

Enhanced nodulation and symbiotic effectiveness of *Medicago truncatula* when co-inoculated with *Pseudomonas fluorescens* WSM3457 and *Ensifer (Sinorhizobium) medicae* WSM419

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Abstract

Aims Low numbers of rhizobia in soil or inoculants delay nodulation and decrease symbiotic legume productivity. This study investigated the effect of co-inoculation with a helper bacterium, *Pseudomonas fluorescens* WSM3457 on the *Medicago truncatula* - *Ensifer (Sinorhizobium) medicae* WSM419 symbiosis challenged by a low inoculum dose.

Methods In a glasshouse experiment the effect of co-inoculation with WSM3457 on the kinetics of nodule initiation and development was assessed 5, 7, 10, 14, 17, 21, and 42 days after inoculation of *M. truncatula* cv. Caliph with 10^3 cells/plant of *E. medicae* WSM419.

Results Co-inoculated plants had enhanced rate of nodule initiation and development, greater numbers of larger crown nodules, and by day 42 accumulated more N than plants inoculated with *E. medicae* WSM419 alone. Nodule development was altered by co-inoculation. Approximately 25% of nodule initials on co-inoculated plants formed in closely associated pairs, young nodules were larger with multiple meristems and developed into cluster-like multi-

lobed nodules compared to those on WSM419 inoculated plants. Molecular typing showed WSM3457 occupied a significant proportion of root nodules on co-inoculated plants.

Conclusion Co-inoculation with *P. fluorescens* WSM3457 enhanced symbiotic effectiveness of *M. truncatula* when inoculated with a low inoculum dose of *E. medicae* WSM419.

Keywords PGPR · Rhizobia · Nodulation enhancement · Co-inoculation · *Ensifer (Sinorhizobium) medicae* · *Medicago truncatula*

Abbreviations

PGPR Plant Growth Promoting Rhizobacteria
WSM Western Soil Microbiology
DAI Days After Inoculation

Introduction

Legumes and their associated nitrogen-fixing bacterial symbionts, the rhizobia, are an integral part of many agricultural systems (Howieson et al. 2007). In addition to being valuable high protein food crops and sources of fodder, and contributing to the sustainable nitrogen economy of soils (Peoples et al. 2009), symbiotic legumes can also improve soil structure and fertility, and assist in controlling weeds, pests and diseases through crop rotations (Howieson

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et al. 2000). The productivity of nitrogen-fixing crop and pasture legumes is often challenged by environmental conditions that are detrimental to the survival of rhizobia in the soil, such as pH, aridity, high temperatures, low clay content, excess of heavy metals and salinity (Brockwell 2001; Peoples et al. 2009; Zhang and Smith 1995). Moreover, when rhizobia inocula are used during pasture or crop establishment, mishandling of the inoculum before sowing, dry sowing, and adverse environmental conditions can contribute to poor survival of inoculant rhizobia on seed and in the soil (Howieson and Ballard 2004; O'Hara 2001). Low numbers of rhizobia in the soil or in inoculants can result in delayed nodulation and an overall loss of legume productivity (Denton et al. 2007).

Plant growth promoting rhizobacteria (PGPR) are soil bacteria which, when grown in association with a host plant, result in stimulation of growth of their host (Vessey 2003). It has been reported PGPR can improve the growth and productivity of a variety of agricultural crops, including canola, wheat and rice (Dobbelaere et al. 2003) and legumes (Bai et al. 2002b; De Leij et al. 2002; Marek-Kozaczuk et al. 2000). Since the 1970's there have been an increasing number of reports of 'helper' PGPR enhancing a variety of legume-rhizobia symbioses (Vessey 2003). The effects of PGPR co-inoculation on legume symbioses include increases in nodule number and/or nodule weight, and in some cases an enhancement of nitrogen fixation or N accumulation. A variety of mechanisms have been proposed for the observed responses of symbiotic legumes to PGPR co-inoculation including phytohormonal stimulation of root growth (Vessey and Buss 2002), increased production of *nod* gene products inducing flavonoids by the legume host (Andrade et al. 1998), stimulation of root hair development (Lucas Garcia et al. 2004) and secretion of B vitamins by the PGPR enhancing rhizobial growth in the rhizosphere (Marek-Kozaczuk and Skorupska 2001). Of particular interest to PGPR enhancing nodulation is the production of the phytohormone indole acetic acid (IAA). This plant hormone stimulates root elongation and increased density of both root hairs and lateral roots (Arshad and Frankenberger 1991; Gray and Smith 2005). As roots are the initiation point for nodule formation increased growth could result in more colonisation sites for rhizobia.

