ECOLOGICAL AND MOLECULAR STUDIES ON RHIZOBIAL RHIZOPINES

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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*-MSI; however, they contained a smaller quantity of 3-*O*-MSI 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*-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.