Mycorrhiza (2006) 16: 167–173
DOI 10.1007/s00572-005-0028-3

ORIGINAL PAPER

Pedro M. Antunes · Deanna Deaville · Michael J. Goss

Effect of two AMF life strategies on the tripartite symbiosis with *Bradyrhizobium japonicum* and soybean

Received: 4 May 2005 / Accepted: 21 October 2005 / Published online: 16 December 2005
© Springer-Verlag 2005

Abstract This study is the first in assessing the effect of soil disturbance on the contribution of arbuscular mycorrhizal fungi (AMF) with different life-history strategies to the tripartite symbiosis with soybeans and *Bradyrhizobium japonicum* (Kirchner) Jordan. We hypothesized that *Gigaspora margarita* Becker and Hall would be more affected by soil disturbance than *Glomus clarum* Nicol. and Schenck, and consequently, the tripartite symbiosis would develop more rapidly and lead to greater N_2 fixation in the presence of the latter. Soil pasteurization allowed the establishment of treatments with individual AMF species and soil disturbance enabled the development of contrasting root colonization potentials. In contrast, the colonization potential of *B. japonicum* was kept the same in all treatments. Soil disturbance significantly reduced root colonization by both AMF, with *Gi. margarita* being considerably more affected than *G. clarum*. Furthermore, the tripartite symbiosis progressed faster with *G. clarum*, and at 10 days after plant emergence, there was 30% more nodules when *G. clarum* was present compared to that when the bacterial symbiont alone was present. At flowering, the absence of soil disturbance stimulated N_2 fixation by 17% in mycorrhizal plants. However, this response was similar for both AMF.

Keywords Tripartite symbiosis · Early interaction · Arbuscular mycorrhizal fungi · Life-history strategies · N_2 fixation

P. M. Antunes (✉) · D. Deaville · M. J. Goss
Department of Land Resource Science,
University of Guelph,
Guelph, Ontario, N1G 2W1, Canada
e-mail: pantunes@uoguelph.ca
Tel.: +1-519-8244120
Fax: +1-519-7671991

Present address:
P. M. Antunes
Department of Integrative Biology, Axelrod Building,
University of Guelph,
Guelph, Ontario, N1G 2W1, Canada

Introduction

Soybean [*Glycine max* (L.) Merr.] plants form associations with arbuscular mycorrhizal fungi (AMF) and *Bradyrhizobium japonicum* (Kirchner) Jordan simultaneously, resulting in a tripartite symbiosis which promotes nodulation and N_2 fixation. It has been assumed that the significance of the tripartite symbiosis is solely associated with increased P supply to the nodules through the AMF (e.g. Ganry et al. 1982; Kawai and Yamamoto 1986; Vejsadová et al. 1993; George et al. 1995; Ibijbijen et al. 1996; Xavier and Germida 2003). However, a number of studies have suggested that signalling mechanisms regulate the early interaction between the three symbionts (e.g. Bayne and Bethlenfalvay 1987; Ames and Bethlenfalvay 1987; Goss and de Varennes 2002; Antunes et al. in press).

AMF families vary widely in the pattern of root colonization and life strategy (Hart and Reader 2002). Before root penetration, some rely on their large spores and are slow root colonizers, as compared to others that use delicate and diffuse hyphae as their most infective propagule. Species belonging to the Gigasporaceae fit the former strategy, whereas Glomaceae are associated with the latter (e.g. Morton 1993; Klironomos and Hart 2002). Once root colonization is established, hyphae of the Gigasporaceae do not spread very profusely within the root system but form an extensive and densely aggregated external mycelium, which is not infective when disrupted. Conversely, the hyphae of the Glomaceae colonize the root system extensively but produce only a limited extra-radical mycelial network, which is highly infective.

The variation in life strategies among the AMF may be linked to host–symbiont compatibility phenomena which represents an important aspect of mycorrhizal soil ecology, particularly regarding the interaction with *B. japonicum* and soybean. Ianson and Linderman (1993) found variation in the nodulation and N_2 fixation response of Pigeonpea [*Cajanus cajan* (L.) Millsp.] to seven different isolates of AMF. While some AMF stimulated nodule growth and function, others did not, particularly at flowering when the demand for carbon increases. The variation among isolates

was independent of taxonomic ranking. However, the initial AMF potential established for each isolate was high (determined on a saturation of infection level), which might lessen differences related to life-history strategy at the early stage. A recent study by Scheublin et al. (2004) showed that root nodules contained AMF communities that were different from those in the legume roots, which strongly supports the hypothesis that AMF functional diversity is important in the tripartite symbiosis. However, in agricultural fields, continuous and aggressive cultural practices, such as tillage, have a negative impact on the AMF extraradical hyphal network (Miller et al. 1995). Consequently, tillage may compromise the survival of AMF whose hyphae become not infective after disruption (e.g. *Gigaspora* sp.). Moreover, there is indication that *Glomus* species have become more abundant in agroecosystems (Helgason et al. 1998; Oehl et al. 2003).