Pseudomonas fluorescens WSM3457 (previously reported as *Alcaligenes xylosoxidans* WSM3457), a gram-negative rhizosphere bacterium isolated in Western Australia, was reported to enhance root mass and nodulation of *Trifolium subterraneum* (sub clover) when co-inoculated with *Rhizobium leguminosarum* bv. *trifolii* WSM409 (Flores-Vargas and O'Hara 2006). We have since used 16s SrRNA sequencing to identify this strain as *P. fluorescens* (NCBI Genbank database, accession number JF423119). Subsequently it was shown that *P. fluorescens* WSM3457 can increase shoot yields and nodulation scores of *Medicago truncatula* when used as a co-inoculant with *Ensifer* (*Sinorhizobium*) *medicae* WSM419 (S. Fox, unpublished Honours Thesis, Murdoch University, 2006).

This study was undertaken to investigate the effect of co-inoculation with WSM3457 on the kinetics of nodule initiation and development in *M. truncatula* when the symbiosis is challenged by a low inoculum dose of rhizobia. In glasshouse experiments legumes are often inoculated with $10^7 - 10^9$ root nodule bacteria (Hirsch et al. 2009; Howieson et al. 1995; Tang et al. 2001; Terpolilli et al. 2008). However, when seeds are inoculated prior to sowing in the field, the inoculum density is much lower, in the order of 10^4 cells per gram of seed. *M. truncatula* and *Medicago sativa* have both been reported to have limited nodulation when inoculated with less than 5×10^3 (Caetano-Anolles and Bauer 1988; Macchiavelli and Brelles-Marino 2004). The use of a rhizobial inoculum containing $10^3 - 10^4$ was adopted to place the symbiosis under pressure and to mimic bacterial populations more likely found in field situations (Ballard and Charman 2000).

Materials and methods

Bacterial strains and growth conditions

P. fluorescens WSM3457 was previously isolated from the roots of annual ryegrass (*Lolium rigidum* G.) growing in a vineyard in the Swan Valley, Western Australia (Flores-Vargas and O'Hara 2006). *E. medicae* WSM419 was isolated from nodules of *Medicago murex* in Sardinia (Howieson and Ewing 1986). Both strains were sourced from the Western Soil Microbiology (WSM) collection held at the Centre for *Rhizobium* Studies at Murdoch University.

E. medicae WSM419 was cultured using ½ LA medium (Howieson et al. 1988) and WSM3457 was cultured in Nutrient Broth (Difco). For solid media the broth was supplemented with 1.5% (w/v) agar. Both strains were grown at 28°C, *E. medicae* WSM419 for 72 h and *P. fluorescens* WSM3457 for 24 h. Broth cultures were grown on an orbital shaker at 200 rev min⁻¹.

Determining indole acetic production

Indole acetic acid production by *P. fluorescens* WSM3457 was quantified using the microtitre plate method described by Sarwar and Kremer (1995). Amounts of IAA produced were expressed as mg IAA equivalents/mg cellular protein (Bio-Rad Laboratories, Inc).

Glasshouse experiment

M. truncatula cv. *Caliph* was subject to four treatments; 1) plants inoculated with *E. medicae* WSM419; 2) plants co-inoculated with *E. medicae* WSM419 and WSM3457; 3) non-inoculated plants with added nitrogen (5 ml of 0.5 M KNO₃ weekly) and; 4) a non-inoculated control with no nitrogen added.

