

09PH
H469



ECOLOGICAL AND MOLECULAR STUDIES ON RHIZOBIAL RHIZOPINES

KEITH HEINRICH

B.Sc. (Hons) University of Adelaide

**A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Agricultural and
Natural Resource Sciences at The University of Adelaide**

Department of Crop Protection

Waite Agricultural Research Institute

The University of Adelaide

February 1999

TABLE OF CONTENTS

Summary	i
Declaration	iv
Acknowledgements	v
Abbreviations	vi
CHAPTER 1 Literature review and project aims	1
1.1 General Introduction	1
1.2 Taxonomy of rhizobia	3
1.2.1 General characteristics	3
1.2.2 Plasmids	4
1.2.3 Classification	5
1.3 Nodulation	8
1.3.1 Introduction	8
1.3.2 Attraction to the root surface	9
1.3.3 Induction of <i>nodD</i> gene expression	9
1.3.4 Attachment to host	10
1.3.5 Infection	11
1.3.6 Nodule development	12
1.3.7 Bacteroids	13
1.3.8 Nodule senescence	14
1.4 Nodulins	14

1.5 Nitrogen fixation.....	15
1.6 Genetic control of nodulation and nitrogen fixation.....	16
1.6.1 Introduction	16
1.6.2 The common nodulation genes.....	16
1.6.3 The specific nodulation genes	17
1.6.4 Nod factors	19
1.6.5 Genes involved in nitrogen fixation	21
1.7 Ecology of rhizobia.....	22
1.7.1 Nodulation competitiveness	22
1.7.2 Factors which affect nodulation competitiveness.....	22
1.7.2.1 Speed of nodulation	22
1.7.2.2 Environmental factors.....	23
1.7.2.3 Biological factors.....	23
1.7.2.4 Other factors	24
1.7.3 Genes involved in nodulation competitiveness	24
1.7.4 Rhizobial inoculants	25
1.8 Rhizopines.....	28
1.8.1 Introduction	28
1.8.2 The occurrence of rhizopines.....	30
1.8.3 Genetics of rhizopines	33
1.8.3.1 Introduction	33
1.8.3.2 The <i>moc</i> locus.....	33
1.8.3.3 The <i>mos</i> locus	35
1.8.4 The function of rhizopines.....	36

1.9 Scope of this thesis.....	37
CHAPTER 2 General materials and methods.....	38
2.1 Bacterial strains, plasmids and cosmids.....	38
2.2 Growth media and conditions	38
2.3 Seed sterilization, plant growth conditions and rhizobial inoculation.....	43
2.3.1 Seed and nodule sterilization.....	43
2.3.2 Plant growth conditions	43
2.3.2.1 Lucerne	43
2.3.2.2 Peas.....	44
2.3.3 Inoculation with rhizobia.....	44
2.4 Gas chromatography-mass spectrometry (GC-MS).....	44
2.4.1 Sample preparation for GC-MS.....	44
2.4.2 GC-MS conditions.....	45
2.5 Triparental matings	46
2.6 Recombinant DNA techniques.....	46
2.6.1 Isolation of plasmid DNA.....	46
2.6.1.1 Small scale alkaline lysis.....	46
2.6.1.2 Cesium chloride-ethidium bromide purification of plasmid DNA.....	46
2.6.2 Restriction digests, fragment isolation and ligations.....	47
2.6.3 Electrophoresis of DNA	48
2.6.4 Eckhardt gels	48
2.6.5 Competent cells and transformation	49
2.6.6 Southern analysis.....	49

2.6.6.1 Southern blotting	49
2.6.6.2 Colony blots.....	50
2.6.6.3 Radioactive labelling of DNA	50
2.6.6.4 Digoxigenin labelling of DNA	50
2.6.6.5 Hybridization and washing conditions	51
2.6.6.6 Colorimetric detection of Digoxigenin labelled DNA.....	51
2.6.6.7 Autoradiography	51
2.7 High voltage paper electrophoresis (HVPE).....	52
2.7.1 Electrophoresis conditions.....	52
2.7.2 Staining of paper electrophoretograms.....	52
2.8 Preparation and purification of 3-O-MSI	52
2.9 Rhizopine synthesis tests	53
2.10 Rhizopine catabolism tests.....	53
2.11 Column purification of 3-O-MSI	54
CHAPTER 3 Rhizopines and rhizobial competition	55
3.1 Introduction.....	55
3.2 Materials and methods	58
3.2.1 Strains and strain construction.....	58
3.2.2 Single strain nodulation experiments	58
3.2.2.1 Plant culture.....	58
3.2.2.2 Nodulation experiments.....	58
3.2.2.3 Statistical analysis.....	59
3.2.3 Competition experiments.....	59