The objectives of this study were to assess whether (1) the progress of the tripartite symbiosis varies depending on the susceptibility of an AMF to soil disturbance: as the infectivity of *Gigaspora margarita* is more affected by soil disturbance than that of *Glomus*, it was predicted that the tripartite symbiosis would progress more rapidly when *G. clarum* is present; and (2) the earlier the tripartite symbiosis can be detected, the greater the rate of nodule growth and function will be at later periods of plant growth. Therefore, it was predicted that N_2 fixation would be better with *G. clarum* than with *Gi. margarita*.

Materials and methods

Soil and growing conditions

The experiment was conducted in a walk-in growth-chamber programmed to a 16-h day at 25°C, with a light intensity of 250 μmol s^{-1} m^{-2}, and an 8-h night at 18°C. To maximize the visible light spectrum, a combination of three different types of 215-W fluorescent lamps (Sylvania Cool White F96T12/CW/VHO, Industrial F96T12/GL/WS/VHO and Vitalux Duro-Test F96T-12) and one type of 40-W incandescent lamps were used. Infestation by thrips, mites and fungus gnats was prevented by using their respective mite predators *Amblyseius cucumeris*, *Phytoseiulus persimilis* and *Hypoaspis miles*. These biological control agents were applied to the leaves as larvae in a sawdust medium.

Fine sandy loam soil in which soybeans had never grown was collected on 20 July 2003 from the top 20 cm of an arable field on a farm near Belwood (43°45′N 80°15′W), Ontario, Canada. The soil was broken up mechanically and sieved (4 mm) before being stored under cover at ambient temperature. Soil samples were collected for analysis of 2.0 M KCl-extractable NH_4-N and NO_3-N (Keeney and Nelson 1982), 0.5 M $NaHCO_3$-extractable P (Olsen and Summers 1982), 1.0 M ammonium-acetate extractable K and Mg (Knudsen et al. 1982) and pH (1:1 in water). To confirm the absence of *B. japonicum*, soybeans were sown in pots, and all failed to nodulate by the time of flowering. To eliminate the indigenous mycorrhizal fungi, the soil was pasteurized on 8 September 2003, according to the method described by McGonigle and Miller (1996). After pasteurization, the soil was stored in covered plastic bins. Prior to use, the soil was assessed for mineral nutrients as described earlier.

Preparation phase

On 14 October 2003, each of 51 pots (3 L) was packed with the equivalent of 3,050 g of oven-dry soil to a bulk density of approximately 1.3 g cm^{-3}. Each pot was planted with two maize seedlings (*Zea mays* L. cv. Pioneer 3905) that had been grown in sterilized (autoclaved at 121°C for 15 min) moist vermiculite for 6 days to a root length of approximately 20 cm. Seedlings planted in pots assigned to each mycorrhizal fungus treatment were inoculated with 20 spores of *G. clarum* or *Gi. margarita*. The spores were extracted from inoculum supplied by Dr. Joseph B. Morton, INVAM—International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (accession numbers BR147B and WV205A, respectively), using sucrose density-gradient centrifugation and a sieve with a 45-μm mesh (Tommerup 1992). After repeated washing, the spores of each species were collected manually with a micropipette and placed in a watch glass. The spores were then carefully examined under a stereoscopic microscope, and only those with healthy appearance were transferred to microtiter wells of an ELISA plate. The viability of the spores was assessed 1 week prior to inoculation by measuring the proportion of those that germinated on 50 open filter paper squares in contact with wet steamed soil in a Petri dish (see Brundrett and Juniper 1993). The test indicated that more than 90% of the spores were viable. The inoculation procedure consisted of introducing the root in a column opened thorough the soil with a glass rod and pipetting the spores along the length of the root. The glass rod was carefully flamed after each pot was inoculated to minimize potential contamination. A sample of 100 g of the original inoculum was washed with 2 L of 0.1% (w/v) Bacto-Peptone, and then filtered through a 20-μm mesh (Ames et al. 1987). An aliquot of 40 ml of this filtrate was added to each pot to supply the soil with the same microbial populations of non-mycorrhizal organisms. The soil was wetted to a gravimetric water content of approximately 200 mg H_2O g^{-1} dry soil and then restored to that content by irrigating daily with deionised water. The pots were completely randomized before each irrigation.