The pots were arranged in the glasshouse in a block-randomised design, with each block containing four replicates of each treatment. Four replicates (four plants per replicate) from each treatment were harvested at 5, 7, 10, 14, 17, 21 and 42 days after inoculation (DAI).

Surface sterilisation of seed

Seeds of *M. truncatula* cv. *Caliph* were sorted for uniformity and lightly scarified using fine sandpaper. Seeds were surface sterilized by soaking in 70% (v/v) ethanol for 1 min, 3% (v/v) sodium hypochlorite for 3 min and then rinsed six times in sterile deionised water prior to germination on 1.5% (w/v) water agar plates. Plates were wrapped in aluminium foil and left at room temperature for eight h before being inverted to allow the emerging radical to grow straight, facilitating the transplanting process. Plates were left at room temperature for 24–36 h, before germinated seedlings were transferred into a steam-sterilised mix of river sand and yellow sand (1:1). Plants were

grown using the culture system described by (Howieson et al. 1995), where deficiency of N limits legume growth except where they are effectively nodulated or are supplied with nitrogen (Terpolilli et al. 2008).

Preparation of inoculant cultures

Cultures of *E. medicae* WSM419 and the co-inoculant *P. fluorescens* WSM3457 were adjusted to an OD_{600nm} of 0.5 with NaCl (0.89% w/v), to achieve approximately 10⁸ cells ml⁻¹. These cultures were then diluted with sterile DI water to achieve an inoculum dosage of 10³ to 10⁴ cells ml⁻¹. 24 h after the seedlings were transplanted, the soil where seedling was planted was inoculated with 1 ml of the appropriate inoculum. The non-inoculated N+ and N- control plants received 1 ml of sterile DI water. Miles and Misra plates were used to quantify the concentration of each strain in the inocula (Vincent 1970).

Assessing the timing of nodule initiation and nodule development on *M. truncatula*

The roots from plants harvested at 5, 7, 10, 14, 17 and 21 DAI were carefully rinsed clean and then fixed in a solution of 100% ethanol: 7% (v/v) acetic acid (1:3) before storing at 4°C until required for nodule initial studies (O'Hara et al. 1988).

A modified version of the root staining procedure described by Tang et al. (1990) was used to count nodule initials. Roots were rinsed clean of the fixative solution, before being cleared in 10% (w/v) KOH at room temperature for 2 h. The roots were rinsed with water and then acidified in 0.25 M HCl for 5 min. Roots were then stained in 0.1% (w/v) Brilliant green for 30 min and de-stained in water overnight. Nodule initials were distinguished from lateral root initials (Cheng et al. 2002), and were counted under a dissecting microscope at x 20 magnification (Tang et al. 1990).

Assessing the effect of co-inoculation on nodulation at 42 DAI

Shoots and roots of plants harvested at 42 DAI were separated at the hypocotyl. Nodules were counted and individual nodules were scored using a modified version of the system described by Howieson and

Ewing (1989) (Table 1). Briefly, each nodule was scored according to its size and position on the root system. Plant nodule score was calculated by the addition of the individual scores for each nodule on that plant.

The five largest nodules from each replicate were then picked and surfaced sterilised. Briefly, nodules were soaked in 80% (v/v) ethanol for 30s (45 s for nodules >5 mm), 3 min (5 min for nodules >5 mm) in 4% (v/v) sodium hypochlorite and then rinsed 6 times for 30s in sterile DI water. Replicate 0.1 ml samples of the final rinse solutions were plated out onto TY agar (casein enzymic hydrolysate 6 g/L, yeast extract powder 3 g/L, agar 12 g/L) to confirm surface sterilisation. Nodules were then individually crushed in 100 µl of sterile 0.89% (w/v) NaCl₂ and the resulting milky solution streaked onto TY, ½ LA and NA plates, incubated at 28 C, and checked daily for growth. WSM419 was identified based on its typical donut morphology (Garau et al. 2005). Bacteria other than WSM419 were picked and streaked onto both TY and NA until pure cultures were obtained. The 16S rRNA gene of three non WSM419 isolates from three different nodules was sequenced and a sequence homology search conducted as described above.

