EFFECT OF EXOGENOUS APPLICATION OF RHIZOPINE ON LUCERNE ROOT NODULATION

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ABSTRACT

Rhizopine, 3-*O*-methyl scyllo-inosamine was applied to the roots of lucerne seedling inoculated with either rhizopine synthesizing *Sinorhizobium meliloti* strain L530 or the non-rhizopine synthesizing strain Rm1021. There was an initial delay in nodule formation. A significant increase in the number of nodules formed in rhizopine treated roots was observed compared with seedling roots inoculated with the rhizobial strains but not treated with rhizopine. The same numbers of nodules were observed after 30 days in the rhizopine treated rootlets inoculated with either the rhizopine or non-rhizopine rhizobial strain, indicating that rhizopine catabolism had no effect on nodule formation. Slight difference was observed after 30 days in the number of nodules on roots inoculated with the rhizopine or non-rhizopine strains but not treated with rhizopine.

Running title: of rhizopine on nodulation

Introduction

In nitrogen deficient environments plants benefit from legume-*Rhizobium* interaction. Many rhizobia survive in the soil as saprophytes and engage in a symbiotic relationship in the presence of appropriate legume hosts by inducing the formation of nodules on the roots. Rhizobia also benefit from this association as both the bacteroid (i.e. the differentiated form of the bacteria within the nodule) and the free-living bacteria inhabiting the infection thread within nodules are protected form biotic and abiotic environmental factors (Gordon *et al.* 1996). Within the nodules, the bacteroids fix nitrogen for plant growth and in some rhizobia, bacteroids also produce rhizopine. Rhizopines (L-3-*O*-methyl-*scyllo*-inosamine [3-*O*-MSI] and *scyllo*-inosamine) are "opine-like" compounds produced from plant precursors within nodules (Murphy &

Saint, 1992; Rao *et al.* 1995). Ecological and genetic studies of *Sinorhizobium meliloti* rhizopine producing strains suggest that rhizopines are important in the *Rhizobium*-legume symbiosis in both annual and perennial *Medicago* species (Wexler *et al.*, 1995). Rhizopine strains demonstrate an intra-species nodulation advantage over non-rhizopine strains. This phenomenon seems to require the presence of both the rhizopine *mos* (synthesis) and *moc* (catabolic) genes (Gordon *et al.*, 1996). However, the mode and site of rhizopine action are not known. Rhizopines are catabolised by free-living rhizopine strains of rhizobia within the infection thread and the rhizosphere, and are utilised as a growth substrate (Murphy *et al.*, 1987; Murphy and Saint, 1992; Murphy *et al.*, 1995). Competition experiments have shown that in the presence of rhizopine those strains, which can catabolise it, have a nodulation advantage (Murphy *et al.*, 1995). However, it is not known whether rhizopine has any effect on nitrogen fixation or nodulation efficiency. Therefore, the objective of this work is to determine whether the direct application of rhizopine (3-*O*-MSI) to lucerne rootlets affects the number of nodules formed.

Materials and methods

Bacterial strains and media

Bacteria used in this work were *Sinorhizobium meliloti* strains L5-30 (Mos^+Moc^+) (Kowalski, 1967) and Rm1021 (Mos^-Moc^-) (Meade *et al.*, 1982). The strains were grown in TY complex media (Beringer, 1974) or Bergersen minimal media (Bergersen, 1961) supplemented with 1 mg calcium pantothenate ml^{-1} and 2 mg $(NH_4)_2SO_4$ ml^{-1}.

Rhizopine Extraction

Nodules harvested from 4-6 weeks old plants inoculated with rhizopine producing *Rhizobium* strain were crushed in water (2.5 g/ml). The extract was purified by passing it through Dowex 50W-X8 (H) (BDH, Poole, England) cation exchange columns. Columns were left overnight in 0.1 M HCl. Before loading the extract, the pH was increased to approximately 2.5 by running water through the column. The extract was loaded on the column at 1 ml per min and left overnight. The column was washed with 5 bed volumes of water, which was also added at 1 ml per min. The negatively charged compounds were eluted slowly (15 ml/h) with 5 bed volumes of 2 M ammonia solution. The eluate was collected when the pH reached 9. Higher yields were obtained when columns were left overnight.

Seed sterilization and seedling growth

To sterilise the seeds, lucerne (*Medicago sativa* var. Hunter River) seeds were immersed in 2% sodium hypochlorite for 6 min and transferred into 70% ethanol for 2 min. The seeds were then rinsed 6 times in MilliQ H_20. The seeds were placed on water agar in a Petri dish, to germinate for 2 days, in the dark at room temperature (20-25 °C). The rootlets in the 2-day old seedlings were dipped into *S. meliloti* strains L5-30 (Mos^+Moc^+) or Rm1021 (Mos^-Moc^-) suspension, prepared in 0.5 ml Haugland's medium (without nitrogen) (Haugland & Verma, 1981) [see Table 1]

Table 1 Haugland's medium (without nitrogen) per litre (Haugland & Verma, 1981).