Maize plants were excised after 6 weeks of growth. Root samples from each of three inoculated and non-inoculated extra control pots were collected to assess the proportion colonized by AMF. Roots were carefully washed from the soil, fixed in formyl acetic alcohol, cleared in KOH and stained with chlorazol-black E (Brundrett et al. 1984) before being examined for AM colonization by the intersections method described by McGonigle et al. (1990).

Soil disturbance was used to establish contrasting AMF potential treatments. For that, while half the pots was left

undisturbed, the other half was individually inverted, the soil was carefully removed and then sieved (4 mm). All root material separated on the sieve was cut into 2-cm-long segments and re-introduced into the soil which was repacked in the pots to a bulk density of 1.3 g cm^{-3}.

Tripartite symbiosis study phase

The experiment was set out as a 4×2 factorial completely randomized design (CRD) with three replications, comprising four inoculum treatments: *G. clarum* and *B. japonicum* (G + B), *Gi. margarita* and *B. japonicum* (Gi + B), *B. japonicum* (B), non-inoculated control (Control) and two disturbance treatments (undisturbed and disturbed). Twenty-four pots were selected for harvest 10 days after emergence, with the remaining 24 pots to be harvested at flowering 40 days later.

After an initial surface sterilization (95% alcohol for 1 min), Harovinton soybean seeds were germinated in sterilized (autoclaved at 121°C for 15 min) moist vermiculite. Twelve seedlings were then planted into each pot on 27 November 2003. Inoculation with *B. japonicum* consisted of placing 0.25 g of peat-based inoculant of *B. japonicum* strain 532C (MicroBio, Saskatoon, SK, HiStick+) at the bottom of the hole into which each soybean seedling was placed. The same amount of sterilized (autoclaved at 121°C for 15 min) inoculant was placed at the bottom of each seedling hole in pots assigned to treatments without *B. japonicum*, so that edaphic conditions around the newly formed radicles were kept similar for all treatments. In all appropriate pots, each radicle passed through the same amount of *B. japonicum* inoculum, thereby ensuring that the inoculum potential was identical for the two disturbance treatments. Once the seedlings emerged, the plants were thinned to five plants per pot.

Soybean plants from six pots of each treatment were harvested 10 days after emergence. The length of shoots was measured, after which they were dried at 65°C for 48 h and weighed. Root samples were examined for AMF colonization as described above. All root systems were examined for nodules, and those visible with the unaided eye were counted. Three 30-cm soil cores were collected from each pot, and a composite sample was used for analysis of NH_4-N and NO_3-N (Keeney and Nelson 1982).

The remaining pots assigned to study the effect of the tripartite symbiosis on nitrogen fixation were thinned to four soybean plants per pot. Fifty millilitres of a solution of $^{15}NH_4{}^{15}NO_3$ 29.9 mM at 10.6% ^{15}N, which corresponds to a rate of approximately 30 kg N ha^{-1}, was then added to each of those pots. According to Goss et al. (2002), this rate is unlikely to affect nodulation. To avoid absorption of ^{15}N through the shoot, care was taken to avoid direct contact between plants and solution during the procedure. To facilitate the incorporation and distribution of ^{15}N, 100 ml of water was then slowly added to each pot. At 40 days after emergence, the shoots were harvested, oven-dried at 60°C for 48 h and weighed. Each root system was carefully washed out of the soil and fixed in formyl acetic alcohol before being examined for AMF colonization as described earlier. Nodules were detached, counted and the total weight determined after being oven-dried at 65°C for 48 h. Three 30-cm soil cores were collected from each pot, and a composite sample was used for analysis of NH_4-N and NO_3-N (Keeney and Nelson 1982). The shoots were finely ground in a model 3 Willey Mill (Thomas Scientific, Swedsboro, NJ) and then pulverized in a MM300 Brinkman Retsch sphere mill for analysis of their total N content and for the ratio of ^{14}N:^{15}N by mass spectrometry (Mulvaney 1993). The N_2 fixation was estimated by the isotopic dilution method based on the percentage of N derived from atmosphere (%Ndfa), which was calculated according to the equations reported by Fried and Middelboe (1977), with non-inoculated soybeans as the non-fixing control.