The shoots and roots were then dried for 4 d at 70° C prior to weighing. The shoots of the four plants from each replicate were then pooled and analysed for nitrogen.

PCR and DNA sequencing of nodule isolates

Bacteria isolated from nodules were grown on nutrient agar for 24 h and then suspended in sterile 0.89% (w/v) NaCl. Cell suspensions were pelleted at 10,000 x G for 5 min, before the supernatant was

Table 1 Nodule rating system used to evaluate nodulation (adapted from Howieson and Ewing (1989))

Size	Weighting	Position of nodule	Weighting
Large cluster >5 mm	4	On upper tap (0–5 cm) or on lateral roots	2
Large 3–5 mm	3	within 1 cm of upper tap	
Medium 1–3 mm	2		
Small <1 mm	1	Nodules elsewhere on root system	1

removed and the cells resuspended in sterile 0.89% (w/v) NaCl to OD 2 at 600 nm.

Primers used for the amplification of the 16S rRNA sequence were FGPS 6 and FGPS 1509 (Normand et al. 1992). The PCR reaction mixture of 25 µl contained 1 µl of cell suspension, 0.5 µl of 5.5U/µl Taq DNA polymerase (Invitrogen Life Technologies), 0.5 µl of each 50 µM primer, 5 µl of polymerisation buffer containing dNTPs (Fisher Biotech, Australia), 15 µl of UltraPure grade water (Fisher Biotech, Australia) and 2.5 µl of 17 mM MgCl₂ (Promega Corp.). The PCR conditions consisted of an initial denaturation cycle of 94°C for 5 min, then 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, before one final cycle of 72°C for 7 min in a Bio-Rad PCR thermal cycler. PCR products were analysed by electrophoresis on a 1% (w/v) agarose gel at 100 V for 45 min. The gel was stained in a solution of ethidium bromide for 30 min and bands visualised under UV. PCR products were stored at –20°C.

PCR products were purified using a QIAquick® PCR Purification Kit (QIAGEN Pty Ltd) as per the manufacturer's instructions. The purified product was used as DNA template in the sequencing reactions. Sequencing primers used in this study were FGPS 6 and FGPS 1509 (Normand et al. 1992) and the internal primers 800 F, 1,100 F, 520R and 820R (Yanagi and Yamasato 1993). For each primer the following sequencing reaction was prepared: 4 µl of purified DNA template, 4 µl of Big Dye Terminator (version 3.1), 1 µl of 3.2 pmole/l primer and deionised water to make up a final volume of 10 µl per reaction.

The reaction mixtures were amplified for 25 cycles at 96°C for 10 sec, 50°C for 10 sec and 60°C for 4 min. The resulting DNA template was then purified using the ethanol precipitation protocol outlined by Perkin Elmer (protocol P/N 402078: <http://www.perkinelmer.com>). Nucleotide sequences were determined using the DNA sequencer model ABI 377 and the ABI Prism® BigDye™ Terminator v3.0 Ready Reaction Cycling Sequencing Kit with Amplitaq® DNA Polymerase, in accordance with the manufacturers instructions, at the Western Australian State Agricultural Biotechnology Centre (SABC).

The sequence data was aligned using the Geneious Pro 5.0 program (Biomatters Ltd). Sequence homology searches were conducted using the NCBI BLAST algorithm (Zhang et al. 2000).

Statistical analysis

Data were analysed for homogeneity of variance ($P=0.01$) and then subjected to analysis of variance using the multivariate general linear model using the Wilks' Lambda model in SPSS 17.0, using a significance level of $P=0.05$. Significant treatment means were compared using Fisher protected LSD test (SPSS17.0 for Windows (Lead Technology, Inc)).

Results

Effect of co-inoculation on timing of nodule initiation and development

Co-inoculation of *M. truncatula* with *E. medicae* WSM419 and WSM3457 enhanced the rate of nodule initiation and subsequently, nodule development (Fig. 1). Non-inoculated plants did not develop root nodules.