Reagent	Concentration	Volume
$MgSO_4.7H_2O$	1 M	2 ml
KH_2PO_4	1 M	1 ml
KCl	1 M	5 ml
$CaCl_2.H_2O$	1 M	5 ml
FeEDTA	40g/L MilliQ H_2O	1 ml
Micronutrients	H_3BO_4 [2.86 g/L]; $MnCl_2.4H_2O$ [1.81 g/L], $ZnSO4.7H_2O$ [0.22 g/L], $CuSO_4.7H_2O$ [0.08 g/L], $Na_2MoO_4.2H_2O$ [0.025 g/L],	1 ml

and then transferred onto a reduced nitrogen (i.e. Haugland's medium without nitrogen plus 2 mM KNO_3) Haugland's agar slant in large test tubes (20 cm long, 2.7 cm diameter). 2.5 µl 3-*O*-*MSI* [2.5 mg/ml] (sterilised by adding absolute ethanol to achieve 70% ethanol concentration, maintained at room temperature for 30 min and spun in a SpeedVac to remove the alcohol by vacuum evaporation), was added to each seedling rootlet on the agar slants. Two seedlings were placed in each tube. There were three replicates in each experiment. One ml of the nutrient (Haugland's solution without nitrogen) was added to the agar slant at the time of rhizobia inoculation. The shoots of growing plants were manually guided through the cotton wool used to stopper the tubes, two weeks after rhizobial inoculation. After three weeks, 1-2 ml of nutrient solution was added at intervals of 2-4 days as required, to prevent the agar from drying. The seedlings were allowed to grow at 22-24°C for about 5 weeks in a temperature regulated glass house. The control experiment was the lucerne plant grown under the same conditions as the experimental ones but not inoculated with rhizobia. The number of nodules formed on the 3-*O*-*MSI* treated and untreated plant roots, as well as the control, at intervals of 2 days was determined.

Results

The lucerne plants were grown for about 5 weeks and active nodules formed on the inoculated roots were pink due to the presence of leghaemoglobin (Werner, 1992), while the few inactive nodules found on non-inoculated plants were white in colour. The number and size of nodules increased with time until day 30 in both the rhizopine treated and untreated roots of the lucerne plants.

Rootlets inoculated with rhizopine strain

Lucerne rootlets inoculated with rhizopine producing *Sinorhizobium meliloti* strain L5-30 first revealed nodules after 4 days. The plant rootlets inoculated with the rhizopine strain

L5-30 and treated with 3-*O*-MSI, produced the first nodules after 6 days. Thus, there was a 2-day delay in the appearance of nodules in the 3-*O*-MSI treated roots vis-à-vis the untreated roots. The number of nodules gradually increased to 8 within the first 16 days on the rhizopine treated roots but to 4 in untreated roots. This was accompanied by a quick increase to 33 in the next 10 days on the rhizopine treated roots and to 18 on the untreated roots. Thereafter, the rate of nodule formation declined until day 30 after which no more nodules appeared on both the rhizopine treated and untreated roots (see Fig. 1).