Statistical analysis

Comparisons between means for data collected during the preliminary phase were made using *t* tests for independent samples. Data collected in the second phase of the study were examined by analysis of variance (ANOVA) as a CRD. The Shapiro–Wilk's *W* test was used to assess if the observations followed a normal distribution (Shapiro et al. 1968). The assumption of homogeneity of variances was confirmed by the Levene's test (Conover et al. 1981). Non-homogenous data were analysed with the Kruskal–Wallis non-parametric test (Kruskal and Wallis 1952). This test assesses the data by ranking them; therefore, no assumptions were made.

Unless otherwise specified, when the *F* test of the treatment mean square indicated that there were significant variances due to treatment effects, means were compared using the Tukey's honest significance difference (HDS) test ($P<0.05$; Tukey 1949). Tests were performed using the computer software package STATISTICA for the Microsoft Windows operating system (StatSoft, Inc., 1995).

Results and discussion

Preparation phase

The spores of *G. clarum* and *Gi. margarita* germinated and developed hyphae within the soil which colonized the maize roots (Fig. 1). It was not our intention to equalize the colonization potential between *G. clarum* and *Gi. margarita* by using the same number of spores of each species. Instead, the objective was to establish a well-defined reference point from which the growth and role of each AMF in the tripartite symbiosis could be evaluated. Root colonization by *G. clarum* spores was more profuse than by *Gi. margarita*, with approximately 2.5 more arbuscules and almost double the hyphae. Although *Gi. margarita* uses spores as the main propagule, root colonization progressed slower than that of *G. clarum*, which was consistent with the findings reported by Hart and Reader (2002). The family Gigasporaceae is characterized by the absence of vesicles (Morton and Benny 1990). Absence of vesicles was confirmed in plants

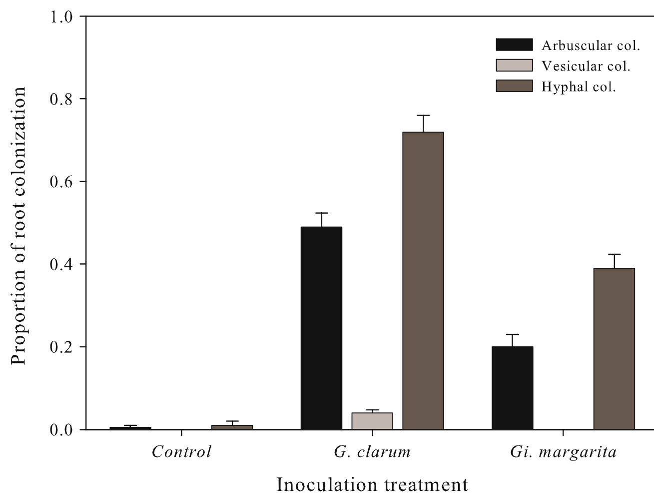

Fig. 1 Proportion of root length colonized by mycorrhizal fungal arbuscules, vesicles and hyphae of maize plants grown in inoculated (*Glomus clarum* or *Gigaspora margarita*) or pasteurized soil (control) after 6 weeks. *Error bars* are standard error of the mean

inoculated with spores of *Gi. margarita*, and only trace amounts of hyphae and arbuscules were detected in non-inoculated control plants. Therefore, the appropriate treatments necessary to test the hypotheses were established, and the second phase of the study was initiated.

Tripartite symbiosis study phase

Soil mineral N and plant growth

The soil concentrations of ammonium and nitrate after pasteurization were 50.3±0.01 and 12.4±0.23 mg kg^{-1}, respectively. After 6 weeks of maize, those concentrations were significantly reduced to 36.3±1.32 and 3.5±0.07 mg kg^{-1}, respectively. At both 10 and 40 days after emergence, the amounts of mineral N present in the soil were, at most, equivalent to 25 kg ha^{-1}, considering a bulk density of 1.3 g cm^{-3} and the top 15 cm. Considering all inoculation treatments and both sampling dates, soil disturbance did not affect the mineral N content in the soil (data not shown), which in any case was unlikely to affect nodula-

Table 1 Effect of soil inoculation and disturbance treatment on the proportion of root length colonized by arbuscules, vesicles and hyphae of AMF, and on nodule formation in soybean plants between 10 and 40 days after emergence

Treatment[a]	Days after emergence	AMF colonization parameters			Nodules	
		Arbuscular colonization	Vesicular colonization	Hyphal colonization	Number nodules $plant^{-1}$	Dry weight mg $plant^{-1}$
Control	10					
+		n.d.	n.d.	n.d.	n.d.	N.A.
−		n.d.	n.d.	n.d.	n.d.	N.A.
B						
+		n.d.	n.d.	n.d.	14.1 (1.05)bc	N.A.
−		n.d.	n.d.	n.d.	16.3 (0.68)bc	N.A.
G + B						
+		0.142 (0.0384)b	0.004 (0.0039)a	0.21 (0.059)b	16.5 (1.77)bc	N.A.
−		0.424 (0.1022)a	0.007 (0.0002)a	0.52 (0.116)a	23.0 (2.60)a	N.A.
Gi + B						
+		0.002 (0.0020)c	n.d.	0.01 (0.002)c	13.9 (0.87)c	N.A.
−		0.129 (0.0239)b	n.d.	0.20 (0.044)b	19.8 (1.33)ab	N.A.
Control	40					
+		n.d.	n.d.	n.d.	0.7 (0.08)b	6.0 (0.50)b
−		n.d.	n.d.	n.d.	3.7 (2.32)b	9.0 (1.76)b
B						
+		0.15 (0.083)c	0.01 (0.006)bc	0.22 (0.112)cd	56.2 (6.19)a	124.3 (1.85)a
−		0.00 (0.000)c	0.00 (0.000)c	0.02 (0.018)d	52.7 (7.05)a	94.1 (12.22)a
G + B						
+		0.38 (0.083)ab	0.05 (0.023)a	0.55 (0.095)ab	54.7 (3.04)a	109.6 (4.96)a
−		0.46 (0.063)a	0.04 (0.014)ab	0.66 (0.088)a	45.7 (4.95)a	117.3 (10.40)a
Gi + B						
+		0.02 (0.008)c	n.d.	0.03 (0.009)d	55.7 (2.58)a	104.8 (5.49)a
−		0.29 (0.053)b	n.d.	0.41 (0.066)bc	49.8 (4.42)a	111.9 (15.07)a

Values in parentheses represent standard error of the mean
For each sampling date, means in a column followed by the same letter are not significantly different ($P<0.05$)
N.A. Not analysed, *n.d.* not detected
[a]Non-inoculated, Control; *B. japonicum* only, B; *Gi. margarita* and *B. japonicum*, (Gi + B); *G. clarum* and *B. japonicum*, G+B.
The + and − symbols correspond to disturbed and undisturbed soil, respectively

Table 2 Effect of soil inoculation and disturbance treatments on the N concentration and N_2 fixation of soybean plants at flowering

Treatment[a]	N concentration (g kg^{-1})	Ndfa (%)	Ndfs (%)	Ndff (%)
Control				
+	13.9 (0.47)a	Trace	72.5 (0.88)a	28.9 (0.30)b
−	10.9 (0.37)a	Trace	68.2 (0.50)a	32.0 (0.20)a
B				
+	28.7 (0.73)a	66.7 (1.58)a	21.5 (1.18)a	11.8 (0.40)a
−	26.0 (0.97)a	69.1 (1.58)a	18.6 (1.13)a	12.3 (0.45)a
G + B				
+	24.2 (0.80)b	66.4 (1.20)b	21.7 (0.90)a	11.9 (0.30)a
−	30.5 (0.80)a	73.5 (1.76)a	15.5 (1.26)b	11.0 (0.50)a
Gi + B				
+	21.3 (0.75)b	62.4 (1.13)b	21.7 (0.84)a	12.9 (0.28)a
−	28.0 (0.92)a	73.6 (1.34)a	15.4 (0.96)b	10.9 (0.38)b

Values in parentheses represent standard error of the mean
For each inoculation treatment, means in a column followed by the same letter are not significantly different ($P<0.05$)
Ndfa Nitrogen derived from the atmosphere, *Ndfs* nitrogen derived from soil, *Ndff* nitrogen derived from fertilizer
[a]Non-inoculated, Control; *B. japonicum* only, B; *Gi. margarita* and *B. japonicum*, Gi + B; *G. clarum* and *B. japonicum*, G+B. The + and − symbols correspond to disturbed and undisturbed soil, respectively

tion. Goss et al. (2002) added 63 kg N ha^{-1} to soil under field conditions before biological N_2 fixation was affected.

Soil disturbance had no effect on plant growth in either microbial inoculation treatment at 10 or 40 days after emergence (data not shown). This result contrasted with those reported by Goss and de Varennes (2002) for the later stage of plant growth, in which plants from undisturbed soil were heavier than those in disturbed soil when indigenous AMF were used. Given this inconsistency, plant growth may not always be a reliable parameter to evaluate tripartite symbiosis dependency. Van der Heijden et al. (1998) found that the mycorrhizal dependency of a plant species could vary greatly because of differential growth responses to specific AMF compared to the growth of the uninoculated plants. They concluded that mycorrhizal dependency, as a measure indicating how much a plant depends on AMF for its growth, is not necessarily a fixed value and therefore cannot be used as a definitive term. At flowering, the control plants weighed significantly less than the plants under any inoculation treatment. This was probably related to the smaller N concentration and content in those plants, for which the absence of N derived from the atmosphere certainly contributed.

Root colonizatidon by AMF and *B. japonicum*

In plants dually inoculated with AMF and *B. japonicum*, soil disturbance significantly affected the development of root colonization by hyphae and arbuscules for both AMF at the early stage (Table 1). Nonetheless, each AMF responded differently to disturbance. While arbuscular and hyphal colonizations by *G. clarum* were, respectively, 66.5 and 60% less in disturbed soil than in undisturbed soil, *Gi. margarita* was considerably more severely affected by soil disturbance. Root colonization by *Gi. margarita* barely developed in disturbed soil, independent of the sampling date. This was likely due to a combination of two factors. First, it is known that hyphae belonging to the Gigasporaceae become ineffective after being disrupted (Klironomos and Hart 2002), and second, 6 weeks of maize and 40 days of soybean growth were probably not sufficient for the *Gi. margarita* to sporulate. In addition, root pieces colonized by AMF that do not form intraradical vesicles, such as *Gi. margarita*, are not infective (Biermann and Linderman 1983). Perhaps the intensity and frequency of tillage practices in real conditions may explain why AMF belonging to the Glomaceae, whose hyphal fragments can serve as propagules, are predominant in agricultural fields. Moreover, for the undisturbed soil treatment, the arbuscular

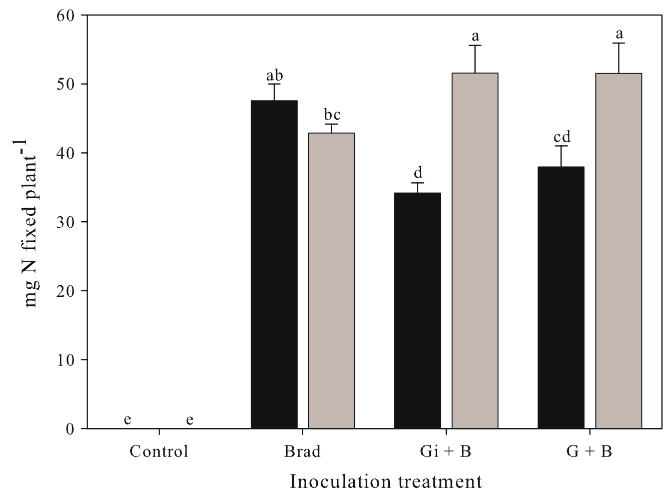

Fig. 2 Effect of microbial inoculation and soil disturbance (*black bars* disturbed and *grey bars* undisturbed) on N_2 fixation by *Bradyrhizobium japonicum* in soybean plants at flowering. Non-inoculated (Control), *B. japonicum* only (B), *Gi. margarita* and *B. japonicum* (Gi + B), *G. clarum* and *B. japonicum* (G + B). *Bars with a different letter* are significantly different as estimated by the least significant difference (LSD) test ($P<0.05$). *Error bars* are standard error of the mean

and hyphal colonizations of plants colonized by *G. clarum* were, respectively, 3.3 and 2.6 times greater than that of plants colonized by *Gi. margarita* at 10 days after emergence, confirming that the development of *Gi. margarita* within the root was much slower. At flowering, colonization by *G. clarum* in disturbed soil equalled that in undisturbed soil for all parameters but was still 1.6 times more extensive than that of *Gi. margarita* from undisturbed soil.

The number of nodules at 10 days after emergence was approximately 40% greater in plants from undisturbed soil than from disturbed soil, but only when one AMF was present, clearly demonstrating the consequences of mycorrhizal colonization on nodulation (Table 1). It is important to emphasise that while the colonization potential of each AMF varied between disturbance treatments, the inoculum potential for *B. japonicum* was constant. Nevertheless, the nodulation was greater in undisturbed soil than in disturbed soil, supporting the hypothesis that this outcome was due to an interaction with the AMF. Plants colonized by *G. clarum* developed more nodules than plants solely colonized by *B. japonicum*. In contrast, nodulation of plants colonized by *Gi. margarita* and those only colonized by *B. japonicum* did not differ. This supports the first hypothesis in that the tripartite symbiosis progressed more rapidly when *G. clarum* was present.

In contrast to the observations at 10 days after emergence, the number and weight of nodules at flowering was not significantly affected by soil disturbance or inoculation treatments. This result paralleled the amounts of AMF colonization by *G. clarum* but not by *Gi. margarita*. It appears that even though the nodulation was faster in undisturbed soil, the maximum in terms of nodulation capacity was attained for each of those treatments prior to 40 days after emergence.

N_2 fixation at flowering

Even though the number and weight of nodules between soil disturbance treatments was similar before 40 days after emergence, the amount of N acquired from biological fixation was significantly smaller for disturbed than for undisturbed soil (Table 2 and Fig. 2). However, this effect was only associated with treatments where one AMF was present. Plants from both mycorrhizal treatments in undisturbed soil fixed approximately 35% more N_2 than did plants from disturbed soil. Moreover, the presence of an intact mycorrhizal mycelium in undisturbed soil stimulated N_2 fixation by approximately 17% relatively to plants solely inoculated with *B. japonicum*. Perhaps these amounts could have been larger if no AMF colonization had occurred in plants solely inoculated with *B. japonicum*.

In contrast to the second hypothesis, N_2 fixation was the same in plants inoculated with *Gi. margarita* and in those inoculated with *G. clarum*. This suggests that there might be compensatory mechanisms within the tripartite symbiosis for a slowly colonizing AMF to have a similar role as a fast colonizer, and these need to be investigated in future research. Variability among the AMF has been reported for (1) mechanisms of phosphate metabolism (Boddington and Dodd 1999), (2) spatial variability in nutrient acquisition and transfer (Jakobsen et al. 1992; Smith et al. 2000, 2004), (3) chemical specificity between host and AMF (van Nuffelen and Schenck 1984; Ianson and Linderman 1993; Saxena et al. 1997; Xavier and Germida 2003) and (4) the microbial composition of the rhizosphere. Meyer and Linderman (1986) demonstrated that AMF colonization selectively favoured pseudomonad growth which often increases nodulation.

Plants from disturbed soil colonized by the AMF fixed less N_2 than did plants from the *B. japonicum* alone treatment. In the study by Ianson and Linderman (1993), some of the AMF that stimulated nodule growth earlier on had a negative effect on their function at flowering. Root colonization by those AMF tended to decrease at flowering, which suggests that they were competing with the *Bradyrhizobium* for carbon. Our results suggest that the demand for carbon was greater in disturbed soil than in undisturbed soil irrespective of the AMF present.

In conclusion, even though AMF life-history strategy and colonization pattern were important for the early progress of the tripartite symbiosis, that did not appear to be relevant for its outcome in terms of total amount N_2 fixation. This indicates that mycorrhizal root colonization cannot be used as a means of predicting the role of individual AMF in the tripartite symbiosis.

Acknowledgements The research was carried out under the Land Stewardship Chair Program at the University of Guelph. P.M.A. thanks the Foundation for Science and Technology of Portugal for the financial support. We are grateful to Julia Zilka and Miranda Labbe for technical support, and to Dr. Miranda M. Hart for her suggestions regarding the content and organization.

References

Ames RN, Bethlenfalvay GJ (1987) Localized increase in nodule activity but no competitive interaction of cowpea rhizobia due to pre-establishment of vesicular-arbuscular mycorrhiza. New Phytol 106:207–215

Ames RN, Mihara KL, Bethlenfalvay GJ (1987) The establishment of microorganisms in vesicular-arbuscular mycorrhizal and control treatments. Biol Fertil Soils 3:217–223

Antunes PM, Rajcan I, Goss MJ (in press) Specific flavonoids as interconnecting signals in the tripartite symbiosis formed by arbuscular mycorrhizal fungi, *Bradyrhizobium japonicum* (Kirchner) Jordan and soybean (*Glycine max* (L.) Merr.). Soil Biol Biochem

Bayne HG, Bethlenfalvay GJ (1987) The Glycine–Glomus–Rhizobium symbiosis. IV. Interaction between the mycorrhizal and nitrogen-fixing endophytes. Plant Cell Environ 10:607–612

Biermann B, Linderman RG (1983) Use of vesicular-arbuscular mycorrhizal roots, intraradical vesicles and extraradical vesicles as inoculum. New Phytol 95:97–105

Boddington C, Dodd J (1999) Evidence that differences in phosphate metabolism in mycorrhizas formed by species of *Glomus* and *Gigaspora* might be related to their life cycle strategies. New Phytol 142:531–538

Brundrett MC, Juniper S (1993) Non-destructive assessment of spore germination and single spore culture isolation of VAM fungi. Poster presented at the 9th NACOM, Guelph

Brundrett MC, Piché Y, Peterson RL (1984) A new method for observing the morphology of vesicular-arbuscular mycorrhizae. Can J Bot 62:2128–2134

Conover WJ, Johnson ME, Johnson MM (1981) A comparative study of tests of homogeneity of variances, with applications to the outer continental shelf bidding data. Technometrics 23:351–361

Fried M, Middelboe V (1977) Measurement of amount of nitrogen fixed by a legume crop. Plant Soil 47:713–715

Ganry F, Diem HG, Dommergues YR (1982) Effect of inoculation with *Glomus mosseae* on nitrogen fixation by field grown soybeans. Plant Soil 47:713–715

George E, Marschner H, Jakobsen I (1995) Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. Crit Rev Biotechnol 15:257–270

Goss MJ, de Varennes A (2002) Soil disturbance reduces the efficacy of mycorrhizal associations for early soybean growth and N_2 fixation. Soil Biol Biochem 34:1167–1173

Goss MJ, de Varennes A, Smith PS, Ferguson JA (2002) N_2 fixation by soybeans grown with different levels of mineral nitrogen, and the fertilizer replacement value for a following crop. Can J Soil Sci 82:139–145

Hart MM, Reader RJ (2002) Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol 153:335–344

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? Nature 394:431–431

Ianson DC, Linderman RG (1993) Variation on the response of nodulating pigeonpea (*Cajanus cajan*) to different isolates of mycorrhizal fungi. Symbiosis 15:105–119

Ibijbijen J, Urquiaga S, Ismaili M, Alves BJR, Boddey RM (1996) Effect of arbuscular mycorrhizal fungi on growth, mineral nutrition and nitrogen fixation of three varieties of common beans (*Phaseolus vulgaris*). New Phytol 134:353–360

Jakobsen I, Abbot LK, Robson AD (1992) External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 2. Hyphal transport of ^{32}P over defined distances. New Phytol 120:371–380

Kawai Y, Yamamoto Y (1986) Increase in the formation and nitrogen fixation of soybean nodules by vesicular-arbuscular mycorrhiza. Plant Cell Physiol 27:399–405

Keeney DR, Nelson DW (1982) Nitrogen-inorganic forms. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil analysis, 2nd edn. Soil Science Society of America, Madison, pp 643–698

Klironomos JN, Hart MM (2002) Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. Mycorrhiza 12:181–184

Knudsen D, Peterson GA, Pratt PF (1982) Lithium, sodium and potassium. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil analysis, 2nd edn. Soil Science Society of America, Madison, pp 225–246

Kruskal WH, Wallis WA (1952) Use of ranks in one-criterion variance analysis. J Am Stat Assoc 47:583–621

McGonigle TP, Miller MH (1996) Mycorrhizae, phosphorus absorption, and yield in response to tillage. Soil Sci Soc Am J 60:1856–1861

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol 115:495–501

Meyer JR, Linderman RG (1986) Response of subterranean clover to dual inoculation with vesicular-arbuscular mycorrhizal fungi and a plant growth-promoting bacterium, *Pseudomonas putida*. Soil Biol Biochem 18:191–196

Miller MH, McGonigle TP, Addy HD (1995) Functional ecology of vesicular arbuscular mycorrhizas as influenced by phosphate fertilization and tillage in an agricultural ecosystem. Crit Rev Biotechnol 15:241–255

Morton JP (1993) