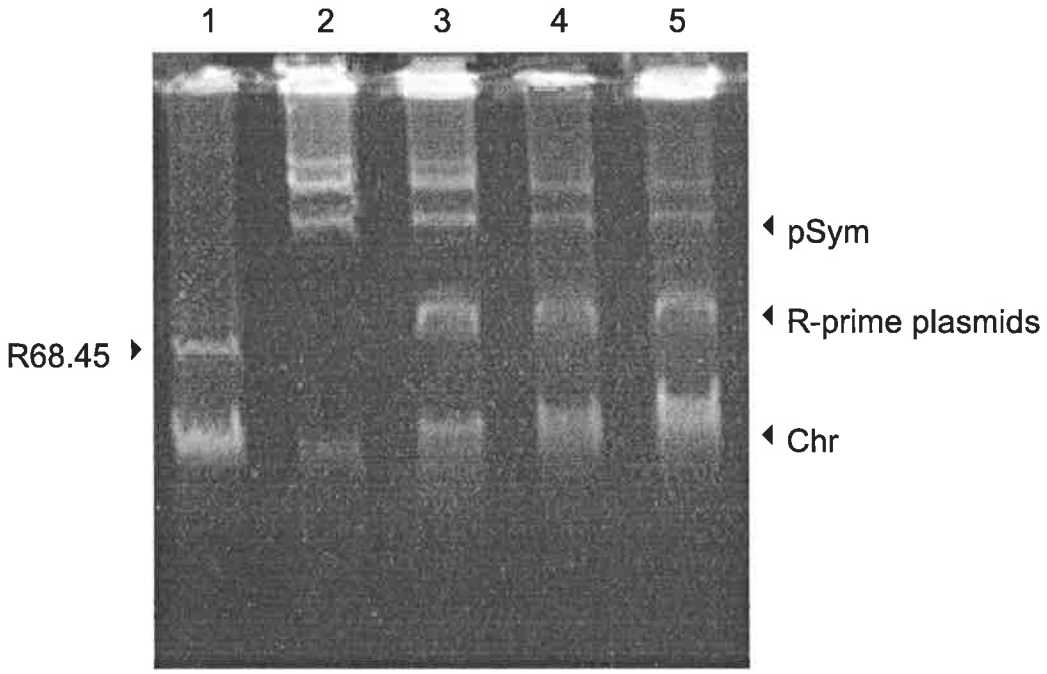
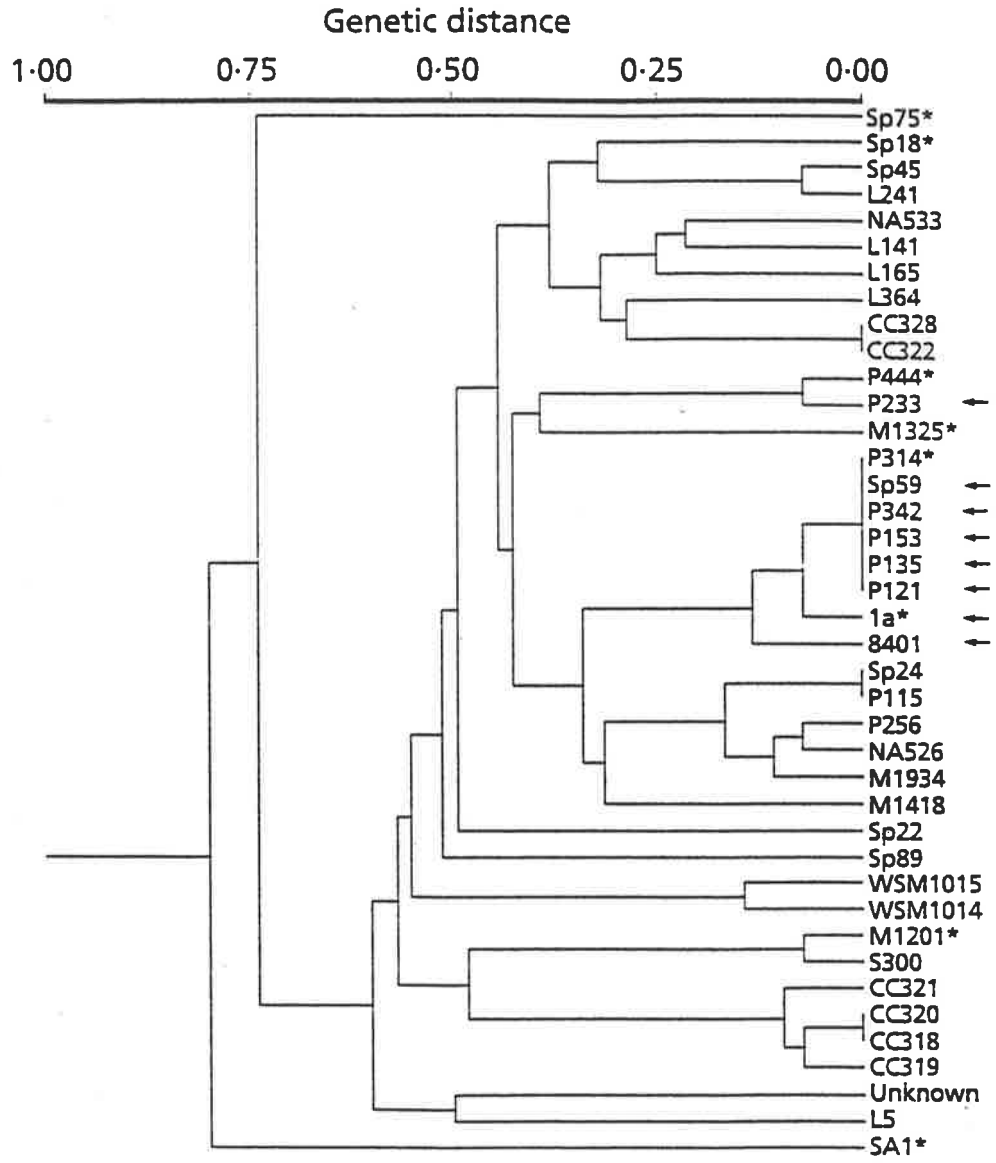

1
2
3
4
5
R68.45
pSym
R-prime plasmids
Chr

Figure 5.5 Phenogram depicting the genetic relationships among *R. leguminosarum* bv. *viciae* strains in terms of chromosomal genotype, as identified by MLEE at 14 loci (from Wexler *et al.*, 1995). The asterisks indicate the rhizopine-producing isolates, and the arrows indicate the isolates which were used in this study.


Genetic distance
1·00
0·75
0·50
0·25
0·00
Sp75*
Sp18*
Sp45
L241
NA533
L141
L165
L364
CC328
CC322
P444*
P233
M1325*
P314*
Sp59
P342
P153
P135
P121
1a*
8401
Sp24
P115
P256
NA526
M1934
M1418
Sp22
Sp89
WSM1015
WSM1014
M1201*
S300
CC321
CC320
CC318
CC319
Unknown
L5
SA1*

Rhizobium strain	Nodulation characteristics	Rhizopine (HVPE)	Rhizopine (GC-MS)	Rep-lications
1a	effective nodules	+++++	+	10
8401(pRL1)	effective nodules	-	-	10
8401(pSym1a::Tn*5-mob*)	effective nodules	++++	+	5
P121	effective nodules	-	nd	3
P121(pSym1a::Tn*5-mob*)	effective nodules but sparse nodulation	+	nd	3
P135	effective nodules	-	-	3
P135(pSym1a::Tn*5-mob*)	effective nodules	++	+	3
P233	effective nodules but sparse nodulation	-	-	3
P233(pSym1a::Tn*5-mob*)	no nodulation	nd	nd	3
P342	effective nodules	-	-	3
P342(pSym1a::Tn*5-mob*)	many nodules, all ineffective	+	+	3

Table 5.3 Nodulation and rhizopine synthesis by some natural isolates of *R. leguminosarum* bv. *viciae*, and by transconjugants of these strains which contain pSym1a in place of their original Sym plasmid. Quantification of rhizopine in HVPE is approximate, and was based on intensity of the silver-staining spot, and by comparison with the amount observed in nodules produced by the rhizopine-producing control, 1a. Note that GC-MS is +/- only and is not semi-quantitative.
nd: not determined

Figure 5.6 Synthesis of 3-*O*-M*S*I in nodules formed by a transconjugant of P135 which contains the Sym plasmid from strain 1a, P135(pSym1a::Tn*5*-*mob*). Results of HVPE in formic/acetic buffer pH 1.7 of crushed nodules. 0.5 µL (1.25 µg) nodule extracts were loaded in each lane, except for lane 3, in which 2 µL (5 µg) was loaded.
Lane 1, purified 3-*O*-M*S*I; lane 2, P135(pSym1a::Tn*5*-*mob*); lane 3, P135(pSym1a::Tn*5*-*mob*); lane 4, 1a; lane 5, P135.

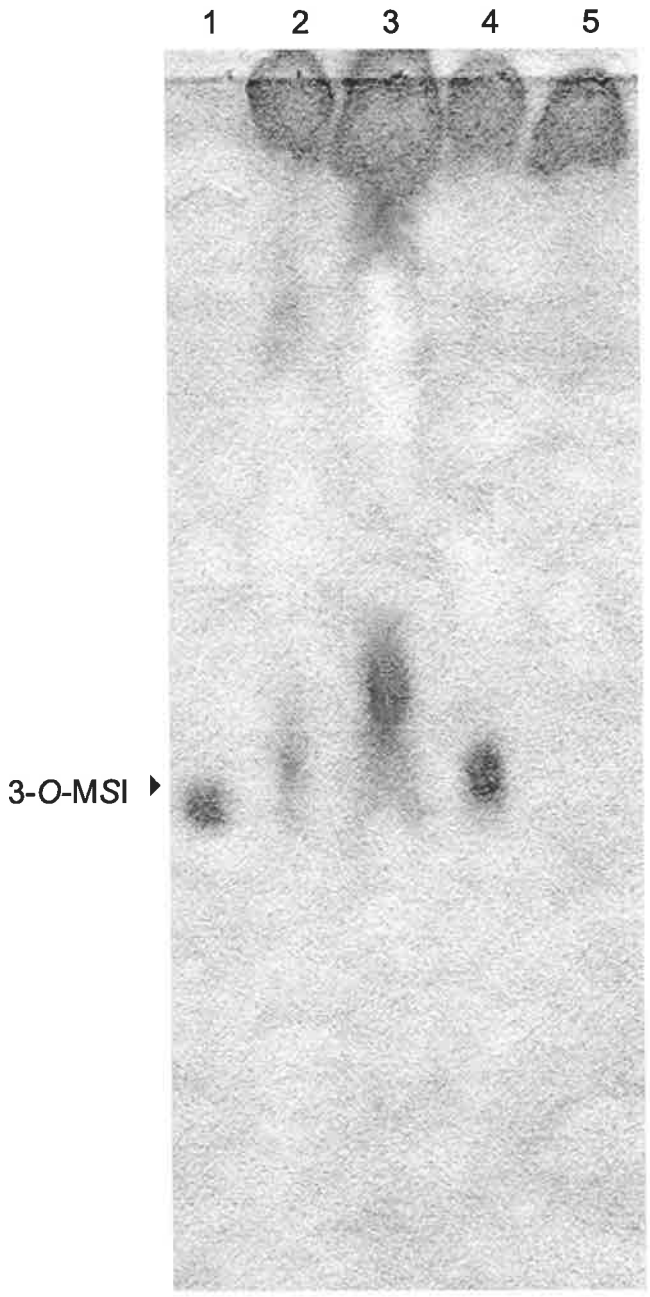

1
2
3
4
5
3-O-MSI

Figure 5.7 Synthesis of 3-*O*-M*S*I in nodules formed by a transconjugant of P342 which contains the Sym plasmid from strain 1a, P342(pSym1a::Tn*5*-*mob*). This strain produces ineffective nodules. Results of HVPE in formic/acetic buffer pH 1.7 of crushed nodules. 0.5 µL (1.25 µg) nodule extracts were loaded in each lane, except for lane 3, in which 2 µL (5 µg) was loaded.

Lane 1, purified 3-*O*-M*S*I; lane 2, P342(pSym1a::Tn*5*-*mob*); lane 3, P342(pSym1a::Tn*5*-*mob*); lane 4, 1a; lane 5, P342.

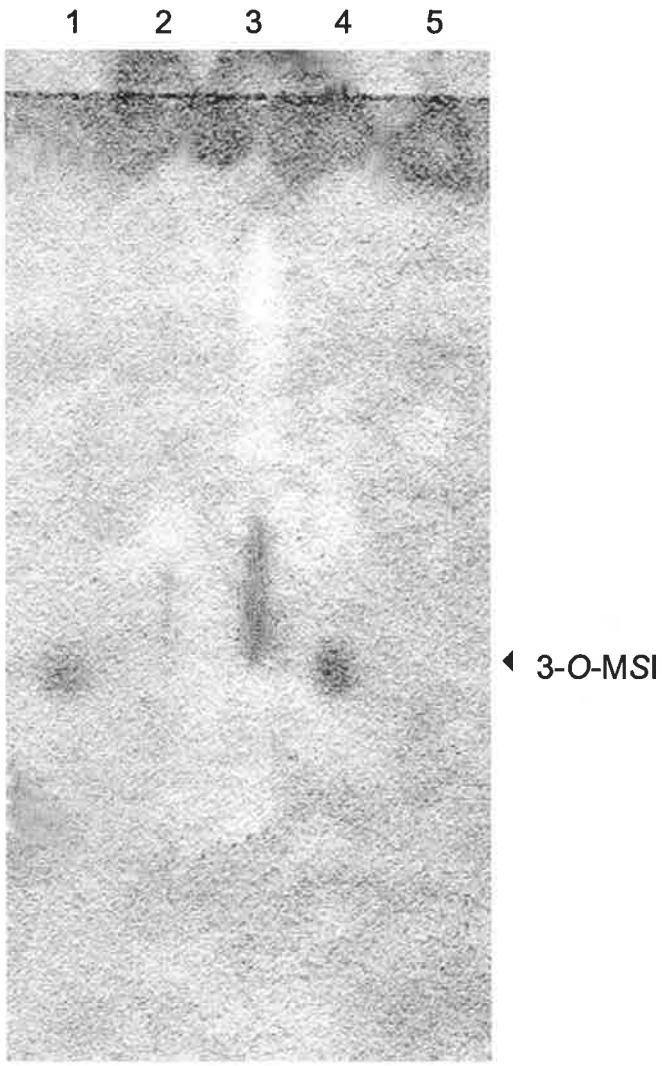
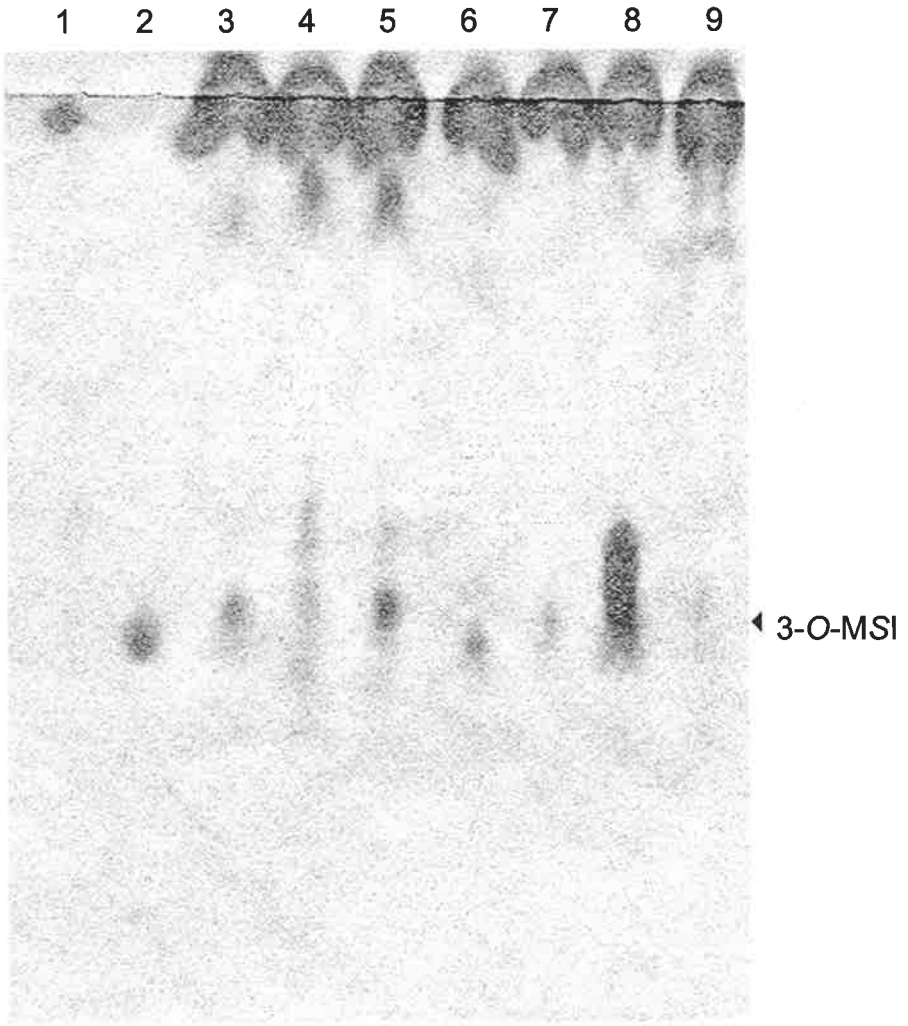

1
2
3
4
5
3-O-MSI

Figure 5.8 Synthesis of a silver staining compound by some R-prime plasmids after transfer to a non-rhizopine strain of *R. leguminosarum* bv. *viciae,* P135. Results of HVPE in formic/acetic buffer pH 1.7 of crushed nodules. 2 μL (5 μg) nodule extracts were loaded in each lane.

Lane 1, standard (mannitol); lane 2, purified 3-*O*-M*S*I; lane 3, P135(pRP1); lane 4, P135(pRP2); lane 5, P135(pRP5); lane 6, P135(pRP6); lane 7, P135(pRP9); lane 8, 1a; lane 9, P135.


1
2
3
4
5
6
7
8
9
3-O-MSI

5.3.3 Cosmid clone bank

5.3.3.1 Construction and nodulation of cosmid clones

A cosmid clone bank was constructed from the 1a Sym plasmid (Section 5.2.5). The Sym plasmid was extracted from *Agrobacterium radiobacter* K749(pSym1a::Tn*5-mob*). It is 230 kb in size (Wexler, 1994), and according to the formula of Sambrook *et al.* (1989), 76 clones of 20 kb in size would be needed to give a 99% probability of covering the entire Sym plasmid. This clone bank was constructed in the knowledge that if the *mos* genes are not closely linked, it might be impossible to obtain all the genes on the one cosmid. However, due to the lack of success in isolating R-primes with the ability to synthesize rhizopine, another approach was necessary.

Cosmids were extracted from *E. coli* and digested with the restriction enzyme *Eco* RI in order to determine size and a partial restriction map. Larger clones, greater than 20 kb in size, were selected for further screening. Approximately 300 different clones were identified for this purpose. This was far in excess of the number calculated to cover the entire Sym plasmid, but it was thought necessary to examine considerably more clones because this formula assumes that a DNA sequence is a point mass rather than considering its size. If the *mos* genes occur as an operon, as they do in *S. meliloti*, they would be expected to be approximately 5 kb in size. Clones greater than 20 kb in size were transferred initially to *R. leguminosarum* strain 8401(pRL1) Cm^r Rif^r by triparental mating. Cosmids which yielded promising results were also transferred to other Mos^- strains of *Rhizobium*, such as P135 Cm^r Rif^r and P121 Cm^r Rif^r.

Transconjugants were isolated on selective media. Transfer of the correct cosmid was verified by its digestion with *Eco* RI after extraction from *Rhizobium*. Once the rhizobia were confirmed to be carrying the clone of interest, they were inoculated onto pea seeds and the plants were grown for 5 - 6 weeks. At this time, nodules were removed from the roots, crushed, and analyzed by HVPE for the presence of rhizopines in the nodule extracts.

Nodules containing silver-staining compounds were analyzed by GC-MS in order to elucidate the chemical structure and identity of these compounds.

Rhizobia were simultaneously re-isolated from some of the nodules, and confirmed by correct antibiotic resistance and *Eco* RI restriction digest to be the strain that was originally inoculated. No contaminants were detected, as in every case the digests revealed restriction patterns identical to those of the bacteria which had been inoculated. The roots of uninoculated plants contained no nodules.

All transconjugants except for two were able to induce an effective symbiosis on pea roots. Strains 8401(pRL1)(pKH070) and 8401(pRL1)(pKH133) were unable to elicit any nodules. The introduced DNA may have contained a region of incompatibility with the cosmid. Due to selection for the incoming cosmid, the Sym plasmid, rather than the cosmid, may have been expelled from the cells, rendering these bacteria unable to nodulate. These two clones did not share any common fragments, nor did they show any homology to *mos*-hybridizing clones identified previously by M. Wexler (1994) (data not shown).

Four cosmids were able to induce the production in nodules of silver-staining compounds which migrated in the same direction as rhizopine in HVPE at pH 1.7. Two of these, pKH085 and pKH104, produced a compound with a similar relative mobility to 3-*O*-M*S*I, while compounds produced by two other strains, pKH227 and pKH246, had electrophoretic mobilities which were greater than that of 3-*O*-M*S*I. These cosmids were subsequently transferred to various rhizobial strains for further investigation. Digests of these four clones with various restriction enzymes showed that they had few, if any, common fragments, and therefore they do not appear to overlap. Analysis of these clones is presented in the following section.

5.3.3.2 Analysis of pKH085 for rhizopine synthesis

After running HVPE for 15 minutes, a silver-staining spot from 8401(pRL1)(pKH085) nodule extracts appeared at the same position as 3-*O*-M*S*I (Figure 5.9a), but after a longer run (45 mins) it was clear that this compound had an electrophoretic mobility slightly less than 3-*O*-M*S*I (Figure 5.9b). The intensity of the silver-staining spot indicated that production of this compound was lower than the level of synthesis of 3-*O*-M*S*I by strain 1a. GC-MS analysis indicated that this compound was not 3-*O*-M*S*I, as no GC peaks were observed with the retention time anticipated for 3-*O*-M*S*I, and mass spectral analysis did not reveal the expected mass spectrum. The unknown compound has not been identified.

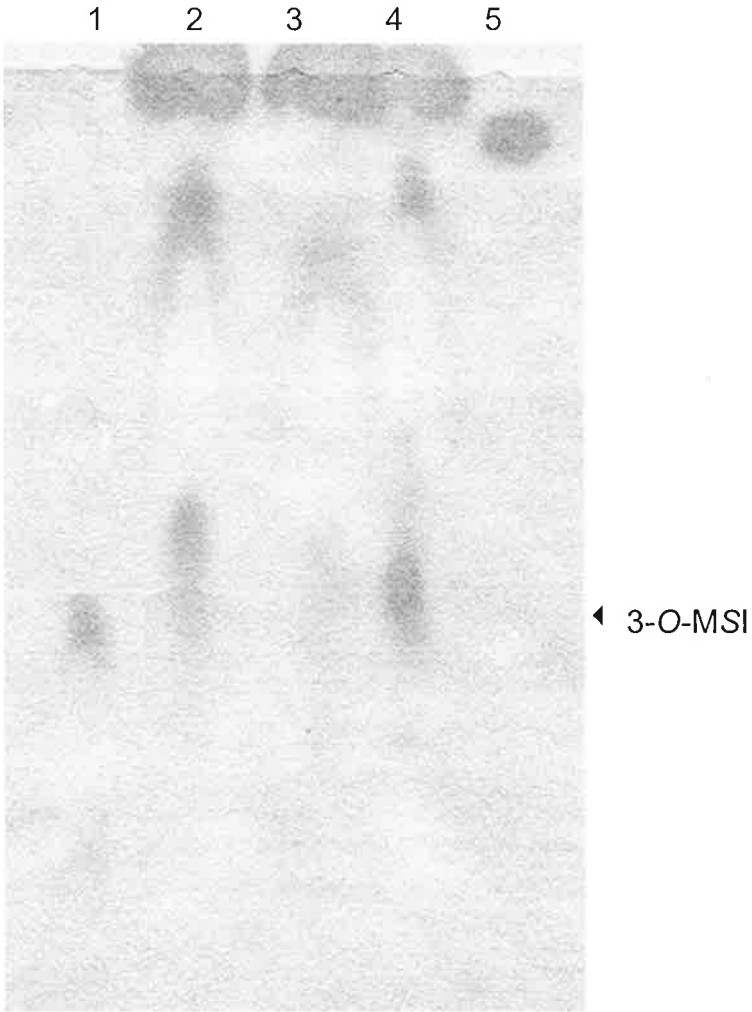
Inoculation of pea plants with strain 8401(pRL1) containing pKH085 was replicated several times over the course of more than a year, but detection of the unknown compound found in the first extraction was not repeated even when plants were grown under exactly the same growth conditions, including time of year, light and temperature. Each time the experiment was repeated, bacteria were re-isolated from some nodules. These rhizobia had the correct antibiotic resistance, and restriction digests of the cosmid with *Eco* RI revealed that they contained the correct cosmid, pKH085. Control plants which were not inoculated were not nodulated. Plants which had been inoculated with strain 1a were included with each experiment, and the nodules produced by this strain always contained abundant 3-*O*-M*S*I.

When pKH085 was transferred into other Moc$^-$ Mos$^-$ strains of *R. leguminosarum* bv. *viciae* (see Table 5.4), neither rhizopine nor the unknown compound was detected in nodules produced by these strains. This was despite the healthy nodulation and presence of effective nodules on plants inoculated with strains P121(pKH085) and P135(pKH085).

Figure 5.9 Synthesis of a compound with similar electrophoretic mobility to 3-*O*-MSI by a transconjugant of strain 8401(pRL1) carrying the cosmid pKH085. Results of HVPE in formic/acetic buffer pH 1.7 of crushed nodules.

(a) HVPE run at 3000V for 15 mins showing the compound produced by 8401(pRL1)(pKH085) has an electrophoretic mobility almost identical to 3-*O*-MSI. 0.5 µL (1.25 µg) nodule extract was loaded in each lane.

Lane 1, purified 3-*O*-MSI; lane 2, 8401(pRL1)(pKH085); lane 3, 8401(pRL1); lane 4, 1a; lane 5, standard (mannitol).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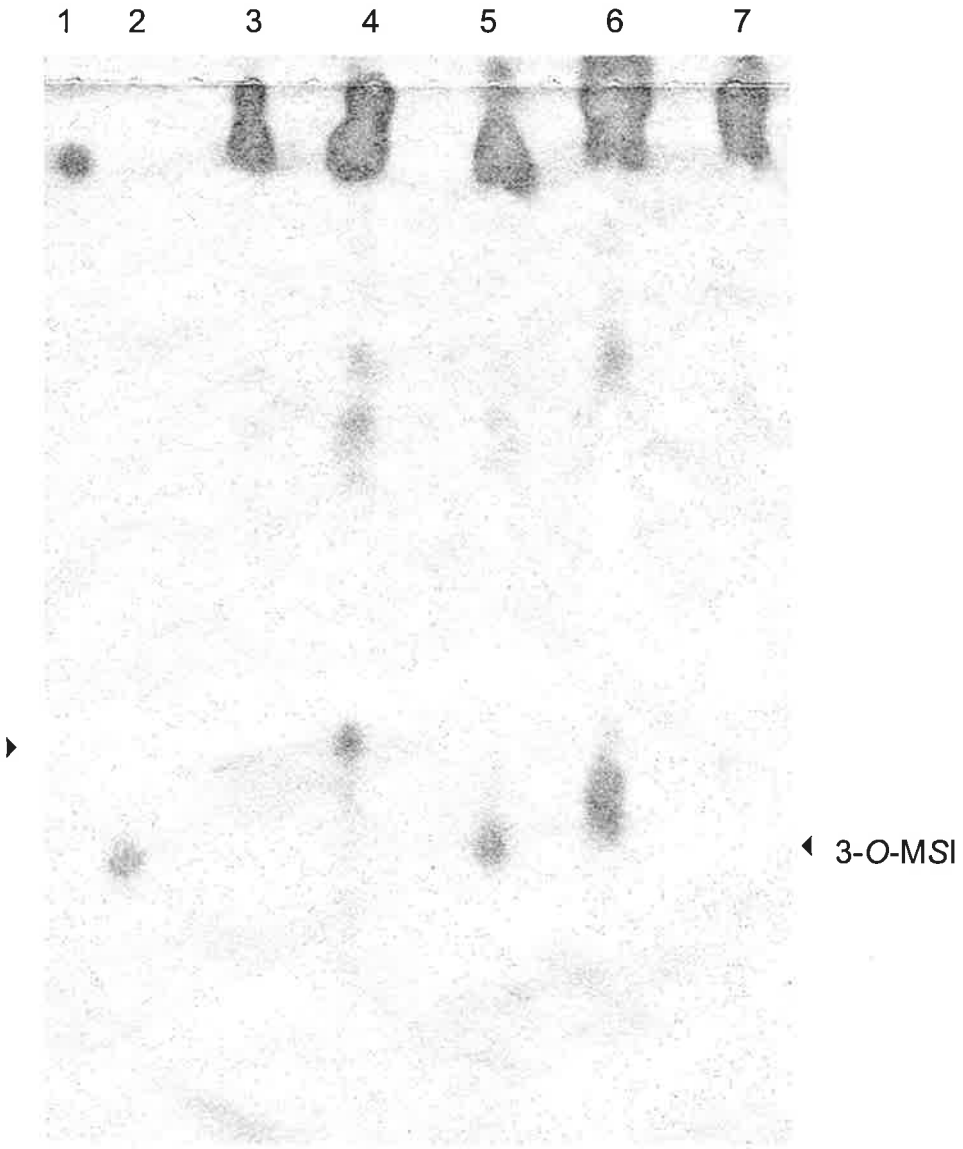
3-O-MSI

Figure 5.9 cont. Synthesis of a compound with similar electrophoretic mobility to 3-*O*-M*S*I by a transconjugant of strain 8401(pRL1) carrying the cosmid pKH085. Results of HVPE in formic/acetic buffer pH 1.7 of crushed nodules.

(b) HVPE run at 3000V for 45 mins, showing that this compound (indicated by the arrow on the left-hand side) migrates slightly more slowly than 3-*O*-M*S*I. 0.5 µL (1.25 µg) nodule extract was loaded in each lane, except for lanes 4 and 6, in which 2 µL (5 µg) was loaded.

Lane 1, standard (mannitol); lane 2, purified 3-*O*-M*S*I; lane 3, 8401(pRL1)(pKH085); lane 4, 8401(pRL1)(pKH085); lane 5, 1a; lane 6, 1a; lane 7, 8401(pRL1).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3-O-MSI

Rhizobium strain	Nodules	Silver-staining compounds (HVPE)	Rhizopine (GC-MS)	Rep-lications
8401(pRL1)(pKH085)	effective	+	-	7
P121(pKH085)	ineffective	-	-	2
P135(pKH085)	effective	-	-	3
P233(pKH085)	no nodulation	nd	nd	2
P342(pKH085)	no nodulation	nd	nd	2

Table 5.4 Nodulation characteristics, the detection of silver-staining compounds by HVPE, and the identification of rhizopine by GC-MS, in the extracts of nodules produced by rhizobia containing the cosmid pKH085.

nd: not determined

It is noted that the cosmid pKH104 may carry most, but not all, of the *mos* genes. If some of the *mos* genes were already present in certain Mos^- strains, such as P135 and 8401(pRL1), this could account for the Mos^+ phenotype of these strains after the introduction of pKH104. Alternatively, P135 may contain necessary precursors for rhizopine synthesis that the other rhizobia used in this study do not have. This is supported by the presence of a small amount of a silver staining compound in strain P135 in the absence of any plasmid introduction.

5.3.3.3 Analysis of pKH104 for rhizopine synthesis

Nodules produced by strains 8401(pRL1) and P135 carrying pKH104 contained a silver-staining compound which migrated to the same position as 3-*O*-M*S*I in paper electrophoresis. Production of this compound by 8401(pRL1)(pKH104) was extremely low and could barely be detected by HVPE, but production by P135(pKH104) was greater and could be detected easily. Nevertheless, this was only approximately 10% of the amount of 3-*O*-M*S*I found in nodules produced by strain 1a (Figure 5.10). GC-MS analysis of nodule extracts from both strains revealed a small GC peaks at the expected position for 3-*O*-M*S*I, which contained the characteristic highly abundant ions of m/z 181 and m/z 223 (Figures 5.11a and 5.11b). Mass spectral data confirmed that the rhizopine 3-*O*-M*S*I was present in these nodules (Figure 5.11c). Both strains were re-isolated from their respective nodules, and no contaminant rhizobia were detected. This result was repeated using three different isolates of P135 containing pKH104. The cosmid pKH104 therefore carries all of the genes required for rhizopine synthesis. However, none of the other strains tested were able to produce rhizopine after the introduction of pKH104. Table 5.5 summarizes nodulation characteristics and the results of rhizopine synthesis tests for rhizobia containing pKH104.

pKH104 is a 22kb clone. After digestion with several different restriction enzymes (*Eco* RI, *Bam* HI, *Pst* I and *Hin*d III), it appeared that this cosmid had a number of fragments in common with pPM1161. pPM1161 is a cosmid isolated previously from pSym1a by M. Wexler, which contains fragments displaying homology to the *S. meliloti mosA* gene (Wexler, 1994). However, the common fragments between pKH104 and pPM1161 were not the *mosA*-hybridizing fragments. The *mosA* hybridizing fragment was a 1.4 kb *Hin*d III fragment, whereas the shared *Hin*d III fragment was 10 kb in size. The shared *Eco* RI fragment was 4 kb. The 4 kb *Eco* RI fragment from pKH104 was extracted from an agarose gel after electrophoresis, radiolabelled, and used as a probe for southern blots of restriction digests of the two plasmids. Hybridization studies showed that this probe had

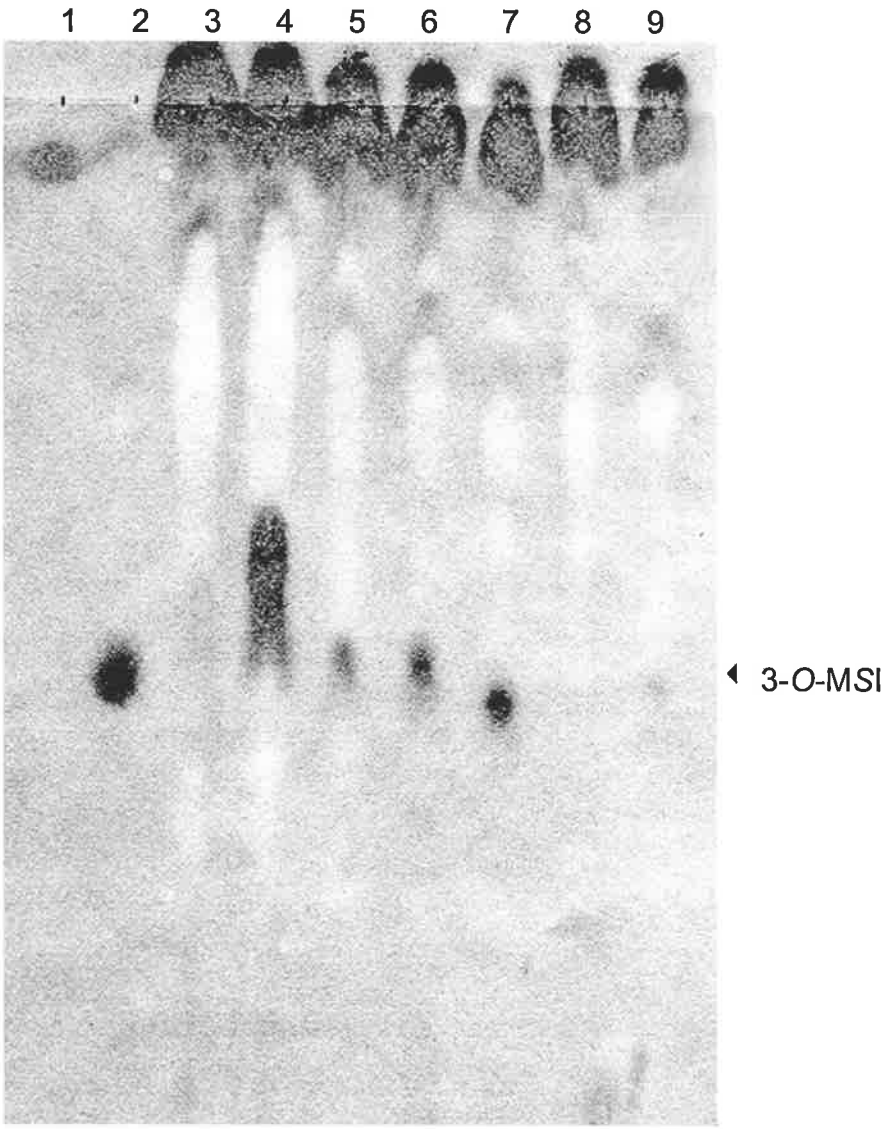
no homology to any fragments in pPM1161, but hybridized strongly to the equivalent fragment from pKH104. pKH104 and pPM1161 are therefore not overlapping clones.

5.3.3.4 Analysis of pKH227 and pKH246 for rhizopine synthesis

Nodules formed by variants of strain 8401(pRL1) carrying pKH227 and pKH246 contained silver-staining compounds which had greater electrophoretic mobility than both 3-*O*-M*S*I and *S*I (Figure 5.12). These compounds were thought to be of potential interest, even though they clearly were neither of the rhizopines. The biosynthetic pathway for rhizopine is unknown, and it was considered possible that perhaps some, but not all, of the rhizopine genes had been cloned, resulting in the formation of a rhizopine precursor. The identity of these compounds could not be elucidated by GC-MS and therefore they may have a structure which precluded their detection by the method chosen, or their molecular weight or base ions may have been larger than the maximum ion scan of m/z 350. As with analysis of pKH085, the problem encountered was a lack of reproducible results in detecting this compound.