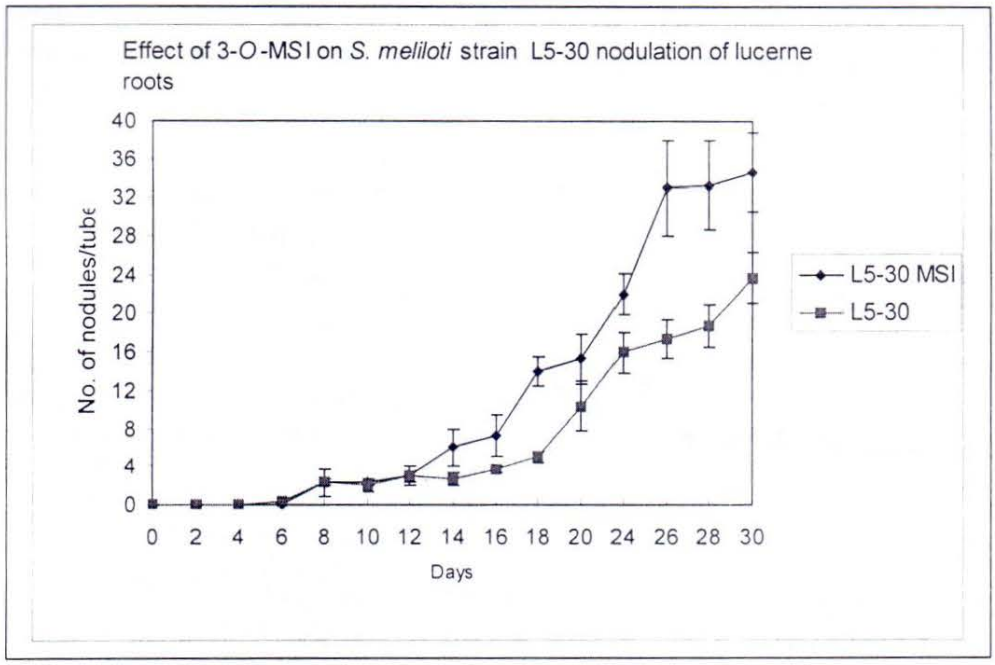

Fig. 1. Number of nodules formed as the days increased on 3-*O*-MSI treated (L5-30 MSI) and untreated (L5-30) lucerne roots inoculated with L5-30. Error bars indicate standard error.

The average number of nodules observed on lucerne roots treated with 3-*O*-MSI and inoculated with rhizopine producing strain L5-30 (L5-30/3-*O*-MSI) (Fig. 1) was higher than the number of nodules present on plants inoculated with the strains but not treated with 3-*O*-MSI.

Rootlets inoculated with non-rhizopine strain

Similarly, lucerne plants inoculated with non-rhizopine producing *S. meliloti* strain Rm1021 started producing nodules after 4 days. When the plant rootlets were inoculated with the non-rhizopine strain Rm1021 and treated with 3-*O*-MSI, nodules first appeared in the roots after 6 days. The number of nodules slowly increased to 7 in the first 16 days on the rhizopine treated roots but to 2 in untreated roots. This was followed by a rapid increase within the next 10 days to 34 on the rhizopine treated roots and to 24 on

untreated roots. Subsequently, the rate of nodule appearance decreased gradually until day 30 after which no more nodules developed on both the rhizopine treated and untreated roots (see Fig. 2).

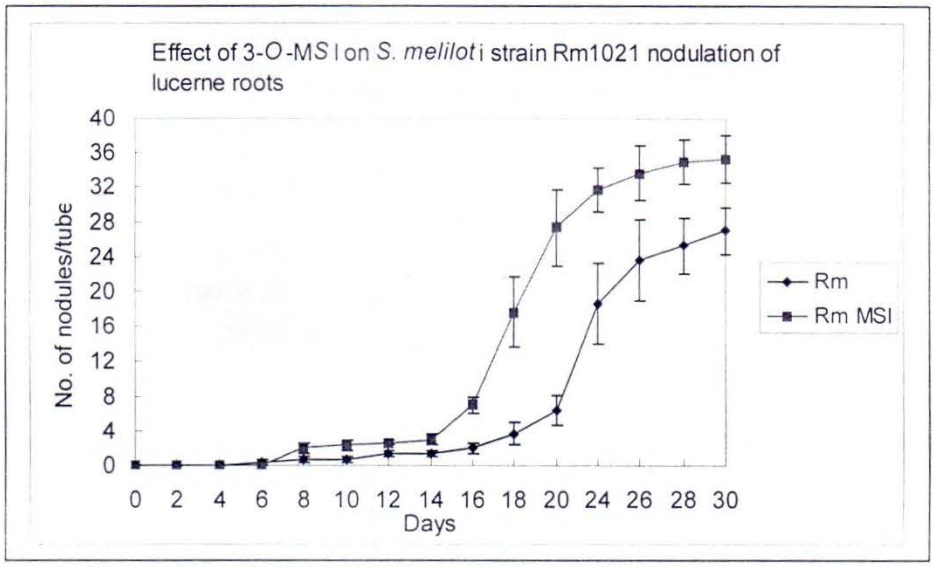

Fig. 2. Number of nodules formed as the days increased on 3-*O*-MSI treated (Rm MSI) and untreated (Rm) lucerne roots inoculated with Rm1021. Error bars show standard error.

The average number of nodules formed on lucerne roots inoculated with non-rhizopine producing strain Rm1021 and treated with 3-*O*-MSI (Rm1021/3-*O*-MSI) was higher than the number of nodules observed on plants inoculated with the Rm1021 strain but not treated with 3-*O*-MSI (see Fig. 2).

However, the number of nodules (35) in L5-30/3-*O*-MSI and Rm1021/3-*O*-MSI treated roots was the same after 30 days. Slight difference was observed in the number of nodules in day 30 in roots inoculated with strains L5-30 (24 nodules) and Rm1021 (27 nodules) but not treated with rhizopine (see Fig. 1 & 2). In the control experiment few (less than 4) inactive white nodules were observed after 14 days (not shown).