Nodule initials were evident in roots of inoculated plants at 5 DAI (Fig. 1). By 14 DAI there were significantly more nodule initials on plants co-inoculated with *E. medicae* WSM419 and WSM3457 compared with those inoculated with *E. medicae* WSM419 alone (significant at $P=0.032$). Subsequently, the rate of nodule initiation in co-inoculated roots decreased, whereas in WSM419 inoculated roots it increased such that there was no significant difference in number of nodule initials between the two inoculated treatments at 17 and 21 DAI.

The rate of nodule development was enhanced by co-inoculation of *M. truncatula* with *E. medicae* WSM419 and *P. fluorescens* WSM3457. Root nodules were present on the co-inoculated plants at 5 DAI whereas nodules did not emerge on *E. medicae* WSM419 inoculated plants until day 10 (Fig. 1b). There were significantly more nodules on co-inoculated plants at both 7 DAI ($P=0.002$) and 10 DAI ($P=0.045$). At the final harvest at 42 DAI there were more nodules on co-inoculated plants ($P=0.019$) than on plants inoculated with WSM419 alone. A larger proportion of co-inoculated plants had crown nodulation, 68.75%, compared to those inoculated with only rhizobia, 31.2%, which was reflected in the significantly enhanced nodulation scores ($P=0.002$) on co-inoculated plants (Table 2).

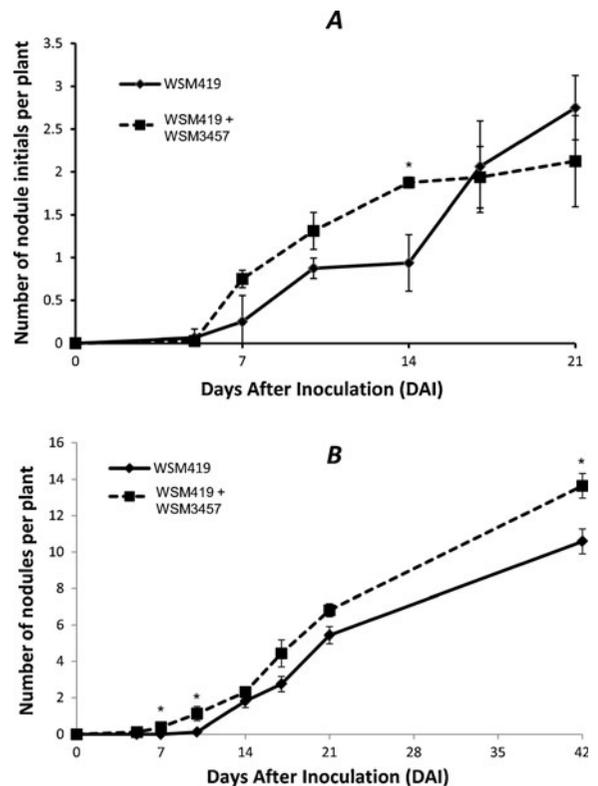


Fig. 1 Number of nodule initials (a) and root nodules (b) on *Medicago truncatula* cv. Caliph inoculated with *Ensifer medicae* WSM419 alone or with *E. medicae* WSM419 and the helper PGPR WSM3457. * Indicates treatment differences that were significant at $P=0.05$

Nitrogen analysis revealed that at 42 DAI the nitrogen concentrations (% N) were similar in shoots of *M. truncatula* plants from the N added (4.01% N), WSM419 inoculated (3.83% N), and WSM419 plus WSM3457 co-inoculated (3.92% N) treatments (Table 2). The N minus control shoots had significantly smaller % N concentrations (1.51% N) than plants from the other three treatment regimes. The total nitrogen accumulated in plant shoots was calculated from the % N and the Dry shoot mass data. Plants in the N added treatment accumulated significantly more shoot nitrogen than plants from the other three treatments, whereas plants in the N minus treatment had significantly less total nitrogen in their shoots than the other three treatments. Plants co-inoculated with *E. medicae* WSM419 and WSM3457 accumulated significantly more shoot N than those inoculated with WSM419 alone ($P=0.004$).