Figure 5.10 Synthesis of 3-*O*-M*S*I by strain P135 containing the cosmid pKH104. Results of HVPE in formic/acetic buffer pH 1.7 of nodule extracts. 2 μL (5 μg) of nodule extract was loaded in each lane.

Lane 1, standard (mannitol); lane 2, purified 3-*O*-M*S*I; lane 3, P135; lane 4, 1a; lanes 5 - 7, 3 isolates of P135(pKH104).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3-O-MSI

Figure 5.11 Detection by GC-MS of 3-*O*-M*S*I in nodules formed by 8401(pRL1)(pKH104). In figures (a) and (b), the x axis indicates retention time on the GC column. The y axis indicates abundance. The scale on the left represents relative abundance (relative ion current, RIC), the scale on the right provides absolute values. In figure (c), the x axis shows mass to charge ratio (m/z) and the y axis represents ion abundance.

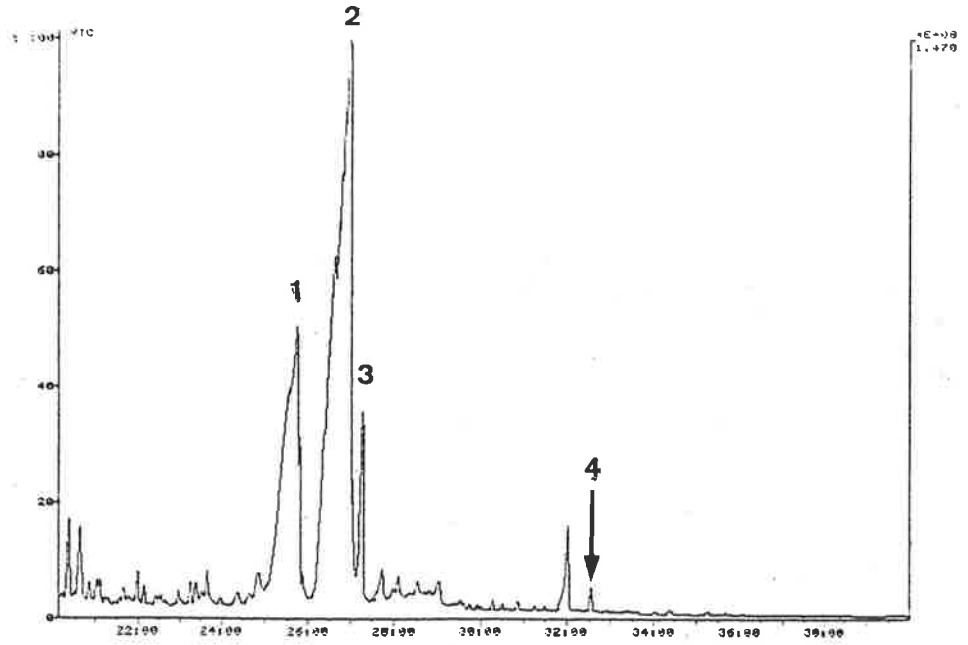
(a) The multiple ion trace shows a small peak with the retention time expected for 3-*O*-M*S*I.

Peak 1, an unidentified mono-methyl inositol; peak 2, ononitol; peak 3, *myo*-inositol; peak 4, 3-*O*-M*S*I.

(b) Single ion traces for m/z 181 and m/z 223, showing discrete peaks which correspond with the peak identified in (a). All three chromatograms in (b) are to the same scale and are directly comparable.

(c) Mass spectrum of the compound identified in (a) and (b), showing characteristic highly abundant ions of m/z 139, 181 and 223, thus confirming that this compound is 3-*O*-M*S*I.

a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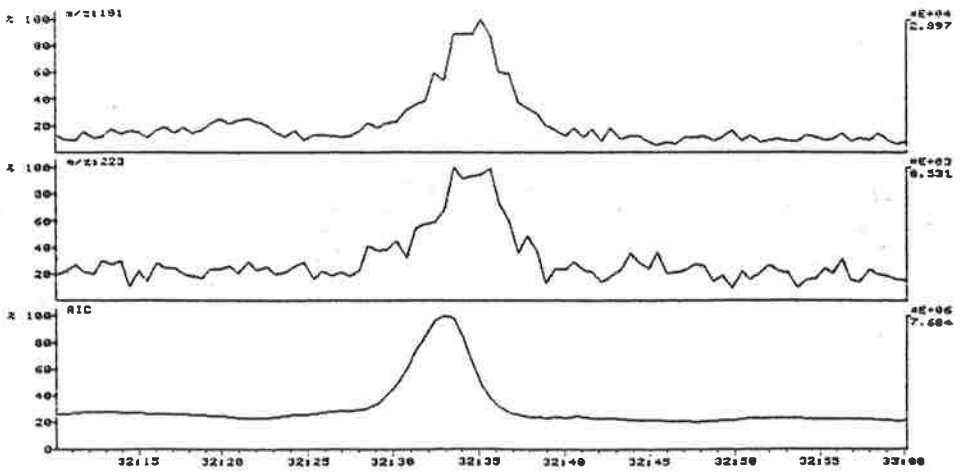
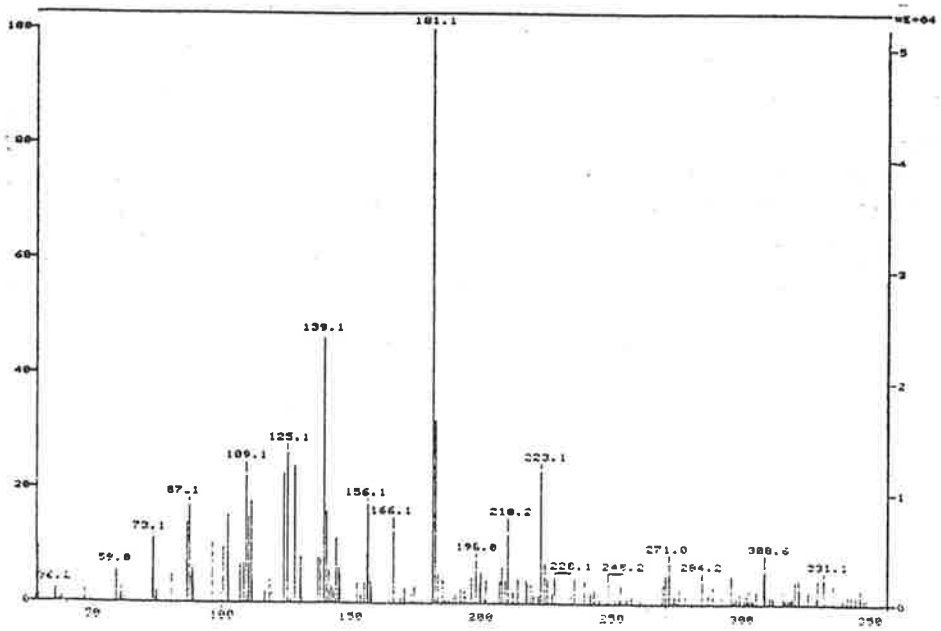
b


BIC

c


181.1
139.1
125.1
109.1
87.1
73.1
59.0
156.1
166.1
223.1
210.2
196.0
271.0
388.6

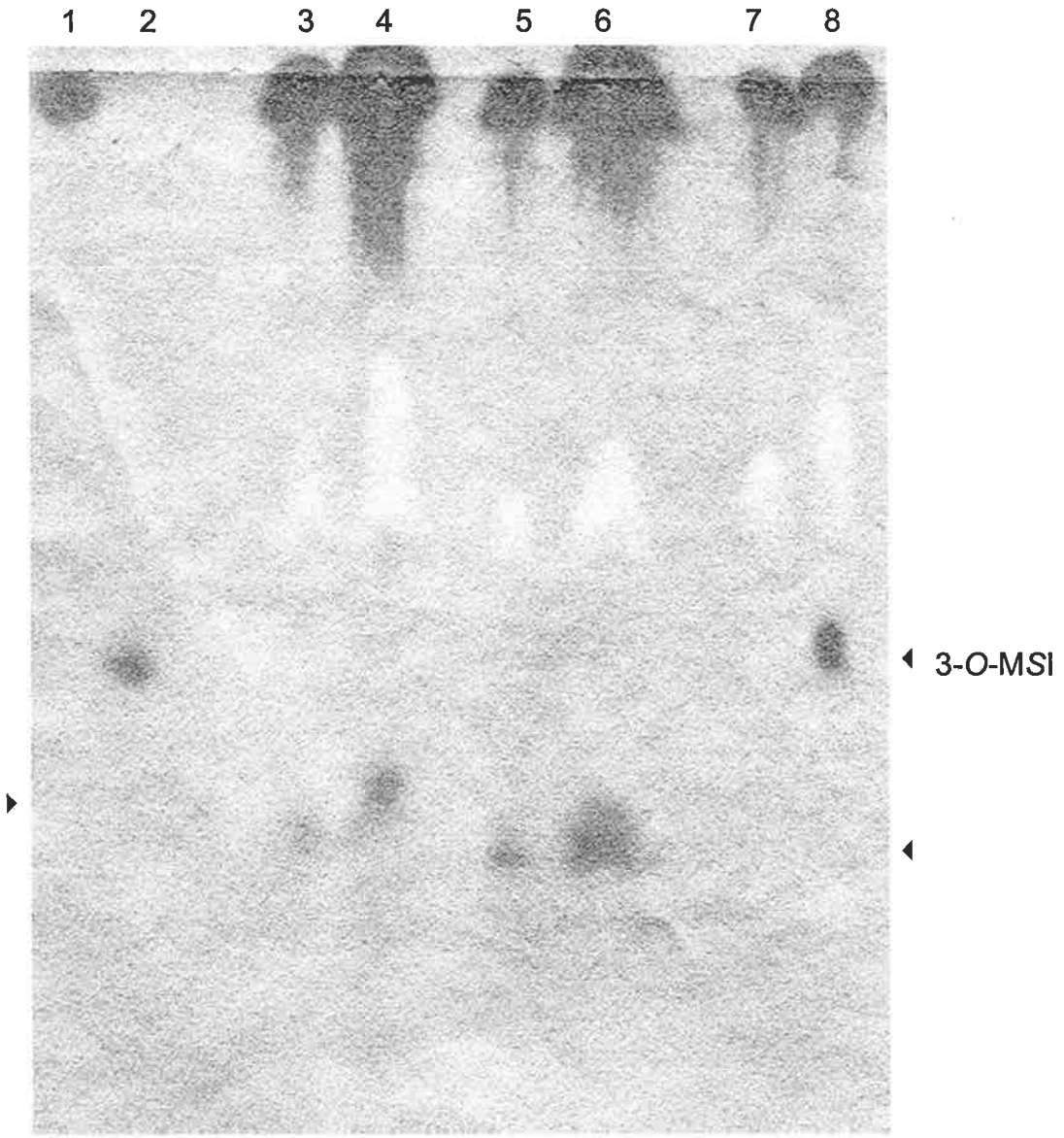
Rhizobium strain	Nodules	Silver-staining compounds (HVPE)	Rhizopine (GC-MS)	Rep-lications
8401(pRL1)(pKH104)	effective	+	+	8
P121(pKH104)	effective	-	-	5
P135(pKH104)	effective	+	+	6
P153(pKH104)	effective	-	nd	1
P233(pKH104)	no nodulation	nd	nd	1
P342(pKH104)	ineffective	-	-	5
Sp59(pKH104)	effective	-	nd	1

Table 5.5 Nodulation characteristics, the detection of silver-staining compounds by HVPE, and the identification of rhizopine by GC-MS, in the extracts of nodules produced by rhizobia containing the cosmid pKH104.

nd: not determined

Figure 5.12 Synthesis of a silver-staining compound by strains 8401(pRL1)(pKH227) and 8401(pRL1)(pKH246). Results of HVPE in formic/acetic buffer pH 1.7 of crushed nodule extracts. 0.5 µL (1.25 µg) of nodule extract was loaded in each lane, except for lanes 4 and 6, in which 2 µL (5 µg) was loaded. The arrow on the left shows the electrophoretic position of the compound produced by 8401(pRL1)(pKH227), and the arrow on the right shows the position of the compound produced by 8401(pRL1)(pKH246).

Lane 1, standard (mannitol); lane 2, purified 3-*O*-MSI; lane 3, 8401(pRL1)(pKH227); lane 4, 8401(pRL1)(pKH227); lane 5, 8401(pRL1)(pKH246); lane 6, 8401(pRL1)(pKH246); lane 7, 8401(pRL1); lane 8, 1a.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8
3-O-MSI

5.4 Discussion

5.4.1 Introduction

This chapter described the isolation of the rhizopine synthesis genes from *R. leguminosarum* bv. *viciae* strain 1a. Preliminary experiments showed that they are located on the Sym plasmid. R-primes and cosmid clones of the Sym plasmid were then constructed in order to more precisely delineate the genes. One cosmid clone was found which contained all of the genes necessary for synthesis of the rhizopine 3-*O*-MSI.

In all experiments, plasmids and cosmids of pSym1a DNA were transferred to non-rhizopine producing rhizobia, and nodules produced by the transconjugants were analyzed for the presence of rhizopine. This approach was used because of the lack of success of previous studies, which involved cloning and examining regions of homology to the *S. meliloti mos* genes (e. g. Wexler, 1994; Bahar, 1997).

5.4.2 The *R. leguminosarum* bv. *viciae mos* genes are located on the Sym plasmid in strain 1a

This study has shown that the *mos* genes of *R. leguminosarum* bv. *viciae* are located on the Sym plasmid in strain 1a. They are also located on the Sym plasmid in all *S. meliloti* strains that have been examined (Murphy *et al.*, 1987; Saint *et al.*, 1993; Wexler, 1994). The plasmid location of these genes in *R. leguminosarum* was demonstrated using transconjugant strains. Non-rhizopine strains acquired the ability to synthesize the rhizopine 3-*O*-MSI when their native Sym plasmid was replaced with that of strain 1a, which is a rhizopine-producing strain. This result was confirmed using several different non-rhizopine strains as recipients for pSym1a, and both HVPE and GC-MS analysis were used to confirm and identify the presence of 3-*O*-MSI in all nodule extracts.

5.4.3 Transfer of pSym1a DNA between rhizobia

A variety of different rhizobia were chosen as recipients for Sym plasmid DNA because of the possibility that chromosomal genotype may influence expression of some Sym plasmid genes, and this was indeed observed in this study. Those strains that are most closely related to strain 1a in terms of chromosomal genotype were thought to be the best candidates for pSym1a transfer. The 1a *mos* genes (and other symbiotic genes) might not be expressed in certain chromosomal backgrounds, especially in those which are more distantly related to this strain. Certain Sym plasmids are known to be associated only with one particular chromosomal background (Young and Wexler, 1988), suggesting that some Sym plasmids might not be stably maintained, or expressed efficiently, in strains with particular chromosomal genotypes (Wexler, 1994). Further, for reasons that are unclear, rhizobia differ markedly in their ability to accept foreign plasmids (Brewin *et al.*, 1982). Strain 8401(pRL1) was used initially, because this strain is known to be a good recipient of foreign DNA (Brewin *et al.*, 1983), is relatively closely related to strain 1a (Wexler *et al.*, 1996), and has been used with some success in earlier studies on rhizopines (e. g. Wexler, 1994). However, a number of other non-rhizopine producing isolates, including P121, P135 and P342, were found to be more similar to 1a than 8401(pRL1) on the basis of chromosomal genotype (Wexler *et al.*, 1996).

It was found that the eight strains chosen as recipients in this study differed markedly in their ability to accept foreign Sym plasmid DNA (cosmids, R-primes or entire pSym1a). With the exception of *S. meliloti* Rm1021, all of these strains were very closely related to strain 1a. Five of the seven *R. leguminosarum* strains (excluding 8401(pRL1) and P233), were indistinguishable after multilocus enzyme electrophoresis (MLEE) analysis at 14 chromosomal loci (Wexler *et al.*, 1996) (see Figure 5.5). However, these five strains differed significantly in their ability to express foreign Sym plasmid genes. This indicates perhaps the importance of small genotypic differences in the chromosome or in the non-

symbiotic megaplasmids. It is known that in these closely related strains, the Sym plasmids, at least, are not particularly closely related to one another (Wexler *et al.*, 1996).

Strain 8401(pRL1) was generally a good recipient of Sym plasmid DNA in this study. Even so, after transfer of some of the R-primes to strain 8401(pRL1), the transconjugant strains produced ineffective nodules and occasionally did not nodulate at all (in these cases the Sym plasmid had presumably been lost). Some strains did not retain the R-primes in the absence of selective pressures in the soil. This may have been because of incompatibility between the native Sym plasmid and the pSym1a DNA contained on the R-prime. Strains P121 and P135 were often found to be better recipients than strain 8401(pRL1). These strains readily accepted the pSym1a DNA, however a few of these transconjugants were also unable to produce effective nodules.

None of the transconjugants (containing cosmids, R-primes or pSym1a) constructed using strain P342 as the rhizobial recipient were able to form effective nitrogen-fixing nodules. Some produced ineffective nodules, while others did not succeed in producing any nodules. It was thought possible that this poor nodulation was linked to the antibiotic resistance of the rhizobia. Cases have been cited where spontaneous antibiotic resistant mutants have at the same time lost the ability to nodulate effectively (Dart, 1975). However, strain P342 Cm^r Rif^r produced effective nodules, and therefore in this case the antibiotic resistance was not associated with the loss of effective nodulation.

When P342 strains carrying cosmids or plasmids were able to nodulate, they formed a much larger number of nodules than usual, but all were ineffective. It has often been observed that strains unable to form a nitrogen-fixing symbiosis with an appropriate legume will form many more nodules than normal. The ineffective nodules may be more numerous because they are not fixing nitrogen, and therefore have a lower utilization of photosynthates (Minchin and Pate, 1973). Another possible reason is that these nodules

lack the autoregulatory inhibition of further nodulation that occurs with the first round of effective nodulation.

5.4.4 Expression of the pSym1a *mos* genes after transfer to non-rhizopine-producing rhizobia

All transconjugant strains produced less rhizopine than the wild-type strain, despite in most cases forming nodules that appeared to be fully functional in other respects. The strain 8401(pSym1a::Tn*5-mob*), which formed healthy nitrogen fixing nodules, produced the greatest amount of rhizopine. This was only marginally less than the amount of rhizopine produced by strain 1a. The least amount of rhizopine was found in nodules produced by P342(pSym1a::Tn*5-mob*), which produced ineffective nodules. The reason that the transconjugants produced less 3-*O*-M*S*I than the natural isolate was probably due to chromosomal differences, and suggests that one or more chromosomal genes may influence *mos* gene expression. However, such genes are not directly involved in, and do not directly control, expression of the rhizopine genes. Although the recipient strains were known to be closely related to strain 1a in terms of chromosomal genotype (Wexler *et al.*, 1996), there may still be enough differences in the chromosome to influence expression of Sym plasmid genes. Alternatively, as mentioned in Section 5.4.3, it may be differences in the non-symbiotic megaplasmids, rather than differences in the chromosome, which affect levels of rhizopine synthesis.

The transconjugant which always produced ineffective nodules, P342(pSym1a::Tn*5-mob*), was able to direct rhizopine synthesis, although at a very low level. The fact that these bacteria synthesized much less 3-*O*-M*S*I than the other transconjugants implies that there is a link between rhizopine genes and symbiosis. It is important to note that in this ineffective strain, rhizopine synthesis was not inhibited altogether. This observation provides support for the hypothesis that there can be rhizopine synthesis in the absence of effective nitrogen fixing nodules, and is in agreement with a previous report that a NifH$^-$ strain can synthesize

rhizopine despite being unable to fix nitrogen (Murphy *et al.*, 1988). The synthesis of low levels of rhizopine in nodules which did not seem to be fixing nitrogen is consistent with results reported in Chapters 3 and 4. These chapters described the early effects of rhizopine on rhizobial competition for nodulation, and the detection of rhizopine in the roots of four day old lucerne seedlings, well before the presence of any effective nodules on the roots. The implications of this will be discussed in more detail in Chapter 6.

5.4.5 Rhizopine synthesis by pKH104

One cosmid clone containing the rhizopine biosynthesis genes, pKH104, was isolated. This cosmid was able to direct rhizopine synthesis in root nodules after transfer to strain P135. pKH104 was also recorded to have enabled 3-*O*-*MSI* synthesis after transfer to strain 8401(pRL1), although this was not found consistently in all replications of the experiment. These transconjugants produced small amounts of 3-*O*-*MSI* in root nodules, estimated to be less than 10% of that produced by the wild-type strain, 1a. The reasons for the low level of rhizopine synthesis are probably similar to those cited in Section 5.4.4 for the tranconjugant strains in which pSym1a had been transferred in its entirety.

Confirmation of rhizopine synthesis by strains carrying pKH104 was complicated by the small quantity of 3-*O*-*MSI* recovered from nodules, as HVPE has limits of detection of approximately 250 ng. Another problem was that when the quantity of nodule extract loaded on HVPE was increased, the movement of the rhizopine became retarded by impurities, making it difficult or impossible to identify compounds solely by their electrophoretic movement in comparison with standards. Also, the presence of the rhizopine after staining with silver nitrate may be disguised by other compounds in the sample. GC-MS, which is a much more sensitive technique, and allows for absolute identification of compounds by their mass spectrum, was used to confirm that the silver-staining compound identified in HVPE was 3-*O*-*MSI*.

Due to time constraints, pKH104 has not yet been further characterized, but is the cosmid of greatest interest because it is capable of directing the synthesis of the rhizopine 3-*O*-M*S*I. As it has been shown by hybridization studies and restriction digests that pKH104 has no homology to the *S. meliloti mosA*, *B* or *C* genes (C. Owuama, pers. comm., this work), it would appear that in *R. leguminosarum* these genes are substantially different from those of *S. meliloti*. Regions of homology to the *mos* genes have been found in *R. leguminosarum* bv. *viciae* strain 1a, but these regions both alone and together are not sufficient for rhizopine synthesis. The regions of *mosB* and *mosC* homology are linked to the *moc* locus, but are not linked to the *mosA*-hybridizing region. The *mosB* and *mosC* genes have only 67% and 49% homology respectively with those of *S. meliloti* (C. Owuama, unpublished results) and may in fact be non-functional pseudogenes.

pKH104 will need to be mapped and subcloned, and a mutagenesis study, for example deletion analysis, could be carried out to define the precise regions of DNA which are essential for rhizopine synthesis. The results of this study provide additional evidence that the *mos* genes in *R. leguminosarum* bv. *viciae* are substantially different from those in *S. meliloti*.

5.4.6 Production of a silver-staining compound by pKH085

The biosynthesis pathway of rhizopine is not known. The silver-staining compound produced by strain 8401(pRL1) carrying pKH085 may be a precursor to rhizopine. *S*I is thought to be a precursor in 3-*O*-M*S*I synthesis, but the unknown compound could not be *S*I, as *S*I migrates faster than 3-*O*-M*S*I in HVPE, whereas the unknown compound migrated more slowly. This compound could not be identified under the conditions employed, and further analysis was considered beyond the scope of this project. It is unclear why this compound should have been present in one batch of nodules and not in others.

5.4.7 Analysis of R-prime plasmids

There is insufficient evidence to suggest that any of the R-primes constructed in this study from pSym1a can produce rhizopine. It appears that the region of the Sym plasmid containing the rhizopine biosynthesis genes was not covered by these R-primes. The largest R-prime, pKHRP4, may have contained the genes, but this R-prime could not be comprehensively tested, because rhizobia into which it was transferred did not produce any nodules.

Although in a number of cases, HVPE analysis of nodules formed by strains of P135 containing different R-primes revealed faint silver-staining spots at the electrophoretic position expected for 3-*O*-MSI, the possibility that the compound was rhizopine was eliminated when GC-MS analysis failed to positively identify either rhizopine in these samples. GC-MS is a much more sensitive technique than HVPE, and allows specific detection by the mass spectrum. It is highly unlikely that rhizopine would be detected by HVPE but yet not be detected in the same sample by GC-MS, when using a protocol which is known to be appropriate for the detection of nanogram quantities of rhizopine (see Chapter 4).

One problem encountered in using such large clones was the difficulty in obtaining successful transfer from *E. coli* to *Rhizobium.* In addition, these transconjugants often did not generate effective symbioses. This problem was particularly apparent when the largest R-prime plasmid (pKHRP4, 180kb) was used, as rhizobia containing this R-prime never produced any nodules. The poor nodulation of R-prime-containing rhizobia may be explained if the Tn*5* insertion in the pSym1a from LS2090 was close to an incompatibility region, resulting in the incorporation of this region into some of the R-primes. In this case, when these R-prime plasmids were transferred to rhizobia, the pSym1a incompatibility region would also be transferred. The presence of an incompatibility region may have resulted in the expulsion of the native Sym plasmid, rendering the strain unable to nodulate.

Eckhardt gel analysis showed that the Sym plasmid was present immediately prior to inoculation, so it may have been lost soon after inoculation onto plants, in the absence of selective pressures.

Most of the rhizobia into which cosmid clones or pSym1a were introduced stably retained the foreign DNA, and generally nodulated more effectively than the R-prime-containing strains. The difference could possibly be explained partly in terms of the size and nature of the DNA being transferred. When an entire foreign Sym plasmid was added, the original Sym plasmid was expected to be expelled from the cells, due to selection for the incoming plasmid. This was shown to be true by Eckhardt gel analysis of the transconjugant cells. Natural rhizobia normally contain only one Sym plasmid. Incompatibility between Sym plasmids of different strains is cited as a major reason for this (O'Connell *et al.*, 1984; Hooykaas *et al.*, 1985). In Sym plasmid transfer, the original Sym plasmid was replaced by the introduced Sym plasmid, and so nodulation abilities were generally retained to a large extent. When either R-primes or cosmid clones were introduced, the bacteria would normally be expected to retain their Sym plasmid, unless a region causing incompatibility was present on the incoming DNA. The cosmid clones were smaller than the R-primes, and hence the probability of the cosmids containing a Sym plasmid incompatibility region was lower.

5.4.8 Summary

This chapter has described steps undertaken in the search for the rhizopine synthesis genes in *R. leguminosarum* bv. *viciae* strain 1a. It was first shown that in this strain, the *mos* genes are located on the Sym plasmid. R-primes and cosmid clones were then constructed from pSym1a. A cosmid clone, pKH104, was identified which contains all of the *mos* genes required for the synthesis of 3-*O*-MSI. This clone displays no homology to the *mosA*, *B* and *C* genes of *S. meliloti*. However, after transfer of pKH104 to some strains which normally do not produce any rhizopine, these strains gained the ability to produce 3-

O-MSI in pea root nodules. Characterization of the functional regions within this cosmid clone is incomplete at this stage.

Chapter	Major findings
Chapter 3	Rhizopines affect rhizobial competition for nodulation. When a rhizopine-producing strain of *S. meliloti* was competed against a non-catabolizing mutant (Moc$^-$) of the same strain, 70% of the nodules were occupied by the wild-type. It was shown that the level of dominance of the wild-type remained consistent over a period of four years under suboptimal growth conditions. Single strain nodulation experiments showed that the Moc$^-$ mutant did not have a reduced capacity to nodulate a compatible host plant, it suffered only with a reduced nodulation competitiveness when in competition with a rhizopine synthesizing and catabolizing strain.
Chapter 4	Gas chromatography-mass spectrometry (GC-MS) was used to detect rhizopine in inoculated roots. 3-*O*-MSI was detected in the roots of four day old lucerne plants. This was before any nodules were visible on the plant roots. Synthesis of rhizopine was extremely low until 18 days after inoculation when there was a massive induction of synthesis. It appears that there may be a low level of rhizopine synthesis in free-living rhizobia.
Chapter 5	Extensive studies were carried out to locate the rhizopine synthesis (*mos*) genes of *R. leguminosarum* bv. *viciae* strain 1a. It was shown using transconjugant strains that the *mos* genes are located on the Symbiotic (Sym) plasmid. Strains which cannot normally produce rhizopine develop this ability when the 1a Sym plasmid (pSym1a) is introduced. R-primes and cosmid clones were constructed from pSym1a. No R-primes containing the *mos* genes were found, but one cosmid clone, pKH104, was able to direct rhizopine synthesis after transfer to *R. leguminosarum* bv. *viciae* strain P135. This cosmid has not been fully characterized. Transconjugant strains containing pSym1a DNA which were able to synthesize rhizopine always produced less than the wild-type strain. This indicates that chromosomal background may have some influence on *mos* gene expression. A transconjugant which produced ineffective nodules, P342(pSym1a::Tn*5*-*mob*) was still able to produce a small quantity of 3-*O*-MSI, supporting the hypothesis that there is a low level of leaky expression of the *mos* genes in free-living rhizobia.

Table 6.1 Summary of the major points of each chapter

CHAPTER 6

General discussion

The major findings of this thesis are summarized briefly in Table 6.1.

6.1 Rhizopines and competition for nodulation

The nitrogen fixing bacteria of the Rhizobiaceae are extremely important agriculturally. The application of rhizobial inoculants in the field in order to increase crop yield is common. However, many inoculants are poorly adapted to the specific environmental conditions to which they are introduced, and are often out-competed by indigenous strains which dominate nodulation, but fix nitrogen with low efficiency. In order to optimize nitrogen fixation in the field, the factors which affect nodulation competitiveness need to be more fully understood. Many different factors influence rhizobial competition for nodulation, and rhizopines have been identified as one of these factors.

Several competition studies have demonstrated the competitive benefit of rhizopine catabolism (e. g. Gordon *et al.*, 1996; this work). However, the precise functions which rhizopines play in the *Rhizobium*-legume symbiosis, and how they affect the dynamics of nodule occupancy, have not been elucidated. Rhizopines were originally proposed to be a selective growth substrate (Murphy *et al.*, 1995). When rhizobia are grown in a minimal medium containing rhizopines, strains carrying *moc* (catabolism) genes catabolize the rhizopine (Murphy *et al.*, 1987; Bahar, 1997). However, the dynamics of nodulation seen in competition studies indicate that the primary function of rhizopine is not as a growth substrate, even under nutrient-limiting conditions (Gordon *et al.*, 1996; Heinrich *et al.*, 1999).

When a rhizopine strain, *S. meliloti* L5-30, was competed against a non-catabolizing (Moc^-) mutant of L5-30, the wild-type dominated nodulation, occupying more than 70% of the nodules. However, nodule occupancy by this strain never reached 100%. Instead, a steady state of competitiveness developed within two weeks of inoculation, which remained constant over a period of four years, even when the plants were grown in suboptimal conditions (described in Chapter 3) (Heinrich *et al.*, 1999). Given that this strain had an early competitive advantage and dominated in the first nodules formed, it is odd that the proportion of nodules occupied by this strain did not increase further over time, as there would have been considerable nodule turnover during the period of the experiment (four years). Free-living rhizobia released as the first generation of nodules senesced were expected to alter the ratio of the competitors in the rhizosphere, providing the Moc^+ strain with a numerical advantage in forming new infections. It was thought that this strain would acquire an increasing advantage with each successive generation of nodules.

In another competition experiment, described by Gordon *et al.* (1996), lucerne plants were inoculated with a 1:1 ratio of L5-30 and Moc^- cells. These plants were harvested at intervals, and the pots were resown with fresh lucerne seedlings without further inoculation. The harvest would have caused nodule senescence (see in Chapter 3), and the subsequent release of free-living rhizobia was expected to alter the ratio of the competitors in the rhizosphere. Although the catabolizing strain was expected to have an increased advantage in nodulating the newly sown plants due to numerical dominance, nodule occupancy of these plants was unexpectedly identical to earlier experiments.

In these competition experiments, the reason why the proportion of nodules occupied by L5-30 remained constant may be because the number of viable free-living rhizobia released from senescing nodules was minute in comparison with the total rhizobial population, and did not alter the ratio of the competitors in the rhizosphere. Beringer *et al.* (1979) estimated that each nodule contains only about 10^6 viable rhizobia, and suggested that passage of

rhizobia from nodules into the rhizosphere would not greatly enhance the soil population. A lack of change in the composition of the rhizosphere population is a likely explanation for the competition results, because it is known that the initial frequency of these two strains does affect eventual nodule occupancy. For example, if the inoculum numerically favors the wild-type, the proportion of nodules occupied by this strain approaches 100% (see Gordon *et al.*, 1996).

One hypothesis is that rhizopines influence competition primarily in the confined environment of the infection thread, rather than in the rhizosphere. If rhizopine gave a nutritional advantage in the infection thread, catabolizing strains might grow and reproduce faster than their non-catabolizing competitors, and this would allow them to dominate in doubly infected nodules. If the majority of rhizobia in mixed infections were Moc^+, a small number of Moc^- cells might not be easily detected. Although the proportion of nodules formed by mixed infection in a controlled environment can be higher than 35% (e.g. McDermott and Graham, 1990; Beattie and Handelsman, 1993), the control treatments showed that with the strains used in this study, relatively few nodules result from mixed infections. 2.5% and 2% of nodules were doubly infected in the L5-30 versus Mos^- and L5-30 versus neutral mutant treatments respectively. This does not seem high enough to support the hypothesis that competition is mainly occurring in the infection thread in this situation.

It would appear more likely that rhizopines exert a direct effect on the nodulation process, perhaps by inhibiting, or at least interfering with, an early stage in the nodulation of Moc^- strains. For example, they may decrease the rate of development of root hair infection, or increase the proportion of aborted infection threads.

Possibly, it is not the rhizopines themselves which influence rhizobial competition for nodulation, but an as yet unidentified breakdown product in rhizopine catabolism. The

interaction may either improve the nodulation of catabolizing bacteria, or reduce the nodulation capacity of rhizobia which cannot catabolize the compound.

6.2 Further studies on the effects of rhizopines on rhizobial competition for nodulation

Future work using strain L5-30 might involve competition studies between the Moc$^-$ mutant and a double mutant (Moc$^-$ Mos$^-$). The benefit of rhizopine catabolism has already been established, this experiment would investigate any specific benefit of rhizopine synthesis. In this experiment, neither strain would catabolize rhizopine, but the Moc$^-$ strain would be able to synthesize rhizopine.

Additional competition studies should be carried out using different strains of *Rhizobium*. So far, it has been shown that the nodulation competitiveness of strain L5-30 is reduced if its *moc* genes are not functional. The effects on nodulation of Moc$^-$ mutants of other strains of *S. meliloti,* or of *R. leguminosarum* strains, have not been exhaustively pursued. Also, the effects of introducing *moc* and *mos* genes to non-rhizopine strains have not been extensively examined. Rhizopine genes could be transferred to various non-rhizopine strains of *S. meliloti* and *R. leguminosarum* bv. *viciae*, and transconjugant strains could then be competed against the wild-type to investigate whether introduction of the *moc* genes has increased nodulation competitiveness. If rhizopines are an important mechanism in rhizobial competition, nodulation competitiveness would be expected to improve. Both the *mos* and the *moc* genes would need to be transferred together, and stably maintained in the recipient, to ensure that rhizopine would be present in the experimental environment. *S. meliloti* would be more amenable to experimentation, because in this species the *mos* and *moc* genes are closely linked (Murphy *et al.*, 1987). Experiments involving other species would probably also need to use the *S. meliloti* rhizopine genes. However, these genes cannot be expressed in all species of *Rhizobium* (M. Wexler, unpublished data).

6.3 The timing of rhizopine synthesis in rhizobia

The speed with which the nodulation advantage appeared in competition studies demonstrated that rhizopines affect a very early stage of nodulation. The experiments described in Chapter 4 support this assertion. The rhizopine 3-*O*-M*S*I was detected in inoculated lucerne plants as early as four days after germination. This is earlier than was previously thought, and was well before any effective nodules were visible on the roots. Although root-hair infections had almost certainly already been initiated, it is unlikely that nodule development had progressed sufficiently for there to have been fully functional bacteroids in the incipient nodules at this stage. These results suggest that there may be a low level of rhizopine synthesis in free-living rhizobia, perhaps in those which are in a micro-aerobic environment close to the root surface or in the infection thread. A huge induction of rhizopine synthesis occurred between 18 and 21 days after inoculation, which most likely coincided with the NifA-NtrA induction in the bacteroids of symbiotic genes such as the *nif* and *fix* genes, and hence, the start of the effective nitrogen-fixing symbiosis. Hence it is highly likely that the *mos* genes are also under this symbiotic regulation, as was proposed previously by Murphy *et al.* (1988) on the basis of sequence data of the promoter region.

Detection of 3-*O*-M*S*I at such an early stage of nodule development provides further support for the hypothesis proposed in Section 6.1, that rhizopines influence intraspecific competition for nodulation by directly affecting a very early stage in the nodulation process.

Gas chromatography-mass spectrometry (GC-MS) was used for detection of rhizopine because of its sensitivity and specificity. This technique can detect as little as 1 ng in impure samples, such as the nodule or root extracts. However, it is possible that a very low concentration of rhizopine is present even earlier than was detected in this study. Even very low levels of rhizopine production may be sufficient to influence rhizobial competition for nodulation, particularly if these compounds function as signal molecules. Other signal

molecules, such as the Nod factors, operate at extremely low levels (as little as 10^{-12} M (Roche *et al.*, 1991a)).

The early synthesis of rhizopine could be further investigated by examining actual expression of the *mos* genes. This could be achieved by fusion of a marker gene such as *gus* to the *mos* promoter. This application and its disadvantages were discussed in Section 4.4. Briefly, read-through transcription from *lacZ* into the GUS coding region, followed by translational initiation at the GUS initiator, have often been observed in *Rhizobium* (Jefferson, 1987). Perhaps a better candidate for use in *Rhizobium* would be the green fluorescence protein (GFP). The GFP does not have the same problems associated with its use. Also, the GFP assay is easier to carry out, because no substrate is required. Expression is easy to monitor as neither fixing nor staining of the organism is necessary. GFP has already been used to visualize rhizobial growth in the rhizosphere and infection thread (Gage *et al.*, 1996).

6.4 The function of rhizopines

Inositol-based rhizopines can be synthesized and catabolized by approximately 10% of the strains of *R. leguminosarum* bv. *viciae* and *S. meliloti*, but not by any of the other *Rhizobium* species tested to date. Considering that rhizopine genes are beneficial, it is not known why comparatively few species and strains contain these genes. It has been suggested that this may reflect relatively recent evolution of the rhizopine system (Rao *et al.*, 1995). Also, if all strains of *Rhizobium* were able to synthesize and catabolize the same rhizopine, this would lead to no particular advantage for any given strain (Murphy *et al.*, 1995). The absence of rhizopine genes from biovars *phaseoli* and *trifolii* of *R. leguminosarum* is especially surprising, considering that Sym plasmid rearrangements occur naturally between all three biovars (Laguerre *et al.*, 1993a), and such rearrangements would be expected to facilitate the transfer of rhizopine genes to each of the biovars (Wexler, 1994). Biovars *phaseoli* and *trifolii* do not have the capability to produce

rhizopine even when the *S. meliloti mos* genes are introduced. This may indicate that the host plants of these bacteria are not able to provide an essential precursor for rhizopine synthesis (Wexler, 1994), although it is more likely that the *mos* promoter simply does not work in these biovars (see Watson and Schofield, 1985).

In order to further explore the significance of rhizopines in the *Rhizobium*-legume symbiosis, and specifically, their importance in nodulation competitiveness, it is essential to investigate more thoroughly the occurrence of rhizopine genes in all the root-nodulating genera of the Rhizobiaceae. No species or strains of *Azorhizobium*, *Bradyrhizobium* or the proposed genus of *Mesorhizobium* have been tested, and it would be interesting to identify the distribution of rhizopine genes within these genera. Recently described species of *Rhizobium* and *Sinorhizobium*, such as *R. galegae*, *R. giardinii* and *R. gallicum*, and *S. saheli*, *S. medicae* and *S. teranga*, also have not been examined. It would be particularly interesting to determine the presence of rhizopine genes in newly described species, such as *S. medicae*, which have a similar host range and geographical location to some of the *S. meliloti* strains tested previously. Assuming that rhizopine genes are present in other species, analysis of these genes may provide further evidence of the evolutionary origin of the rhizopine system.

Possibly other types of rhizopines exist, which are not inositol-based compounds, and are not detectable by the methods employed to date. It would be interesting to analyze the nodule extracts of different strains for new rhizopine compounds.

6.5 The rhizopine synthesis genes of *R. leguminosarum* bv. *viciae*

The *mos* genes of *R. leguminosarum* bv. *viciae* appear to be quite different from those of *S. meliloti*. Although regions of homology to the *S. meliloti mosA, B* and *C* genes have been found previously in *R. leguminosarum* bv. *viciae,* the functional *mos* genes had not been located in this species (Wexler, 1994; Bahar, 1997). This study described the

isolation of an unrelated cosmid clone, pKH104, which has the ability to produce 3-*O*-MSI (Chapter 5). pKH104 has no homology to the *S. meliloti mosB* and *mosC* genes, nor to a *mosA*-hybridizing fragment previously isolated from *R. leguminosarum* bv. *viciae*.

mosB and *mosC*-hybridizing fragments identified previously in *R. leguminosarum* display relatively low levels of homology with the *S. meliloti mosB* and *mosC* genes (Wexler, 1994; C. Owuama, unpublished data), and may in fact be non-functional pseudo-genes. However, the *mosA*-hybridizing fragment has very high homology to the *S. meliloti mosA* gene, although it is 30 amino acids shorter (Bahar, 1997). Considering the high degree of similarity, it was expected to be involved in rhizopine synthesis. Results presented in this thesis indicate that this is not the case, but targeted mutagenesis of the fragment would show this with certainty. After mutating with an appropriate agent, the fragment could be transferred to a rhizopine strain, and incorporated into the Sym plasmid by a double crossover followed by resultant loss of the homologous region from the Sym plasmid. If the *mosA*-hybridizing fragment is essential for rhizopine synthesis, transconjugants would have a Mos$^-$ phenotype.

Due to lack of time, pKH104 has not yet been fully characterized. This cosmid is 22kb in size, and will need to be analyzed more thoroughly, including mapping and subcloning to define the functional regions, and finally, DNA sequencing. A mutagenesis technique, such as deletion analysis, may be useful in defining the functional regions.

It is possible that the *mos* genes are not the same in all strains of *R. leguminosarum*. Perhaps in some, they are highly homologous to those of *S. meliloti*, while in other strains, the genes may be quite dissimilar. One strain of *R. leguminosarum*, P444, has no homology to the *S. meliloti mosA* gene, even though it produces 3-*O*-MSI in root nodules. Several other isolates have *mosA* homology on their Sym plasmids, but are not able to produce any rhizopine (Wexler, 1994). This may indicate that there are at least two different biochemical pathways which lead to rhizopine synthesis.

The rhizopine 3-*O*-MSI was detected in ineffective nodules (for example, in those produced by P342(pSym1a::Tn*5-mob*)), and was present in four day old inoculated lucerne roots. In both cases, rhizopine was detected in the absence of nodules which were effective. Nitrogen fixation itself is not a prerequisite for rhizopine synthesis, as a NifH$^-$ mutant, which forms ineffective nodules, produces rhizopine (Murphy *et al.*, 1988). In this study, the amount of rhizopine produced in ineffective nodules was estimated to be approximately 100 times less than that produced by the wild-type strain, 1a. The amount of rhizopine found in lucerne roots remained extremely low between four and 16 days after germination. The evidence suggests that the *mos* genes are subject to symbiotic regulation as proposed previously (Murphy *et al.*, 1988). However, there seems to be a low level of constitutive or "leaky" expression of these genes prior to NifA-NtrA-mediated induction. A more likely explanation, mentioned previously in Section 6.3, is that this early induction is mediated by NifA and NtrA, and occurs at a low level in free living rhizobia in a micro-aerobic environment on the root surface or in the infection thread.

The massive increase in the amount of rhizopine seen approximately 19 days after germination seems likely to coincide with induction of NifA-NtrA-regulated genes in the bacteroids, and hence, the initiation of nitrogen fixation. Before this time, rhizopine has already exerted its effect on rhizobial competition for nodulation.

The experiments demonstrating the appearance of rhizopine in the roots of four day old lucerne seedlings also help to explain the competition results. If even a low level of rhizopine synthesis occurs within a few days of inoculation, and before an effective symbiosis is established, it may be sufficient to affect nodule occupancy in the first generation of nodules. As it has now been shown that rhizopine is present at a very early developmental stage, the hypothesis postulated earlier in this thesis that rhizopines inhibit, influence, or interfere with an early stage in the nodulation process, would seem plausible.

It is possible that it is not the rhizopine itself which affects competition, but perhaps an unidentified breakdown product in the catabolic pathway.

6.6 *nfe* genes

There are some similarities between the rhizopine genes and the *nfe* genes. The *nfe* genes are present in some strains of *S. meliloti* and *B. japonicum,* and also affect nodulation competitiveness. Insertions into the *nfe* region of *S. meliloti* result in reduced nodulation competitiveness (Sanjuan and Olivares, 1989), while transfer of the *nfe* genes to other rhizobia improves nodulation competitiveness (Sanjuan and Olivares, 1991). Correct expression of the *nfe* phenotype depends on strain-specific genetic or physiological characteristics (Sanjuan and Olivares, 1991). From the observations presented here, it would appear that optimum expression of the rhizopine genes is also dependent on various strain-specific characteristics.

A low level of homology was found between the *nfe1* gene and ORF1 of the *mos* operon (Soto *et al.*, 1993), and both *mos* and *nfe* have NifA-NtrA-regulated promoters. Maximal expression of *nfe* is expected to occur in the latter stages of nodule development (Sanjuan and Olivares, 1989). The function and mode of action of most of the *nfe* gene products is unknown. Some are thought to be regulatory proteins, while others show little homology to any known proteins (Soto *et al.*, 1993). Unlike the rhizopine genes, the *nfe* genes are not located on the Sym plasmid but on a cryptic megaplasmid (Sanjuan and Olivares, 1989).

6.7 The rhizopine metabolic pathway

The biosynthesis pathway of the two rhizopines, *S*I and 3-*O*-M*S*I, has not been determined. One rhizopine-producing cosmid was obtained in this study, but other cosmids may have contained some but not all of the rhizopine genes. In this scenario, no

rhizopine would be produced, but the appearance of an unknown compound in HVPE or GC-MS analysis might indicate an intermediate which would ordinarily be converted immediately to the following compound in the pathway. In this study, no candidate compounds were positively identified by HVPE or GC-MS. However, homologies and similarities with the streptomycin biosynthesis pathway in *Streptomyces griseus* have given an indication of some of the steps which might occur in rhizopine metabolism. A proposed pathway for both rhizopine synthesis and catabolism is presented in Figure 6.1.

In the 1960s, *scyllo*-inosamine (*S*I) was discovered as an intermediate in the streptomycin biosynthesis pathway of *Streptomyces griseus* (Walker and Walker, 1967). In this pathway, *myo*-inositol is converted to *scyllo*-inosose, which is then converted to *scyllo*-inosamine via a transaminase. The enzyme catalyzing this conversion, *scyllo*-inosamine-pyruvate transaminase, which also reacts with α-ketoglutarate, adds an amino group to *scyllo*-inosose to create *scyllo*-inosamine. The gene encoding this enzyme, *stsC*, was recently cloned (Ahlert *et al.*, 1997), and has homology to the *mosB* gene of *S. meliloti*. This suggests that rather than being a regulatory gene, *mosB* may encode a transaminase. Certainly, an aminase is likely to be involved in rhizopine synthesis at some point, especially if, as predicted, the precursor is *myo*-inositol or a related compound. Evidence for the involvement of *myo*-inositol in rhizopine metabolism is convincing, as a mutant unable to use *myo*-inositol as a carbon source is unable to catabolize rhizopine (Bahar, 1997). *Myo*-inositol is also a precursor in the streptomycin biosynthesis pathway (Walker and Walker, 1967).

The function of MosC is unknown, and this protein has little homology to any known protein (Murphy *et al.*, 1993). It was proposed to be a transporter (Murphy *et al.*, 1995). As MosC would appear to be a very specialized transporter protein, which is present only in rhizopine strains, it is more likely to transport the rhizopine out of the bacteroids, rather than transporting precursors into the bacteroids. The precursors are likely to be common compounds, such as *myo*-inositol, which are often found in bacteroids and legume root

nodules, and which are probably used in other metabolic pathways in the bacteria besides rhizopine synthesis. It is unlikely that rhizopine strains would require a specialized transporter to import these compounds.

A further similarity between the rhizopine and streptomycin pathways has been found. The MocA protein, which is involved in rhizopine catabolism, has homology to StrI from *S. griseus* (Bahar, 1997). StrI is likely to catalyze a step which is dependent on dehydrogenases in the biosynthesis of hexose-derived moieties of streptomycin (Mansouri and Piepersberg, 1991).

It is possible that the rhizopine genes and the streptomycin biosynthesis genes have the same evolutionary origin. The *nfe* genes may also be of the same ancestry, although the relatively low homologies between *mos* and *nfe*, and the fact that homology with the *mos* operon is limited to ORF1, indicate this is less likely. The *mos* genes may correspond to the early steps in the streptomycin pathway, in which *myo*-inositol is converted to *S*I via *scyllo*-inosose. An additional gene present in rhizobia unlikely to have a homolog in *Streptomyces, mosA,* is responsible for the methylation of *S*I to produce 3-*O*-M*S*I.

The genes *mocDEF* and *mocR* are responsible for the conversion of 3-*O*-M*S*I to *S*I in the catabolic pathway (Bahar *et al.*, 1999). *mocA* has levels of high homology to *strI*. Perhaps in the future, other *moc* genes will be found to correspond to some of the later genes in the streptomycin synthesis pathway. According to this hypothesis, 3-*O*-M*S*I would not be an end-product, but rather an intermediate in the biosynthesis of another compound which has not yet been identified. Despite rhizopine metabolism in *Rhizobium* possibly having the same ancestral origin as streptomycin biosynthesis, it is unlikely that the end-product in *Rhizobium* is streptomycin. As both *Rhizobium* and *Streptomyces* are soil bacteria, this may have facilitated horizontal transfer of the genes between the species.

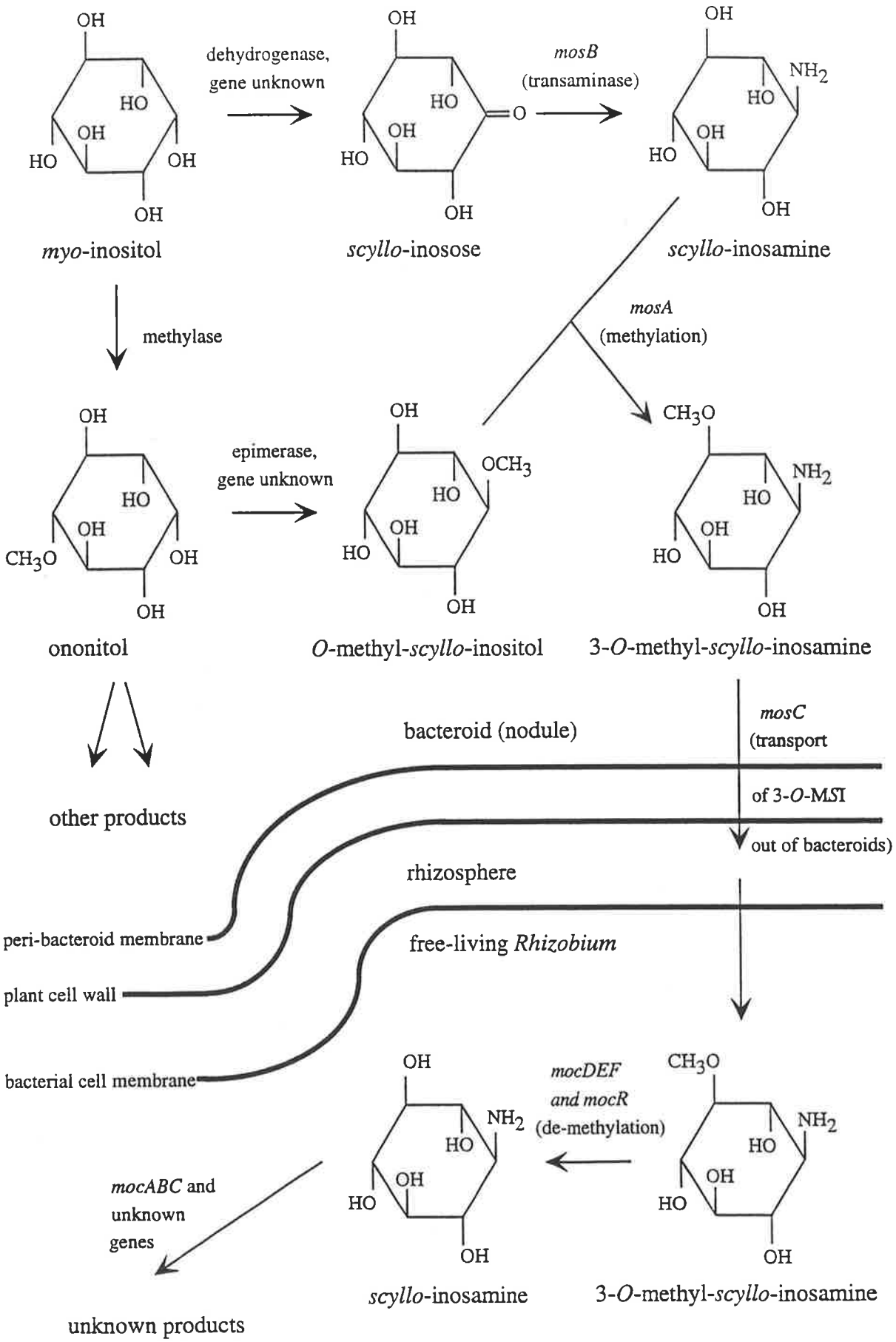

Figure 6.1 Proposed pathway for rhizopine synthesis and catabolism

Subsequent divergent evolution may have resulted in the considerable differences in the latter stages of the two pathways.

6.8 The involvement of cyclitols in rhizopine synthesis

It is curious that MosA shows no homology to methylases or related enzymes, because it appears to play a role in methylation. A methylase or methyl-transferase is required to transfer a methyl group to *S*I in order to produce 3-*O*-M*S*I. The methyl ether, *O*-methyl-*scyllo*-inositol, is a candidate as potential donor of the methyl group. This compound has been found in nodules produced on pea roots by the rhizopine-producing isolate, 1a, but not in nodules produced by strain 1045. Strain 1045 does not produce rhizopine (Skot and Egsgaard, 1984).

The presence of ononitol (4-*O*-methyl-*myo*-inositol) has also been recorded. Ononitol was abundant in nodules produced by strain 1045, but was low in nodules elicited by 1a (Skot and Egsgaard, 1984). This was a small study, and a correlation of the abundance of these compounds in other rhizobia has not been established. Nodules produced by a non-rhizopine strain commonly used in the current study, 8401(pRL1), also contained large quantities of ononitol (Chapter 4), but did not appear to contain any *O*-methyl-*scyllo*-inositol (see Table 6.2).

The amount of ononitol in root nodules appears to be inversely proportional to the amount of *O*-methyl-*scyllo*-inositol. This could be interpreted as a conversion of ononitol to *O*-methyl-*scyllo*-inositol in rhizopine strains. This may suggest that this compound is involved in the production of 3-*O*-M*S*I. As *O*-methyl-*scyllo*-inositol contains one methyl group, it could be the donor of the methyl group in the conversion of *S*I to 3-*O*-M*S*I. This could be investigated by using chemically synthesized *O*-methyl-*scyllo*-inositol incorporating radiolabelled C^{14}, and examining whether the radioactive carbon isotopes become incorporated into 3-*O*-M*S*I. A similar experiment could be done to demonstrate the

involvement of ononitol in rhizopine synthesis. The main problem would be obtaining appropriate *in vitro* conditions to enable rhizopine synthesis.

Throughout this study, the quantities of ononitol and *myo*-inositol in nodules were observed to be inversely proportional. This is in agreement with a report by Streeter (1985), who found that a decrease in ononitol was proportional to an increase in *myo*-inositol in soybean nodules. A decrease in *myo*-inositol may occur as it is being converted to ononitol, and this conversion is known to occur in nature (Kindl, 1969). A decrease in ononitol also appeared to be proportional to the appearance of 3-*O*-M*S*I, further suggesting that ononitol is a rhizopine precursor (see Table 6.2).

One proposal is that in rhizopine strains, as *myo*-inositol is being converted to *S*I via *scyllo*-inosose, another pathway is simultaneously converting *myo*-inositol to *O*-methyl-*scyllo*-inositol via ononitol. *O*-methyl-*scyllo*-inositol may then act as donor of a methyl group in the conversion of *S*I to 3-*O*-M*S*I (see Figure 6.1). *myo*-inositol is a commonly found compound in legume root nodules, and is known to serve many functions in different biochemical pathways in plant metabolism. For example, it is also converted to pectin and various other polysaccharides (see Kindl, 1969; Loewus, 1964).

Due to very small differences in retention time between ononitol and *O*-methyl-*scyllo*-inositol (see Skot and Egsgaard, 1984), it was difficult to determine whether *O*-methyl-*scyllo*-inositol, as well as ononitol, was present in some samples. Although these two compounds have slightly different chemical structures, they have the same chemical composition, and can be difficult to identify on the basis of mass spectrum alone. To ascertain whether both compounds were present, they would need to be separated on the gas chromatography column by a procedure which would allow for greater separation of these two compounds. This could perhaps be done using their trimethylsilyl derivatives (Sherman *et al.*, 1970).

strain of *R. leguminosarum* bv. *viciae*	*myo*-inositol	ononitol (4-*O*-methyl-*myo*-inositol)	*O*-methyl-*scyllo*-inositol	3-*O*-MSI
1a	+++	+	+	+
1045	+	+++	-	-
8401(pRL1)	+	+++	-*	-

Table 6.2 Relative abundance of various cyclitols in the nodules produced on pea roots by three strains of *R. leguminosarum* bv. *viciae.*

* does not appear to be present, but for confirmation, the nodule extracts should be analyzed using trimethylsilyl derivatives, which provide better separation better separation between these two compounds in gas chromatography.

6.9 Final conclusions

This thesis has contributed to the understanding of how rhizopines affect rhizobial competition for nodulation. It has been shown that the ability to catabolize rhizopine increases the nodulation competitiveness of a strain. The effects on competition occur very soon after inoculation. The rapid response is possible because there is a low level of rhizopine synthesis by rhizobia (which may or may not be free-living) which can be detected in inoculated lucerne roots within four days of germination. The level of dominance of a rhizopine-catabolizing strain over a non-catabolizing mutant did not increase beyond 70% over a four year period. This may reflect that although the Moc^+ strain is able to dominate nodulation, there is no concomitant change in its frequency in the rhizosphere.

This thesis also describes experiments which have succeeded in obtaining a cosmid clone from the Sym plasmid of *R. leguminosarum* bv. *viciae* strain 1a which contains the *mos* genes. The rhizopine synthesizing region in *R. leguminosarum* is substantially different from that of *S. meliloti*, but needs to be characterized in more detail.

Finally, a model was proposed for the pathway of rhizopine synthesis based on GC-MS data, gene homologies, and published results and observations of compounds occurring in legume nodules.

Further work is required to fully understand the rhizopine system, and the full significance and importance of rhizopines in the *Rhizobium*-legume symbiosis.

CHAPTER 7

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APPENDICES

Appendix 1 lists the media used throughout this study.

A1.1 Bergersen's minimal media

A1.2 GTS minimal media

A1.3 Hoagland's low nitrogen nutrient solution

A1.4 Luria Bertani media

A1.5 M9 Minimal media

A1.6 SOC media

A1.7 TY media

Appendix 2 lists the antibiotics and concentrations used throughout this study.

Appendix 3 contains a restriction map of the cloning vector used in this study, pLAFR3.

Appendix 4 contains tables describing the nodulation characteristics and results of rhizopine synthesis tests for strains of *Rhizobium* containing R-prime plasmids.

Appendix 5 lists the publications generated from this thesis.

APPENDIX 1 Media

All media and chemicals were handled using the appropriate aseptic technique. All chemicals and reagents were of laboratory or analytical quality.

Bergersen's minimal media

(per liter)

Bergersen's salts solution (x 5)	400 mL
Biotin (0.2 mg/mL)	1 mL
Thiamine (1 mg/mL)	1 mL

Bergersen's salts solution (x 5)

(per liter)

KH_2PO_4	1.1 g
$MgSO_4.7H_2O$	0.5 g
$FeCl_2.6H_2O$ in 0.1M HCl	0.1 g
$CaCl_2.4H_2O$	0.2 g

Adjust pH to 6.8 using 0.1 N NaOH and autoclave.

GTS minimal media

(per liter)

agar (1.5%)	15 g in 767 mL water
Autoclave, cool to 55°C, then add:	
Solution 1	200 mL
Solutions 2, 3 and 5	1 mL
Solutions 4, 6 and 7	10 mL

Solution 1	K_2HPO_4	0.13 g/L
	NaCl	5.0 g/L
	Tris	15.0 g/L
	Na succinate	13.5 g/L
	$(NH_4)SO_4$	10.0 g/L (pH 7.5)
Solution 2	$MgSO_4.7H_2O$	246 g/L
Solution 3	$CaCl_2.2H_2O$	14.5 g/L
Solution 4	$FeCl_3.6H_2O$	27 mg/L
Solution 5	$Na_2MoO_4.2H_2O$	242 mg/L
	H_3BO_3	3 g/L
	$MnSO_4.2H_2O$	1.83 g/L
	$ZnSO_4.7H_2O$	287 mg/L
	$CuSO_4.5H_2O$	125 mg/L
	$CoCl_2.6H_2O$	119 mg/L
Solution 6	Biotin	200 mg/L
Solution 7	Glucose	200 g/L

Sterilize Solutions 1 - 5 by autoclaving and solutions 6 and 7 by filtration.

Hoaglands low nitrogen nutrient solution

(per liter)

1 M $MgSO_4.7H_2O$	2 mL
1 M KH_2PO_4	1 mL
1 M KCl	5 mL
1 M $CaCl_2.2H_2O$	5 mL
micronutrients	1 mL
FeEDTA (4 g/100 mL)	1 mL

Autoclave.

Micronutrients

(per liter)

H_3BO_4	2.86 g/L
$MnCl_2.4H_2O$	1.81 g/L
$ZnSO_4.7H_2O$	0.22 g/L
$CuSO_4.5H_2O$	0.08 g/L
$Na_2MoO_4.2H_2O$	0.025 g/L

Autoclave.

Luria Bertani media

(per liter)

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	5 g

Adjust pH to 7.2 with NaOH and autoclave.

M9 minimal media

(per liter)

$NaHPO_4$	6 g
KH_2PO_4	3 g
NH_4Cl	1 g
NaCl	0.5 g

Adjust pH to 7.4, add 15 g (1.5%) agar. Autoclave, cool to 55°C, then add:

1 M $MgSO_4.7H_2O$	1 mL
0.1 M $CaCl_2.2H_20$	1 mL
1 M thiamine HCl	1 mL
20% (w/v) glucose	10 mL

$CaCl_2.2H_2O$ and $MgSO_4.7H_2O$ should be autoclaved prior to use, thiamine and glucose should be filter-sterilized.

SOC media

(per liter)

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	5 g

100 mM $MgSO_4.7H_2O$

10 mM $MgCl_2$

20 mM glucose

Adjust pH to 7.0 with NaOH and autoclave.

TY media

(per liter)

Bacto-tryptone	5 g
Bacto-yeast extract	3 g
$CaCl_2.2H_2O$	0.9 g

Adjust pH to 6.8 and autoclave.

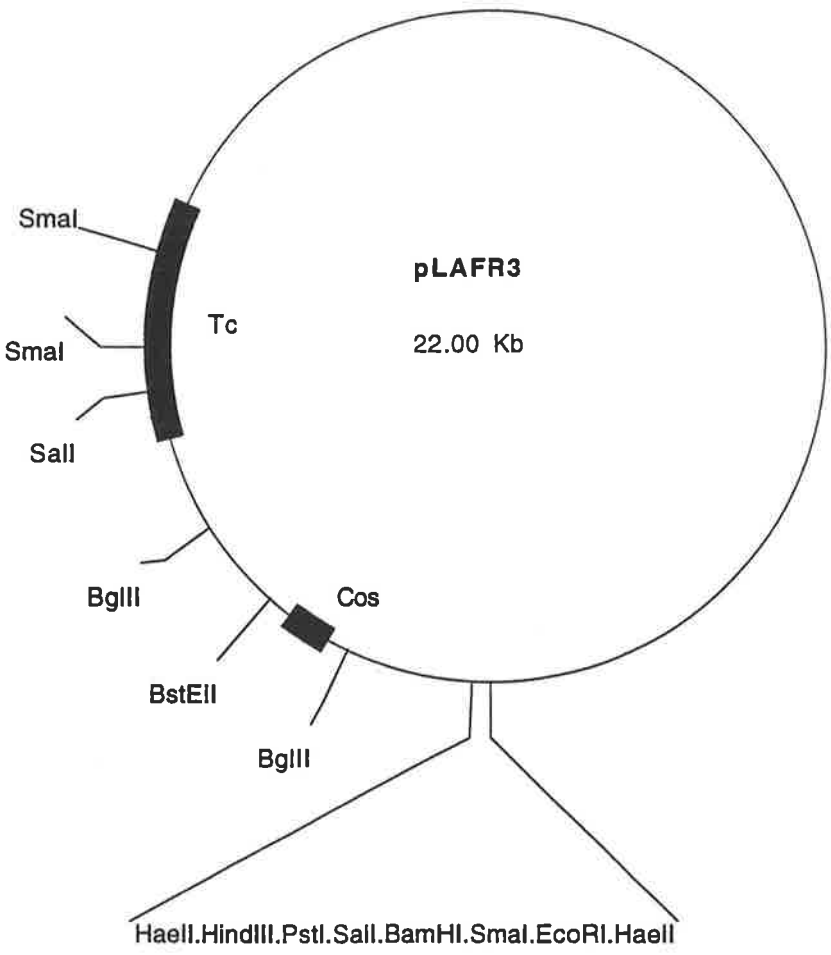
APPENDIX 2 Antibiotics

The concentrations of antibiotics used throughout this study are given in Table A2.1.

Antibiotic	**Concentration** (*Rhizobium* and *Agrobacterium*) (µg/mL)	**Concentration** (*E. coli*) (µg/mL)	**Stock solution** (mg/mL)	**Solvent**
Chloramphenicol	50	50	50	ethanol
Kanamycin monosulfate	250	50	50	water
Naladixic acid	-	200	50	water
Rifampicin	100	100	10	methanol
Tetracycline hydrochloride	10	10	10	50% ethanol
Streptomycin sulfate	250	-	250	water

Table A2.1 Antibiotic concentrations used throughout this study. When different concentrations were used, this is specified in the text.

APPENDIX 3 Cloning Vector



Plasmid name: pLAFR3
Plasmid size: 22.00 kb
Constructed by: Staskawicz et al.
Construction date: 1987
Comments/References: Broad host range cloning vector (cosmid). (Staskawicz et al., 1987)

APPENDIX 4 Results of *mos* tests and nodulation studies on transconjugant rhizobia

Tables A4.1 - A4.10 describe the nodulation characteristics and the presence of silver-staining compounds (detected by HVPE) and rhizopines (detected by GC-MS) in nodules produced by rhizobia containing R-prime plasmids.

Strain	Nodulation	Silver-staining compounds (HVPE)	Rhizopine (GC-MS)	Rep-lications
8401(pRL1)(pKHRP1)	effective	-	-	5
P121(pKHRP1)	effective	-	-	2
P135(pKHRP1)	effective	+	-	3
P233(pKHRP1)	ineffective	-	nd	1
P342(pKHRP1)	no nodulation	nd	nd	1
Rm1021(pKHRP1)	effective	-	-	1

Table A4.1 Nodulation characteristics, and the presence of silver-staining compounds (detected by HVPE) and rhizopines (detected by GC-MS), in nodules produced by rhizobia containing pKHRP1.

Strain	Nodulation	Silver-staining compounds (HVPE)	Rhizopine (GC-MS)	Rep-lications
8401(pRL1)(pKHRP2)	ineffective	-	nd	3
P121(pKHRP2)	effective	-	nd	1
P135(pKHRP2)	effective	+	-	2
P233(pKHRP2)	effective	-	nd	1
P342(pKHRP2)	no nodulation	-	nd	1

Table A4.2 Nodulation characteristics, and the presence of silver-staining compounds (detected by HVPE) and rhizopines (detected by GC-MS), in nodules produced by rhizobia containing pKHRP2.

Strain	Nodulation	Silver-staining compounds (HVPE)	Rhizopine (GC-MS)	Rep-lications
8401(pKHRL1)(pRP3)	ineffective	-	nd	3
P121(pKHRP3)	ineffective	-	nd	1
P135(pKHRP3)	ineffective	-	-	1
P233(pKHRP3)	effective	-	nd	1
P342(pKHRP3)	no nodulation	nd	nd	1
Rm1021(pKHRP3)	no nodulation	nd	nd	1

Table A4.3 Nodulation characteristics, and the presence of silver-staining compounds (detected by HVPE) and rhizopines (detected by GC-MS), in nodules produced by rhizobia containing pKHRP3.

Strain	Nodulation	Silver-staining compounds (HVPE)	Rhizopine (GC-MS)	Rep-lications
8401(pRL1)(pKHRP4)	no nodulation	nd	nd	3
P121(pKHRP4)	no nodulation	nd	nd	1
P135(pKHRP4)	no nodulation	nd	nd	1
P233(pKHRP4)	no nodulation	nd	nd	1
P342(pKHRP4)	no nodulation	nd	nd	1
Rm1021(pKHRP4)	no nodulation	nd	nd	1

Table A4.4 Nodulation characteristics, and the presence of silver-staining compounds (detected by HVPE) and rhizopines (detected by GC-MS), in nodules produced by rhizobia containing pKHRP4.

Strain	Nodulation	Silver-staining compounds (HVPE)	Rhizopines (GC-MS)	Rep-lications
8401(pRL1)(pKHRP5)	effective	-	-	3
P121(pKHRP5)	effective	-	nd	1
P135(pKHRP5)	effective	+	-	1
P233(pKHRP5)	no nodulation	nd	nd	1
P342(pKHRP5)	ineffective	-	nd	1

Table A4.5 Nodulation characteristics, and the presence of silver-staining compounds (detected by HVPE) and rhizopines (detected by GC-MS), in nodules produced by rhizobia containing pKHRP5.

Strain	Nodulation	Silver-staining compounds (HVPE)	Rhizopines (GC-MS)	Rep-lications
8401(pRL1)(pKHRP6)	effective	-	-	3
P121(pKHRP6)	effective	-	nd	1
P135(pKHRP6)	effective	+	-	1
P233(pKHRP6)	effective	+	-	1
P342(pKHRP6)	ineffective	-	nd	1

Table A4.6 Nodulation characteristics, and the presence of silver-staining compounds (detected by HVPE) and rhizopines (detected by GC-MS), in nodules produced by rhizobia containing pKHRP6.

Strain	Nodulation	Silver-staining compounds (HVPE)	Rhizopines (GC-MS)	Rep-lications
8401(pRL1)(pKHRP7)	ineffective	-	nd	3
P121(pKHRP7)	effective	-	nd	1
P135(pKHRP7)	effective	-	nd	1
P233(pKHRP7)	no nodulation	-	nd	1
P342(pKHRP7)	no nodulation	-	nd	1

Table A4.7 Nodulation characteristics, and the presence of silver-staining compounds (detected by HVPE) and rhizopines (detected by GC-MS), in nodules produced by rhizobia containing pKHRP7.

Strain	Nodulation	Silver-staining compounds (HVPE)	Rhizopines (GC-MS)	Rep-lications
8401(pRL1)(pKHRP8)	effective	-	nd	3
P121(pKHRP8)	effective	-	nd	1
P135(pKHRP8)	effective	+	-	1
P233(pKHRP8)	no nodulation	nd	nd	1
P342(pKHRP8)	effective	-	nd	1

Table A4.8 Nodulation characteristics, and the presence of silver-staining compounds (detected by HVPE) and rhizopines (detected by GC-MS), in nodules produced by rhizobia containing pKHRP8.

Strain	Nodulation	Silver-staining compounds (HVPE)	Rhizopine (GC-MS)	Rep-lications
8401(pRL1)(pKHRP9)	effective	-	nd	3
P121(pKHRP9)	ineffective	-	nd	1
P135(pKHRP9)	effective	+	-	1
P233(pKHRP9)	effective	-	nd	1
P342(pKHRP9)	no nodulation	-	nd	1

Table A4.9 Nodulation characteristics, and the presence of silver-staining compounds (detected by HVPE) and rhizopines (detected by GC-MS), in nodules produced by rhizobia containing pKHRP9.

Strain	Nodulation	Silver-staining compounds (HVPE)	Rhizopine (GC-MS)	Rep-lications
8401(pRL1)(pKHRP10)	ineffective	-	nd	2
P121(pKHRP10)	effective	-	nd	1
P135(pKHRP10)	effective	+	-	1
P233(pKHRP10)	effective	-	nd	1
P342(pKHRP10)	effective	-	nd	1

Table A4.10 Nodulation characteristics, and the presence of silver-staining compounds (detected by HVPE) and rhizopines (detected by GC-MS), in nodules produced by rhizobia containing pKHRP10.

APPENDIX 5 Publications arising from this thesis

Journal Publications

Gordon, D. M., Ryder, M. H., **Heinrich, K.** and Murphy, P. J. 1996. An experimental test of the rhizopine concept in *Rhizobium meliloti*. *Appl. Environ. Microbiol.* **62:**3991-3996.

Heinrich, K., Gordon, D. M., Ryder, M. H. and Murphy, P. J. 1999. A rhizopine strain of *Sinorhizobium meliloti* remains at a competitive nodulation advantage after an extended period in the soil. *Soil Biol. Biochem.* In press.

Conference Papers

Heinrich, K., Turner, A. M., Ophel Keller, K., Ballard, R., Ryder, M. H., Wexler, M., Gordon, D. G. and Murphy, P. J. 1996. The genetic relationships and competitiveness of rhizopine strains of *Rhizobium*. The Eleventh Australian Nitrogen Fixation Conference. Perth, Australia.

Heinrich, K., Ryder, M. H. and Murphy, P. J. 1997. The effects of rhizopines on rhizobial competition for nodulation. Australian Society for Microbiology Conference. Adelaide, Australia.

Poster Abstracts

Bahar, M., Wexler, M., Gordon, D., **Heinrich, K.** and Murphy, P. 1996. Ecology and evolution of rhizopines in the *Rhizobium*-legume symbiosis. 8th International Congress. Molecular Plant-Microbe Interactions, Knoxville, USA.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1996, p. 3991–3996
0099-2240/96/$04.00+0

Vol. 62, No. 11

An Experimental Test of the Rhizopine Concept in *Rhizobium meliloti*

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In some *Rhizobium*-legume symbioses, compounds known as rhizopines are synthesized by bacteroids and subsequently catabolized by free-living cells of the producing strain. It has been suggested than rhizopines act as proprietary growth substrates and enhance the competitive ability of the producing strain in its interactions with the diverse microbial community found within the rhizosphere. Wild-type, rhizopine-producing *Rhizobium meliloti* L5-30 and mutant L5-30 strains deficient for either rhizopine synthesis or catabolism were inoculated onto lucerne host plants in competition experiments. These experiments demonstrated that no apparent advantage resulted from the ability to synthesize a rhizopine, whereas the ability to catabolize rhizopine provided a clear advantage when an organism was in competition with a strain without this ability. The results suggest that when an organism is in competition with a catabolism-deficient mutant, the ability to catabolize rhizopine results in enhanced rates of nodulation. The results of the experiments were not consistent with the hypothesis that the sole role of rhizopines is to act as proprietary growth substrates for the free-living population of the producing strain.

In nitrogen-deficient environments the benefits accruing to the plant partner in rhizobium-legume associations are well understood. The plant gains from the bacterium's ability to convert atmospheric nitrogen into a form that can be assimilated. This additional nitrogen results in increased plant growth and reproductive success, which may in turn enhance the plant's competitive ability in its interactions with other members of the floral community. The benefits gained by the bacteria involved in these associations are much less well understood. This is particularly true in rhizobial species, such as *Rhizobium meliloti* and *Rhizobium leguminosarum*, in which the formation of a bacteroid represents an irreversible developmental event (7, 12). Thus, although the bacteroid stage is the stage responsible for nitrogen fixation, the formation of every non-reproductive bacteroid represents a loss to the bacterial population. The free-living bacteria inhabiting infection threads within nodules may obtain some degree of protection from biotic and abiotic environmental factors. However, a large fraction of the free-living rhizobial population is found in the rhizosphere (1). Competition in this nutrient-rich environment is intense as there is a diverse and abundant microbial community exploiting these resources.

One manner by which the rhizobial population could benefit from its association with plants would be if the bacteroid subpopulation produced a compound that could be exploited as a growth substrate exclusively by members of the free-living rhizobial population. The specific production of a growth substrate could confer a competitive advantage for the producers over other saprophytic microorganisms in the rhizosphere. Rhizopines may be such compounds.

Rhizopines (L-3-*O*-methyl-*scyllo*-inosamine, and *scyllo*-inosamine) are produced from plant precursors by bacteroids within nodules (3, 8, 10). Significantly, rhizopines can be catabolized only by the free-living cells of the producing strain (3). Not all strains of a species synthesize rhizopines (13). In an extensive survey, about 12% of the *R. meliloti* and *R. leguminosarum* strains tested were found to produce rhizopines (13). The genes for synthesis and catabolism of rhizopines are coincident and have been found only on the symbiotic plasmid. In *R. meliloti* the genes are closely linked, and rhizopine synthesis is regulated via the *nifA* gene (4). No species of bacteria other than some *Rhizobium* species has been found to have the ability to catabolize rhizopines (9). These factors suggest that rhizopines play a role in symbiosis, and it has been advocated that rhizopines represent proprietary growth substrates that enhance the population growth of the producing strain in the soil (5, 6).

The purpose of this study was to test the rhizopine concept in planta. From a naturally isolated L-3-*O*-methyl-*scyllo*-inosamine-synthesizing and -catabolizing *R. meliloti* strain, mutants were constructed by using a transposon-insertion technique. One of these mutants is defective for the ability to synthesize rhizopine (Mos^-), another mutant is unable to catabolize the rhizopine (Moc^-), and a third mutant contains the transposon but is unimpaired with respect to rhizopine synthesis or catabolism and served as a neutral control. Plants growing in pots were inoculated with each combination of wild type and mutant, and the relative frequencies of the strains were monitored through time.

MATERIALS AND METHODS

Strains and strain construction. The mutants produced for these experiments were all derived from *R. meliloti* L5-30 (Mos^+, Moc^+). The Mos^+ Moc^- mutant (strain 2129) and the Mos^+ Moc^+ neutral mutant (strain 2126) were prepared by mutating plasmid pPM1031 with Tn*5* and then incorporating the mutated fragment into the genome of *R. meliloti* L5-30 by marker exchange as previously described (3). The same procedure was used to obtain the Mos^- Moc^+ mutant (strain 2168), except that in this case the starting plasmid was pPM1062. The

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wild-type strain, L5-30, was modified so that it harbored plasmid pPH1JI, which was used in the other strains as part of the marker exchange protocol (strain 2338).

In vitro growth experiments. To determine if the Tn*5* insertion resulted in any significant change in the growth of the mutant strains, the exponential-phase growth rates of the three mutants and the wild-type strain were estimated for cultures growing in liquid medium and on agar medium. The medium used was TY (10 g of tryptone per liter, 5 g of yeast extract per liter, 0.9 g of $CaCl_2 \cdot H_2O$ per liter), and agar (15 g $liter^{-1}$) was added for the surface growth experiments. Liquid culture experiments were carried out in 250-ml square bottles containing 50 ml of medium. For the surface cultures, 2 ml of liquid agar medium was spread onto sterile glass microscope slides and allowed to harden (11). The growth cultures were started from stationary-phase cultures, serially diluted to produce an initial density of about 10^4 cells ml^{-1}. The inoculum (100 μl) was spread uniformly on the agar-coated slides. All cultures were incubated at 28°C, and the liquid cultures were shaken at 150 rpm. The cultures were sampled at regular intervals until the stationary phase was achieved (for liquid cultures, 56 h; for surface cultures, 72 h). Cell densities were determined by serial dilution and plating.

Single-strain nodulation experiments. Lucerne (variety Hunter River) seeds were germinated on agar. After germination (3 days) the seedlings were transplanted into steam-sterilized glass tubes (15 by 2 cm) containing a mixture of equal parts of sand and loam. The tubes were then randomly assigned to one of four groups. Immediately after transplanting, each seedling was inoculated with 100 μl (10^8 cells) of a stationary-phase culture of either the wild-type strain (2338) or the Moc^- (2129), Mos^- (2168), or neutral (2126) mutant. The inoculum was applied to the base of the stem. At regular intervals after inoculation 15 plants were randomly selected from each of the four groups of tubes and the number of nodules per plant was determined.

Plant culture. Experiments were carried out in tall-form 1-liter pots. The soil medium consisted of equal parts of loam and coarse sand supplemented with superphosphate (1.12 kg m^{-3}), $CaCO_3$ (600 g m^{-3}), and K_2SO_4 (600 g m^{-3}). The soil was steam pasteurized prior to use. The variety of lucerne (*Medicago sativa*) used was Hunter River, and Tamor was the variety of white clover (*Trifolium repens*) used. Seeds were sterilized by soaking them for 2 min in 100% ethanol and then for 2 min in a sodium hypochlorite solution (125 g $liter^{-1}$); this was followed by five rinses in sterile distilled water. Multiple seeds were sown per pot, and seedlings were thinned to one plant per pot prior to inoculation at 2 weeks after planting. The plants were watered as required with filtered water and were maintained under natural light conditions at 22 to 26°C during the day and 16 to 18°C at night.

Competition experiments. The basic design of the experiment consisted of inoculating plants with equal numbers of one of the mutant strains and the wild-type strain. The frequencies of both cell types were then monitored through time by isolating bacteria from nodules taken from the lucerne host plant. The following three strain combinations were tested: Moc^- mutant and wild-type strain (strain 2129 versus strain 2338), Mos^- mutant and wild type strain (strain 2168 versus strain 2338), and neutral mutant and wild-type strain (strain 2126 versus strain 2338). For each of the three strain combinations the changes in the relative frequencies of the mutant and wild-type strains were monitored in three ways.

The first method, designated the lucerne harvest experiment, involved inoculating a large number of pots containing young lucerne plants with one of the three strain combinations. At regular intervals, a number of pots were randomly selected, the plants were removed, and the nodules were harvested. This harvest method allowed the relative frequencies of the mutant and wild-type strains in the nodule-inhabiting population of bacteria to be estimated.

The second method, the lucerne resown experiment, involved inoculating another group of pots containing the lucerne host with one of the three strain combinations. At regular intervals, a number of pots were randomly selected, and the plants, including the roots, were removed and discarded. The soil from each pot was mixed and returned to the original pot, and the pots were then resown with lucerne seed. After 5 weeks had elapsed, the host plants were removed and the nodules were harvested. In the second planting, the lucerne served as a "trap" host. The bacteria present in the soil were responsible for nodule formation on these plants.

For the third method, referred to as the clover-lucerne experiment, a group of pots containing white clover were inoculated with one of the three strain combinations. At regular intervals, a number of pots were randomly selected and the clover plants were removed and discarded. The soil was returned to the pots, and the pots were resown with lucerne seed. The lucerne plants were allowed to grow for 5 weeks, and then they were removed and their nodules were harvested. The purpose of the clover was to deny the bacteria a host while allowing for a nonspecific rhizosphere effect. As described above, the lucerne served as a trap host.

Initiation of the experiment. A total of 450 pots were seeded with lucerne, and 180 pots were seeded with white clover. At 2 weeks after seeding equal numbers of plants were inoculated with one of the three strain combinations. The mutant and wild-type strains were cultured separately in TY for 48 h. Each of the mutant strain cultures was thoroughly mixed with an equal volume of the wild-type culture. For each of the three strain combinations, the relative frequencies of the mutant and wild-type strains in the initial inoculum mixture were determined by replicated serial dilution and plating ($n = 6$). Each plant was inoculated with 1 ml (10^9 cells) of the appropriate strain combination. The inoculum was applied to the base of the stem.

TABLE 1. Sample sizes in the in planta competition experiments and the proportions of nodules cooccupied by mutant and wild-type strains

Expt	Mutant compared with wild type	Mean no. of plants per sample	Mean no. of nodules sampled per plant	Mean % of nodules with both strains
Lucerne harvest	Neutral	10	19	9.6
	Mos^-	7	21	10.2
	Moc^-	9	22	9.3
Lucerne resown	Neutral	8	6	2.2
	Mos^-	8	11	2.1
	Moc^-	8	9	2.4
Clover-lucerne	Neutral	7	7	3.7
	Mos^-	7	9	4.0
	Moc^-	7	11	1.5

Nodule and bacterial sampling. Nodules were removed from the plants such that a small amount of root remained on either side of the nodule. The nodules were surface sterilized for 2 min in 2% sodium hypochlorite, and this was followed by five washes in sterile distilled water. The nodules were placed individually in the wells of a microtiter plate containing 40 μl of sterile distilled water per well. They were then crushed with a sterile wooden applicator stick, and 30 μl of the mixture from each well was streaked onto a TY plate containing streptomycin (250 μg ml^{-1}). The plates were incubated for 3 to 4 days at 28°C. From each plate 10 well-isolated colonies were transferred with a toothpick onto a TY plate containing streptomycin, and a TY plate containing streptomycin and kanamycin (250 μg ml^{-1}). These plates were incubated at 28°C for 3 to 4 days, and the numbers of Kan^+ (mutant) and Kan^- (wild-type) colonies were recorded. The purposes of the streptomycin were to select for the L5-30 strains and to prevent the growth of potential *R. meliloti* contaminant strains. Less than 2% of naturally isolated strains of this species are streptomycin resistant ($n = 117$) (unpublished data).

Statistical analysis. A plant was considered the experimental unit. For each plant the total number of wild-type and mutant colonies was determined and expressed as the ratio of wild-type colonies to total colonies. The total number of L5-30 colonies per plant equaled the number of colonies tested per nodule (10) times the number of nodules examined per plant. For various reasons it was not always possible to examine the same number of pots per sample or to test the same number of nodules per plant. The average number of plants examined per sample and the mean number of nodules examined per plant are presented in Table 1. Overall, less than 10% of the nodules examined were occupied by more than one strain (Table 1).

The analyses used were one-way analysis of variance and least-squares linear regression. For every treatment combination the data were first analyzed to determine if there was any significant change through time in the frequency of the wild-type strain following inoculation. If no such trend was detected, then the average frequency of the wild-type strain after inoculation was compared with the frequency of the wild-type strain in the inoculum.

Test for frequency dependence. Competitive interactions are often frequency dependent (that is, a situation in which the fitness advantage conferred by a trait depends on the frequency of the trait in the population). To test for this possibility, an additional experiment was conducted. TY cultures of the wild-type strain and the Moc^- strain were mixed to produce three inocula that varied in the proportion of wild-type cells present; these inocula contained 5, 36, and 85% wild-type cells. These cultures were used to inoculate pots containing five lucerne seedlings (10^9 cells per pot). At 4, 7, and 13 weeks after inoculation three pots were selected at random from each treatment, the nodules were harvested from the plants, and the relative frequencies of the wild-type strain were determined in the manner described above. The data were analyzed by a two-way analysis of variance.

RESULTS

In vitro growth experiments. In liquid culture the Moc^- mutant (2129) was the only mutant to grow significantly more slowly than the wild-type strain (2338) and exhibited a growth rate 7% less than the growth rate of the wild-type strain (Table 2) ($P < 0.001$). This result was not observed for the Moc^- mutant growing on a surface culture. On surfaces, no significant differences in the growth rates of the four strains were detected (Table 2) ($P > 0.5$).

TABLE 2. Exponential-phase growth rates of the mutant and wild-type L5-30 strains in TY under different culture conditions

Strain	Phenotype	Growth rate (h^{-1})	SE (h^{-1})	% of wild-type value
Liquid cultures				
2338	Wild type	0.33	0.003	100
2126	Neutral	0.33	0.004	100
2168	Mos$^-$	0.33	0.002	101
2129	Moc$^-$	0.30	0.002	93
Surface cultures				
2338	Wild type	0.28	0.014	100
2126	Neutral	0.27	0.014	97
2168	Mos$^-$	0.30	0.011	107
2129	Moc$^-$	0.29	0.012	102

Single-strain nodulation experiments. The number of nodules per plant increased through time in a similar manner for all four strains ($P < 0.001$) (Fig. 1). No differences between the strains in the number of nodules recovered per plant were detected ($P > 0.65$), nor was any interaction between strain and time detected ($P > 0.32$).

In planta competition experiments. When the wild-type strain was in competition with the neutral mutant, a significant decline in the level of the wild-type strain over time was observed in the nodule-inhabiting population of bacteria (Fig. 2A) ($P < 0.001$). In the lucerne resown experiment the frequency of the wild-type strain in the nodules of the trap hosts also declined when the wild-type strain was in competition with the neutral mutant (Fig. 2B) ($P < 0.022$). The rates of decline were comparable in the two experiments (0.7 and 0.8%/week, respectively). In the clover-lucerne experiment, no change in the frequency of the wild-type strain in the nodules of the trap hosts was detected when the wild-type strain was in competition with the neutral mutant (Fig. 2C) ($P > 0.54$).

The frequency of the wild-type strain also declined when this strain was in competition with the Mos$^-$ mutant. This decline was observed in the lucerne harvest experiment (Fig. 3A) ($P <$ 0.001) and in the nodules of the trap host in the lucerne resown experiment (Fig. 3B) ($P < 0.006$). The frequency of the wild-type strain declined at comparable rates in the two experiments (0.6 and 0.7%/week, respectively). However, in the clover-lucerne experiment no change in the frequency of the wild-type strain in the nodules of the trap hosts was detected (Fig. 3C) ($P > 0.08$).

Quite different results were obtained in the experiments involving the wild-type strain and the Moc$^-$ mutant. Subsequent to inoculation no change in the frequency of the wild-type strain in the nodule population of bacteria was detected through time ($P > 0.66$). However, the average frequency of

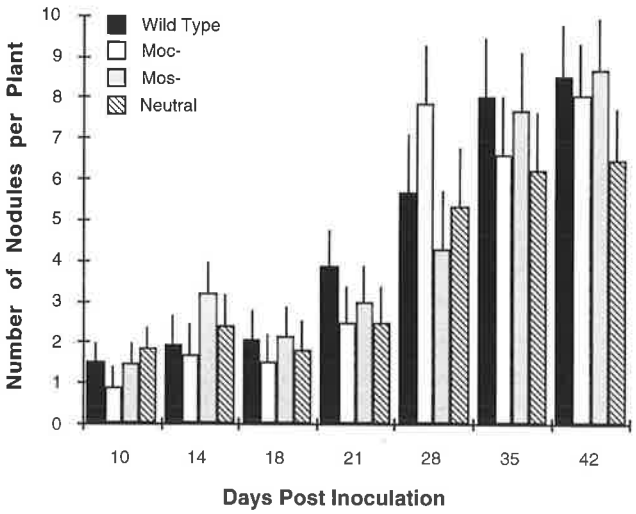

FIG. 1. Accumulation on lucerne plants of nodules produced by wild-type *R. meliloti* L5-30 and different rhizopine Tn*5*-induced mutants. The number of nodules per plant increased in a linear manner (note that the *x* axis is not an interval scale). The vertical lines indicate the 95% confidence limits of the means.

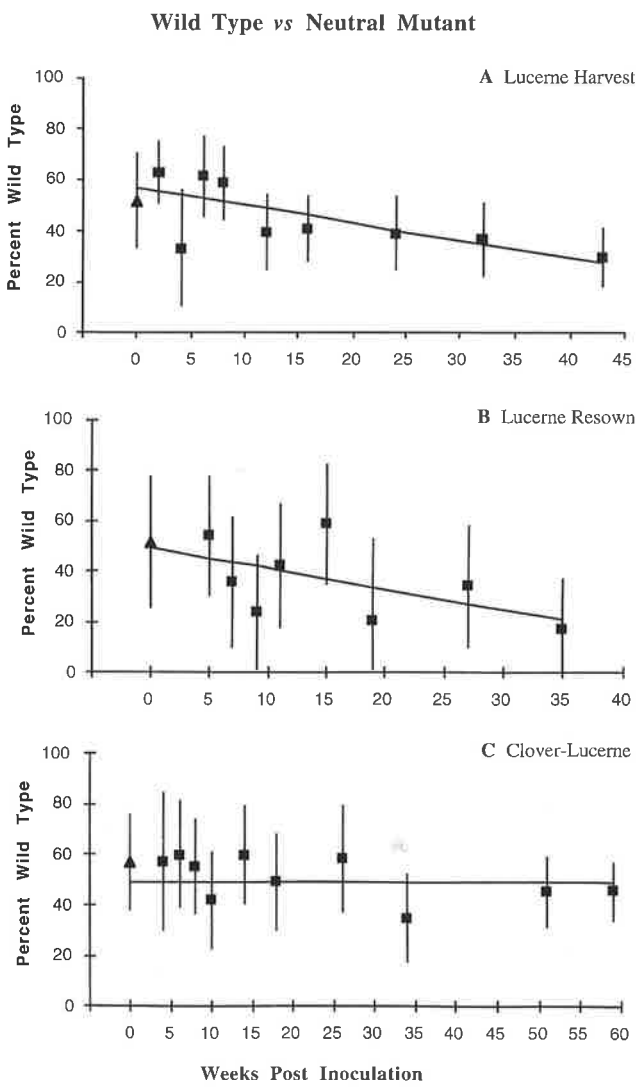

FIG. 2. Change in frequency through time of rhizopine-producing wild-type *R. meliloti* (Mos$^+$ Moc$^+$) cells occupying nodules when they were in competition with the neutral Tn*5*-induced mutant (Mos$^+$ Moc$^+$). (A) Frequency of the wild-type strain in the nodule population of the primary lucerne crop. (B) Frequency of the wild-type strain in the nodules of the lucerne trap hosts when the primary crop was lucerne. (C) Frequency of the wild-type strain in the nodules of lucerne trap hosts when the primary crop was clover. See Materials and Methods for details of the three experimental treatments. The solid triangle indicates the frequency of the wild-type strain in the inoculum. The solid squares indicate the frequency of the wild-type strain in free-living cells occupying nodules of lucerne hosts. The vertical lines indicate the 95% confidence limits of the mean frequency. The solid lines indicate the change through time in the frequency of the wild-type strain after inoculation.

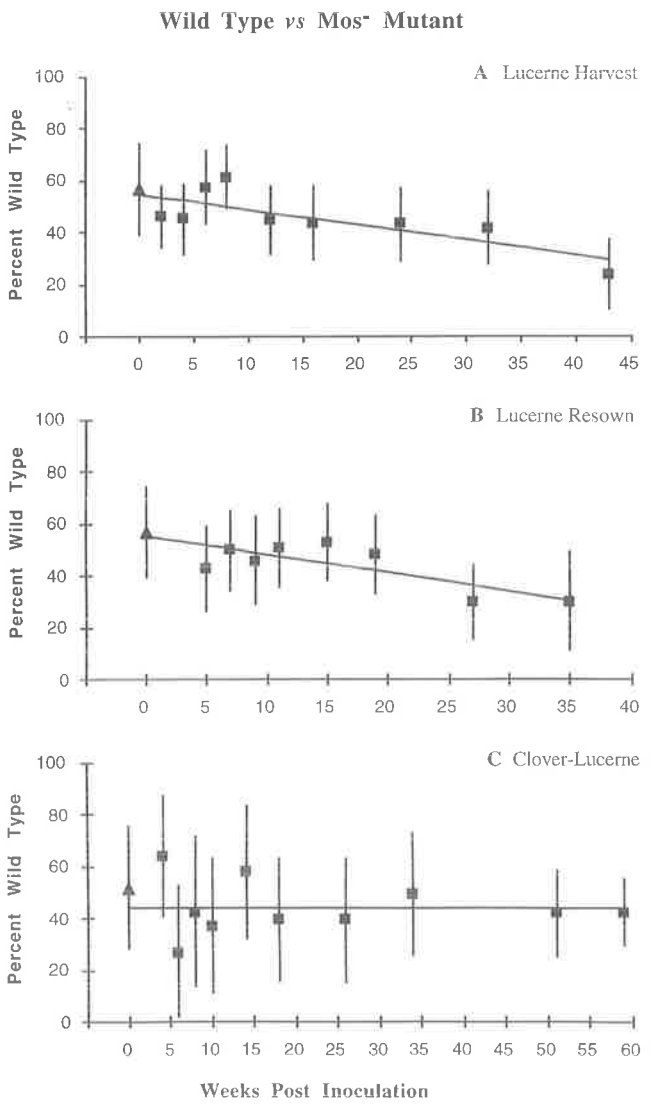

FIG. 3. Change in frequency through time of rhizopine-producing wild-type *R. meliloti* (Mos^+ Moc^+) cells occupying nodules when they were in competition with the Mos^- Tn*5*-induced mutant (Mos^- Moc^+). (A) Frequency of the wild-type strain in the nodule population of the primary lucerne crop. (B) Frequency of the wild-type strain in the nodules of the lucerne trap hosts when the primary crop was lucerne. (C) Frequency of the wild-type strain in the nodules of lucerne trap hosts when the primary crop was clover. See Materials and Methods for details of the three experimental treatments. The solid triangle indicates the frequency of the wild-type strain in the inoculum. The solid squares indicate the frequency of the wild-type strain in free-living cells occupying nodules of lucerne hosts. The vertical lines indicate the 95% confidence limits of the mean frequency. The solid lines indicate the change through time in the frequency of the wild-type strain after inoculation.

the wild-type strain after inoculation was 73%, a value significantly greater than the frequency in the inoculum (47%) (Fig. 4A) ($P < 0.001$). Comparable changes in the frequency of the wild-type strain when it was in competition with the Moc^- mutant were seen in the nodules of the trap hosts in the lucerne resown experiment (Fig. 4B). After inoculation, no change in the frequency of the wild-type mutant over time was detected ($P > 0.54$). However, the average frequency of the wild-type strain across all samples subsequent to inoculation (67%) was significantly greater than the frequency of this strain in the inoculum (47%) ($P < 0.015$). Again, similar changes in the frequency of the wild-type strain when it was in competition with the Moc^- mutant occurred in the clover-lucerne experiment (Fig. 4C). Following inoculation no change in the frequency of the wild-type strain in the nodules of the trap hosts was observed ($P > 0.40$). The average frequency of the wild-type strain after inoculation was 72%, a value significantly greater than the frequency of this strain in the inoculum (47%) ($P < 0.002$).

Test for frequency dependence. On average, the wild-type strain was recovered at a significantly higher frequency with respect to the Moc^- mutant than would be expected on the

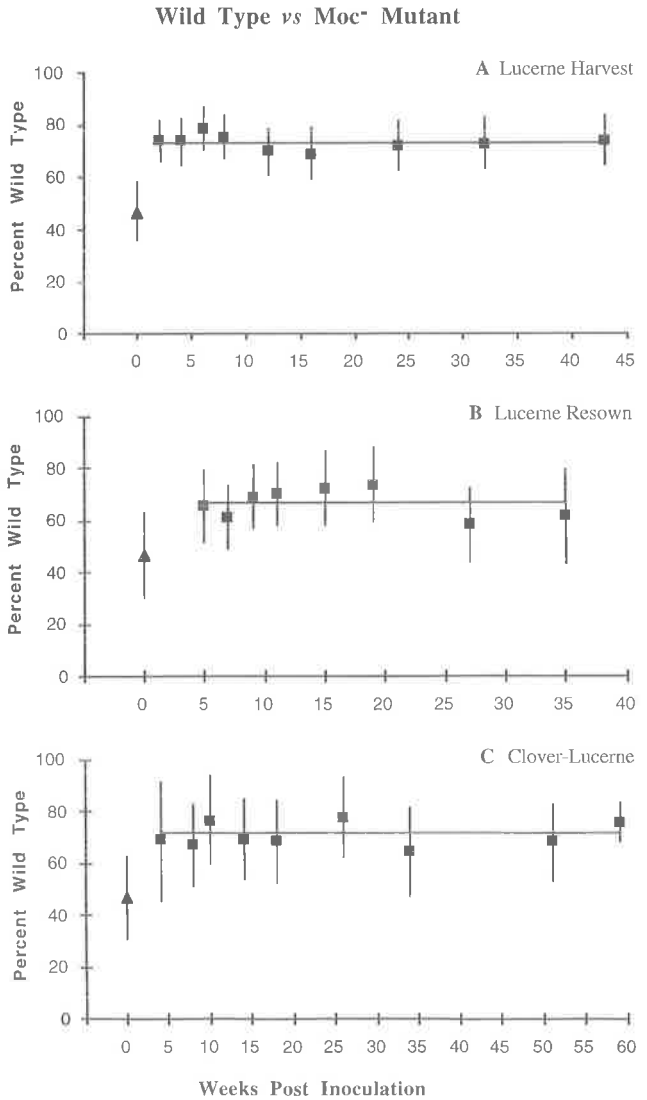

FIG. 4. Change in frequency through time of rhizopine-producing wild-type *R. meliloti* (Mos^+ Moc^+) cells occupying nodules when they were in competition with the Moc^- Tn*5*-induced mutant (Mos^+ Moc^-). (A) Frequency of the wild-type strain in the nodule population of the primary lucerne crop. (B) Frequency of the wild-type strain in the nodules of the lucerne trap hosts when the primary crop was lucerne. (C) Frequency of the wild-type strain in the nodules of lucerne trap hosts when the primary crop was clover. See Materials and Methods for details of the three experimental treatments. The solid triangle indicates the frequency of the wild-type strain in the inoculum. The solid squares indicate the frequency of the wild-type strain in free-living cells occupying nodules of lucerne hosts. The vertical lines indicate the 95% confidence limits of the mean frequency. The solid lines indicate the change through time in the frequency of the wild-type strain after inoculation.

basis of its frequency in the inoculum ($P < 0.001$). The frequencies of the wild-type strain in the inocula were expected to be 5, 36, and 85%, but the observed frequencies of the wild-type strain were 48, 77, and 99.5%, respectively. The frequency of the wild-type strain did not change with the time of harvest ($P > 0.45$), and no initial frequency–time of harvest interaction was detected ($P > 0.39$).

In all experiments nodules occupied by both wild-type and mutant strains represented less than 10% of the nodules examined (Table 1). Thus, the results presented in Fig. 2 through 4 not only represent the relative frequencies of wild-type cells but also reflect the relative frequencies of wild-type nodules (that is, nodules in which the wild-type strain represents at least 90% of the population of free-living cells inhabiting the nodule) (10 colonies were examined per nodule).

DISCUSSION

Expected experimental outcomes. What results would be expected from the competition experiments if the function of rhizopine is to provide a proprietary growth substrate for the free-living population of the producing strain? The following scenarios assume that the Tn*5* insertion has no nonspecific effect on the fitness of the mutants (that is, that mutant and wild-type cells have identical demographic characteristics except with respect to rhizopine synthesis and catabolism). Given this assumption, the frequencies of wild-type and mutant cells recovered from the nodules of the trap hosts (in lucerne resown and clover-lucerne experiments) should reflect the frequencies of wild-type and mutant cells in the soil. Thus, the trap hosts are assumed to provide a sample of the soil population at a particular point in time. In reality, this point in time was about 3 weeks. The time from sowing to harvest was 5 weeks, and it took somewhat less than 1 week for germination to occur, while any nodules formed within about 1 week of harvest were too small to be sampled.

In wild-type strain versus neutral mutant experiments the frequency of the wild-type strain should equal its frequency in the inoculum and should not change through time as the two strains are identical with respect to rhizopine synthesis and catabolism. Similar results are expected in wild-type strain versus Mos$^-$ mutant experiments. While the Mos$^-$ mutant is incapable of synthesizing rhizopine, it is capable of catabolizing the rhizopine produced by the wild-type strain. The total amount of rhizopine being synthesized by the bacteroid population should be reduced because a certain fraction of bacteroids are incapable of synthesis. However, the reduction in the amount of rhizopine present in the rhizosphere should affect the wild-type and Mos$^-$ strains equally. Thus, while the total population of free-living cells might be smaller, the relative frequencies of the two strains should equal their frequencies in the inoculum and should not change through time. These results are expected in lucerne harvest, lucerne resown, and clover-lucerne experiments for both wild-type strain versus neutral mutant and wild-type strain versus Mos$^-$ mutant competition experiments.

In experiments involving the wild-type and Moc$^-$ strains, the initial frequency of the wild-type strain in the soil and nodule populations should equal its frequency in the inoculum. Once symbiotically effective nodules are formed (1 to 2 weeks), the bacteroids of both strains should start producing rhizopine, but only the wild-type strain should be able to exploit the rhizopine as an additional carbon source. As a result, the frequency of the free-living wild-type cells should increase through time in the rhizosphere, and this should in turn be reflected by an increase in the frequency of the wild-type strain in the nodule-inhabiting population of bacteria.

In clover-lucerne experiments no bacteroids are formed and no rhizopine is produced while the clover is present. As a result, no change in the frequency of the wild-type strain relative to the Moc$^-$ mutant should occur no matter how long the strains are in the presence of the nonhost species. Each crop of lucerne trap hosts is in the soil for less than 5 weeks. In addition to the time required for germination, there is the time required for symbiotically effective nodules to develop and for rhizopine synthesis to commence. Furthermore, the nodules formed on the trap hosts should be small and few in number given the time available. As a consequence, little rhizopine should be produced, and at most only a small increase in the frequency of the wild-type strain relative to its frequency in the inoculum is expected. This effect, if detectable, should be the same for each crop of trap hosts, and therefore no change in the frequency of the wild-type strain relative to the Moc$^-$ mutant over time is expected.

Some differences between the results of lucerne harvest and lucerne resown experiments might be expected when the wild-type strain is competing with the Moc$^-$ mutant. In both experiments rhizopine is produced in the presence of the primary lucerne crop, and therefore the frequency of the wild-type strain in the rhizosphere is expected to increase through time. However, in lucerne harvest experiments the rate at which the wild-type strain increases in frequency in the nodule population may be influenced by the rate of nodule formation on the primary crop. Nodules do not accumulate on a host in an unrestricted manner; the rate of nodule formation declines as the number of nodules per plant increases (2). As a consequence, the frequency of the wild-type strain in the nodule population of bacteria at a particular point in time may not reflect the frequency of the wild-type strain in the rhizosphere at that time. The extent to which this influences the results depends on the rates at which old nodules senesce and new nodules are formed. This phenomenon should not be a factor in lucerne resown experiments. The second crop of lucerne does not acquire a sufficient number of nodules in the brief time that it is in the soil.

Observed experimental outcomes. The results suggest that the Tn*5* insertion did not affect the demographic characteristics of the mutant strains (except rhizopine synthesis and catabolism). The results of the in vitro growth experiments suggest that the Tn*5* insertion had no effect on the exponential-phase growth rates of the mutant strains when they were growing on surfaces. The environmental conditions of the surface experiments are most like those of a soil environment.

The results of the single-strain nodulation experiments suggest that the Tn*5* insertion did not affect the nodulation ability of any of the mutant strains relative to the wild-type strain. All strains formed nodules on the host plant at the same rate, and over 5 weeks there was no indication of any decline in the rate of nodule formation (note that the x axis in Fig. 1 is not an interval scale). The results of these experiments suggest that an important underlying assumption of the experiments is justified; this assumption is that the relative frequencies of wild-type and mutant cells recovered from the nodules of the trap host reflect the relative frequencies of mutant and wild-type cells in the soil. Furthermore, these results suggest that density dependence in the rate of nodule formation is unlikely to be a significant factor in determining the number of nodules recovered from plants used as trap hosts as, on average, there were less than 10 nodules per trap host (Table 1).

Contrary to expectations, the wild-type strain declined in frequency with respect to both the neutral and Mos$^-$ mutants

in the lucerne harvest and lucerne resown experiments. The reasons for the decline in the frequency of the wild-type strain are unknown. The decline in the frequency of the wild-type strain was not observed in the clover-lucerne experiment, a result which suggests that when no host plant is present, the survival and/or growth characteristics (fitness) of the wild-type strain and the neutral or Mos$^-$ mutants in the soil are similar. The different outcomes of these experiments imply that the decline in the frequency of the wild-type strain was due to events occurring in the nodule-inhabiting populations of free-living cells rather than in the soil population. Although the decline in the frequency of the wild-type strain is unexplained, the results demonstrate that small changes through time (0.7%/week) in the frequency of the wild-type strain relative to the mutants could be detected in these experiments. The ability to observe small changes in frequency is relevant to the outcome of the wild-type strain versus Moc$^-$ mutant experiments.

In all in planta experiments the average frequency of the wild-type strain in nodules relative to the Moc$^-$ mutant (~70%) was significantly greater than would be expected on the basis of the frequency of the wild-type strain in the inoculum (47%). The increase in frequency was observed within 2 weeks after inoculation, and no further change in the frequency of the wild-type strain through time was detected. These results are contradictory to the predicted outcome of these experiments. If the rhizopine acts solely as a growth substrate, then the frequency of the wild-type strain should gradually and continuously increase through time. An increase in the frequency of the wild-type strain should not be observed within 2 weeks after inoculation. At this time symbiotically effective nodules are only just appearing, and these nodules are small and few in number. Thus, very little rhizopine is being produced. Furthermore, no increase in the frequency of the wild-type strain through time was observed.

The results of the wild-type strain versus Moc$^-$ mutant experiment designed to test for frequency dependence support the results of the main experiment. The wild-type strain was consistently recovered at a higher frequency from the nodules than would be expected on the basis of its frequency in the inoculum (Fig. 4). This result was observed within 4 weeks after inoculation, and the frequency of the wild-type strain did not change thereafter.

These experiments demonstrated that there is a clear fitness advantage associated with the ability to catabolize a rhizopine. However, none of the results are consistent with an advantage resulting from the rhizopine acting as a specific growth substrate. The results do suggest that when a strain with the ability to catabolize rhizopine is in competition with a strain lacking this ability, the strain with the ability nodulates plants at a higher rate. The enhanced nodulation ability of the Moc$^+$ (wild-type) strain is not a by-product of the presence of Tn*5* in the mutants. The results of the single-strain nodulation experiments do not support such a conclusion. The fitness advantage occurs only when the strain is in competition with the Moc$^-$ mutant. A competitive advantage resulting from enhanced nodulation ability, rather than a change in numbers in the soil, would produce outcomes consistent with those observed in these experiments. In particular, these outcomes are consistent with the observation that the advantage is expressed within 2 weeks of host plant inoculation.

The results of our experiments demonstrate that it is unlikely that the only role of rhizopines is to act as proprietary growth substrates. Rather, the ability to catabolize a rhizopine appears to enhance the rate at which a strain is able to form nodules when it is in competition with a strain that is unable to catabolize a rhizopine. The mechanism(s) responsible for this enhanced nodulation ability is unknown and will require investigation.

ACKNOWLEDGMENTS

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CORRIGENDUM

Chapter 1

p 25, line 7, should read "a *nfe* probe".

p 30, line 25, the word "*mos*" should be replaced with "the rhizopine synthesis genes".

Chapter 2

p 40, Table 2.1, PM2338 is strain L5-30(pPH1JI), and not the native isolate L5-30.

p 42, Table 2.2, line 9 should be "Gram negative".

Chapter 3

p 65, Table 3.2, the following sentence should be added at the end of the legend: "The +/- values indicate the standard error of the mean".

Chapter 4

p 75, the first sentence of Section 4.2.2 should read "60 g of whole plant tissue was collected from each sample".

p 78, line 17, "m/z 209" should be replaced with "m/z 210".

p 89, line 19, the phrase "from the *lacZ* transcript" should be deleted.

p 96, line 6, the word "helper" should be deleted.

Chapter 5

p 99, Section 5.2.6, the words "for rhizopine production" should be added to title.

p 100, Table 5.1, the *S. meliloti* strain Rm1021 referred to in this table should be correctly named strain Rm1021 Cm^r". This is a chloramphenicol-resistant derivative of the native isolate *S. meliloti* Rm1021.

p 103, line 5, "strain 1a (pSym1a)" should read "strain 1a (pSym1a::Tn*5-mob*)."

p 120, Figure 5.8, the legend should read: "Lane 1, standard (mannitol); lane 2, purified 3-*O*-MSI; lane 3, P135(pKHRP1); lane 4, P135(pKHRP2); lane 5, P135(pKHRP5); lane 6, P135(pKHRP6); lane 7, P135(pKHRP9); lane 8, 1a; lane 9, P135."

p 122, paragraph 3, the second sentence should read "The introduced DNA may have contained a region of incompatibility with the Sym plasmid".

p 127, second paragraph, it is noted that the cosmid pKH104 may carry most but not all of the *mos* genes. If some of the *mos* genes were already present in certain Mos^- strains, such as P135 and 8401(pRL1), this could account for the Mos^+ phenotype of these strains after the introduction of pKH104. Alternatively, P135 may contain necessary precursors for rhizopine synthesis that the other rhizobia used in this study do not have. This is supported by the presence of a small amount of a silver staining compound in strain P135 in the absence of any plasmid introduction.

Chapter 6

p 158, Table 6.2, the words "better separation" should be deleted.