Regression analysis was used to determine whether there were significant differences between the number of nodules formed on roots treated with 3-*O*-MSI and the untreated ones. The square root of the mean number of nodules formed was used in the analysis so as to create a constant variation or make the residuals more even. Using this transformation, the variation in the number of nodules on roots treated with L5-30 and L5-30/3-*O*-MSI was significant at $p<0.05$. Similarly, there was a significant difference ($p<0.05$) in nodule number between roots treated with Rm1021 and Rm1021/3-*O*-MSI.

The results indicate that the application of the rhizopine, 3-*O*-MSI enhances nodulation on lucerne roots.

Discussion

Rhizobia were added at levels vastly in excess (4×10^8) of that required for nodulation and the experiments did not involve competition between strains, it is unlikely that differences in the number of bacteria outside of the nodule were responsible for the effects observed. In the experiment, there was an enhanced nodulation when rhizopine was added to rootlets inoculated with either rhizopine producing *S. meliloti* strain L5-30 or non-rhizopine producing *S. meliloti* strain Rm1021. As Rm1021 can not catabolise the rhizopine (Murphy *et al.*, 1995), this suggests that catabolic activity on rhizopine is not responsible for the enhanced nodulation rather the application of rhizopine to plant roots is accountable for the improved nodulation.

The number of nodules produced in this experiment is higher than in a similar work (Owuama & Murphy, 2002) in which the plants were restrained within the tubes with non-absorbent contain wool so as to minimize contamination. The plants in the previous report (Owuama & Murphy, 2002), were invariably stressed by restricting them within the test tubes in which they were grown. Stressing the plants may have also influenced the plant signals. Biological and environmental stresses are known to affect the levels of flavonoids and isoflavonoids produced by plants (van Etten and Pueppke, 1976; Koes et al., 1994).

The addition of rhizopine initially resulted in a delay in nodulation between days 4-6. This occurred in all strains regardless of whether they are Moc^+ or Moc^-. It is possible that the delay in nodule initiation on roots treated with rhizopine may be due to rhizopine modulation of plant signals such as flavonoids and isoflavonoids that induce the expression of nodulation genes in rhizobia and production of Nod factors. As well, a rhizopine-induced delay in the early phases of nodulation would be consistent with previous observations that a Moc^- strain (incapable of catabolising rhizopine) is at a competitive disadvantage in competition for nodulation (Gordon *et al.*, 1996). Gordon *et al.* (1996) proposed a model whereby the early phases of nodulation are inhibited by rhizopine. Thus, a Moc^+ strain catabolises the rhizopine giving these strains an advantage over a Moc^- strain by reducing/eliminating the initial inhibitory effect of rhizopine on nodulation. Competing Moc^- strains would have a delayed nodulation. When two strains are competing, this initial delay in nodulation would be important in determining which strain nodulates, as the initiation of nodulation by one strain will trigger feedback inhibition which would restrict subsequent nodulation events. However, there will be no effect on nodulation when bacteria are not competing since the number of initiation events greatly exceeds the number of nodules that form (Caetano-Anollés and Gresshoff, 1991). The later effects of enhanced nodulation by exogenous addition of rhizopine appear to be different from that proposed in the early events in nodulation. When bacteria produce rhizopine during nodulation (as opposed to its exogenous addition outlined here) there is no difference between the level of nodulation for individual isolates (Gordon *et al.*, 1996). The differences are only observed when the bacteria are competing for nodulation, whereby the rhizopine producing strain shows competitive nodulation advantage over the non-rhizopine producing strain (Gordon *et al.*, 1996).

The enhanced nodulation on legume roots by externally applied rhizopine in this work could result from the effect of rhizopine on the diffusible signal from the shoot to the root and/or the changes in the root hairs, and/or by interacting with the Nod factor. The rate of nodule formation has been reported to decline as the number of nodules on a plant increased (Caetano-Anollés and Gresshoff, 1991). This is due to a feedback inhibition mechanism, which regulates the infection events in nodule formation and limits the number of nodules formed on a root, so as to permit the metabolic efficiency of the plant (Calvert *et al.*, 1984). A diffusible shoot factor has been postulated to be important in this decline in nodule formation as the number increases on a plant (Caetano-Anollés *et al.*, 1990). Caetano-Anolles *et al.* (1990) suggested that the diffusible signal may be modified or transmuted into another signal, which represses the formation of new nodule premordia in the roots. Rhizopine may be suppressing the effect of the diffusible signal from the shoot to the root. Secondly, rhizopine may cause changes in the root hairs and/or interact with the Nod factor. Microinjection of Nod factors from *R. elti* into living root hairs of bean (*Phaseolus vulgaris*) have been shown to alter the organization of actin microfilaments in root hair cells, a likely prelude to the formation of infection threads (Cardenas *et al.*, 1998). Also, preinfection threads differentiation (a stage necessary for successful infection) in alfalfa, requires in addition to Nod factors, complementary signaling (Timmers *et al.*, 1999).

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