It was observed during the microscopic assessment of nodule initiation that there were differences

Table 2 Nodule number, nodule score, shoot mass, root mass, %N and Total N of *Medicago truncatula* cv. Caliph inoculated with *Ensifer medicae* WSM419 alone or with *E. medicae* WSM419 and the helper PGPR WSM3457

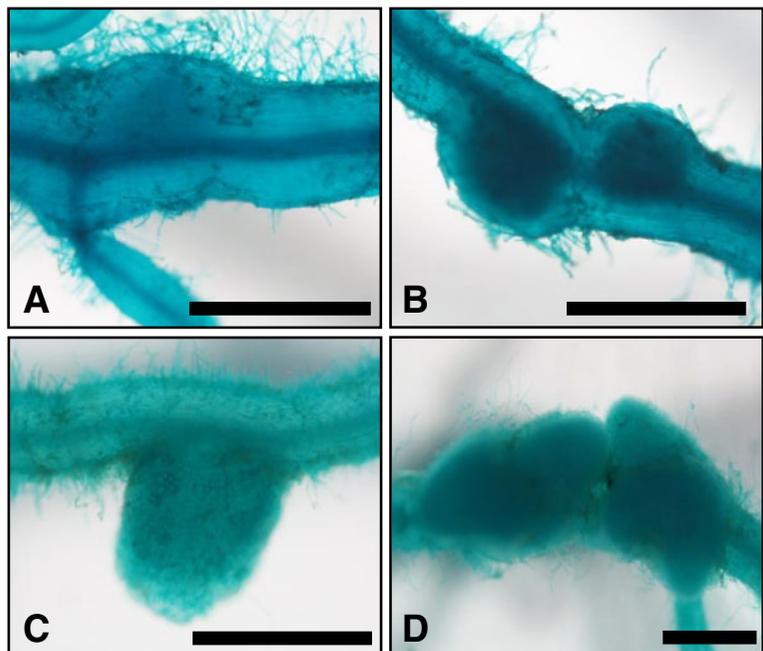
Treatment	Nodule number	Nodule score	Shoot mass (mg/plant)	Root mass (mg/plant)	% N	Total N (mg/plant)
Nitrogen added			177.14a	92.68a	4.01a	7.10a
WSM419	10.58b	16.1b	154.92b	74.81a	3.83a	5.88c
WSM419+WSM3457	13.645a	27.5a	167.03ab	91.86a	3.97a	6.61b
Uninoculated control			13.49c	13.28b	1.51b	0.21 d

between the two inoculated treatments in the morphology of nodule initials and young root nodules. From 7 DAI, up to 25% of the nodule initials on the co-inoculated plants formed in closely associated pairs (Fig. 2b). This was not observed in plants inoculated with WSM419 alone where nodule initials always occurred as single zones of meristematic cells in the root cortex (Fig. 2a). The young nodules on co-inoculated roots at 10 to 21 DAI were often more developed with multiple meristems and were generally larger in size (Fig. 2d) than those on roots inoculated with WSM419 (Fig. 2c). Similarly, at 42 DAI nodules on co-inoculated plants were larger cluster-like multi-lobed nodules (Fig. 3b) compared to those on WSM419 inoculated plants (Fig. 3a).

DNA sequencing of 16S rRNA gene of nodule occupants

There was no bacterial growth on the plates spread with water from the final nodule rinse solution, which shows that the nodule sterilisation process was successful. WSM419 was isolated from all nodules examined. In addition, 40% of the nodules from the co-inoculated treatment also contained a bacterium that based on visual assessment of colony morphology, colour and colony size was WSM3457. The 16S rRNA sequences from three representative isolates were identical to WSM3457, confirming the co-inoculant bacterium was present in a significant proportion of root nodules sampled from the co-inoculation treatment.

Fig. 2 Seedling roots of *Medicago truncatula* cv. Caliph after inoculation with with *Ensifer medicae* WSM419 alone or with *E. medicae* WSM419 and the helper PGPR WSM3457. Nodule initials five days after inoculation with WSM419 (a) and, WSM419 plus WSM3457 (b). Nodules 17 days after inoculation with WSM419 (c) and, WSM419 plus WSM3457 (d). Scale bars 1 mm



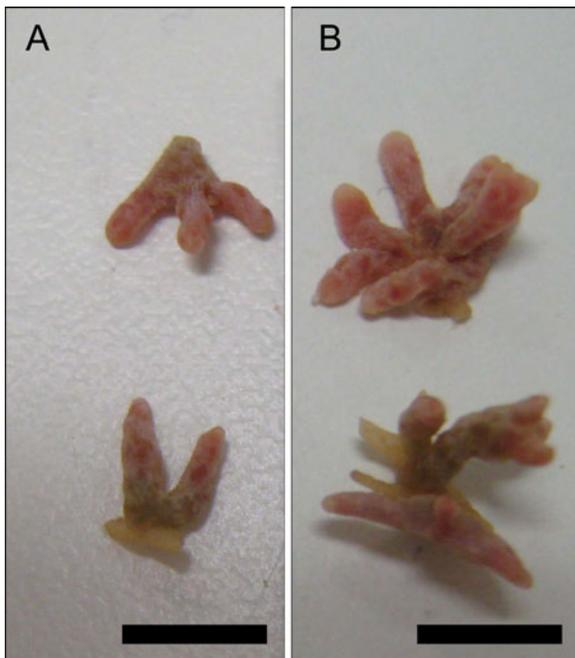


Fig. 3 Representative root nodules on *Medicago truncatula* cv, Caliph 42 days after inoculation with *Ensifer medicae* WSM419 alone (a) or with *E. medicae* WSM419 and the helper PGPR WSM3457 (b). Scale bars 5 mm

IAA production of WSM3457

WSM3457 produced 0.103 ± 0.02 and 0.36 ± 0.07 mg of IAA equivalents per mg of cellular protein in the – tryptophan and +tryptophan treatments respectively.

Discussion

Co-inoculation of *M. truncatula* with *E. medicae* WSM419 and *P. fluorescens* WSM3457 enhanced the rate of nodule initiation and development resulting in co-inoculated roots forming greater numbers of larger crown nodules earlier than plants inoculated with *E. medicae* WSM419 alone. Co-inoculation also resulted in increased nodule numbers, nodulation scores and total nitrogen accumulation in co-inoculated plants relative to *M. truncatula* inoculated with 6×10^3 cfu of rhizobia alone. Total dry shoot mass of plants inoculated with WSM419 alone was 85% of the nitrogen added control plants. By comparison total dry shoot mass of plants inoculated with both WSM419 and WSM3457 was 96% of the nitrogen

added control plants. The increase in both plant mass and nitrogen accumulation in co-inoculated plants represents a marked enhancement of symbiotic effectiveness of *M. truncatula*.

Observations of nodule initials and nodules can be used to differentiate between effects on successful infection and initiation from those on nodule development per se (O'Hara et al. 1988; Tang et al. 1990). Accordingly, the earlier nodule initiation observed on *M. truncatula* co-inoculated with *E. medicae* WSM419 and *P. fluorescens* WSM3457 suggests that infection by the rhizobia was enhanced and this conclusion is further supported by the nodule score data. Nodule scores were higher on the co-inoculated plants due to the increase in the proportion of nodules in the root crown and the size of nodules. The relative location of nodules on the root system is a useful index of the time taken for infection, i.e. nodules located on the upper part of the root system are those that were initiated earlier than those on the lower parts of the root system (Bhuvaneswari et al. 1988; Caetano-Anolles et al. 1988). Thus, the increased crown nodulation observed on co-inoculated *M. truncatula* in this study further supports the data demonstrating that root infection occurred earlier with co-inoculation.

The observation that by 7 DAI *M. truncatula* co-inoculated with *E. medicae* WSM419 and *P. fluorescens* WSM3457 had nodule initials that were often present in pairs and appeared to have early signs of cluster-like morphology was mirrored by observations that there were often large cluster-like nodules on the co-inoculated plants harvested at 42 DAI. These observations coupled with the data that nodule initiation is enhanced with co-inoculation, raises some interesting questions about the interactions occurring here between WSM3457 and the two symbiotic partners (*M. truncatula* and WSM419). Namely, does co-inoculation increase the number of *M. truncatula* root hairs infected by *E. medicae* WSM419? Alternatively, is there no increase in the number of root hairs infected by *E. medicae* WSM419 but does co-inoculation decrease the rate of aborted infection threads? Future work involving a non-destructive root infection assay system, such as the Fahraeus slide method (Fahraeus 1957), will be necessary to answer these questions.

Previous studies have demonstrated that PGPR co-inoculation can enhance early nodule initiation and

development on *Phaseolus vulgaris* (bean) (Srinivasan et al. 1997), and soybean (Nishijima et al. 1988). Srinivasan et al. (1997) demonstrated that a PGPR isolate, *Bacillus megaterium* S49, increased the density of root hairs on *P. vulgaris* roots, and concluded that as root hairs are infection sites for rhizobia, that the enhanced early nodulation may be mediated by the increased number of attachment sites. There has also been a recent study demonstrating that a rhizobium strain modified to over produce IAA resulted in increased nodule number on *Medicago* sp. (Pii et al. 2007). While we have reported here that WSM3457 produces IAA in vitro, at this stage it is not known whether this strain produces this compound in the rhizosphere of *M. truncatula*. In a previous study, co-inoculation of *M. truncatula* with WSM419 and *P. fluorescens* WSM3457 did not alter root physiology compared to inoculation with *E. medicae* WSM419 alone (S. Fox, unpublished Honours Thesis, Murdoch University, 2006) indicating the enhancement of nodulation reported here is not a result of changes in root hair density.

The 16S rRNA sequencing of the nodule isolates showed that all three isolates examined were the co-inoculant *P. fluorescens* WSM3457, which indicates that the co-inoculant is gaining access to root nodules. It is known that approximately 10% of nodules can be occupied by multiple rhizobial strains (Denton et al. 2002; Yates et al. 2008). Likewise there are reports of non-rhizobial species being isolated from legume nodules (Bai et al. 2002a; Mhamdi et al. 2005; Mrabet et al. 2006; Sturz et al. 1997). In one study an *Agrobacterium* sp., originally isolated from common bean nodules, was tagged with a *gusA* reporter and then was co-inoculated with rhizobia on soybean. GusA staining confirmed that the *Agrobacterium* sp. was able to colonise the nodule and that over time seemed to increase in the area colonised (Mhamdi et al. 2005). It is possible that the co-inoculant may concurrently colonise an infected root hair during the infection process and inhabit infection threads. Alternatively WSM3457 may be colonising the outer cortical regions of nodules. Further work is necessary to identify the location of the co-inoculant within the *M. truncatula* root nodules.

The experimental system used here may prove useful as a tool to study the biological interactions affecting the outcomes of the *M. truncatula* - rhizobia symbiotic partnership, particularly in light of recent

work by Terpolilli et al. (2008) that has demonstrated that *E. medicae* WSM419 is a highly effective partner for this legume species. Moreover, the recently completed *E. medicae* WSM419 genome (Reeve et al. 2010) provides opportunities toward further understanding of the legume/rhizobia/PGPR interaction reported here. There is now a capacity to assess the effects of co-inoculation on gene expression in WSM419 during the infection process.

The early enhancement of nodule initiation and development reported in this study may offer a significant advantage to the success of nodulation in field grown legumes that are to subject to environmental stressors detrimental to establishment of an effective symbiosis. Helper PGPR, such as *P. fluorescens* WSM3457, may be useful for enhancing early nodule initiation in legumes to increase the success of rhizobial inoculants under environmental conditions that result in rapid death of the inoculum. Some commercial inoculant products are beginning to emerge that contain plant growth promoting micro-organisms as well as rhizobia but these are predominantly focused on phosphate solubilization in the rhizosphere (Leggett et al. 2007). A co-inoculant that enhances nodulation under stressful edaphic conditions, such as the micro-organism described here, could well prove useful in a commercial agricultural setting.

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