3.2.3.1 Plant culture.....	59
3.2.3.2 Competition experiments.....	59
3.2.3.3 Nodule and bacterial sampling	59
3.2.3.4 Statistical analysis.....	60
3.3 Results.....	61
3.3.1 Single strain nodulation studies	61
3.3.2 Competition studies	61
3.4 Discussion.....	66
3.4.1 Single strain nodulation studies.....	66
3.4.2 Competition studies	67
CHAPTER 4 Timing of rhizopine synthesis.....	73
4.1 Introduction.....	73
4.1.1 Regulation of the <i>mos</i> operon.....	73
4.1.2 Rhizobial competition.....	74
4.2 Materials and methods	75
4.2.1 Plant growth conditions	75
4.2.2 Extraction, purification and analysis of plant material.....	75
4.2.3 Preparation of purified 3- <i>O</i> -MSI	76
4.3 Results.....	77
4.3.1 Detection of rhizopine by GC-MS.....	77
4.3.2 Early detection of the rhizopine 3- <i>O</i> -MSI in plant tissue	79
4.4 Discussion.....	88

CHAPTER 5 Isolation of the rhizopine synthesis genes of *R. leguminosarum* bv.

<i>viciae</i>	92
5.1 Introduction.....	92
5.2 Materials and methods	96
5.2.1 Plasmid transfer	96
5.2.2 General recombinant DNA techniques.....	97
5.2.3 Selection of antibiotic resistant strains of <i>Rhizobium</i>	97
5.2.4 Construction of R-primers	97
5.2.5 Construction of a cosmid clone bank	98
5.2.6 Screening of transconjugant strains.....	99
5.3 Results.....	103
5.3.1 The <i>mos</i> genes are located on the Sym plasmid in strain 1a	103
5.3.2 pSym1a R-primers	110
5.3.2.1 Construction of pSym1a R-primers and transfer to strain 8401(pRL1).....	110
5.3.2.2 Transfer of pSym1a to other non-rhizopine rhizobia	111
5.3.2.3 Transfer of R-primers to other non-rhizopine rhizobia.....	113
5.3.3 Cosmid clone bank	121
5.3.3.1 Construction and nodulation of cosmid clones.....	121
5.3.3.2 Analysis of pKH085 for rhizopine synthesis.....	123
5.3.3.3 Analysis of pKH104 for rhizopine synthesis.....	127
5.3.3.4 Analysis of pKH227 and pKH246 for rhizopine synthesis	128
5.4 Discussion	133
5.4.1 Introduction.....	133

5.4.2 The <i>R. leguminosarum</i> bv. <i>viciae</i> <i>mos</i> genes are located on the Sym plasmid in strain 1a.....	133
5.4.3 Transfer of pSym1a DNA between rhizobia	134
5.4.4 Expression of the pSym1a <i>mos</i> genes after transfer to non-rhizopine-producing rhizobia	136
5.4.5 Rhizopine synthesis by pKH104	137
5.4.6 Production of a silver-staining compound by pKH085	138
5.4.7 Analysis of R-prime plasmids	139
5.4.8 Summary.....	140
CHAPTER 6 General discussion	143
6.1 Rhizopines and competition for nodulation	143
6.2 Further studies on the effects of rhizopines on rhizobial competition for nodulation	146
6.3 The timing of rhizopine synthesis in rhizobia.....	147
6.4 The function of rhizopines	148
6.5 The rhizopine synthesis genes of <i>R. leguminosarum</i> bv. <i>viciae</i>	149
6.6 <i>nfe</i> genes.....	152
6.7 The rhizopine metabolic pathway	152
6.8 The involvement of cyclitols in rhizopine synthesis.....	156
6.9 Final conclusions	159
CHAPTER 7 Bibliography	160

APPENDICES	191
Appendix 1 Media	192
Appendix 2 Antibiotics	196
Appendix 3 Cloning vector	197
Appendix 4 Results of <i>mos</i> tests and nodulation studies on transconjugant rhizobia.....	198
Appendix 5 Publications generated from this thesis	204

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::Tn5-*mob*), formed an effective symbiosis. The ineffective nodules produced by P342(pSym1a::Tn5-*mob*) also contained some 3-*O*-MSI; however, they contained a smaller quantity of 3-*O*-MSI than transconjugants which nodulated effectively.

Subsequent work directed at isolating the *R. leguminosarum* *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*-MSI. 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.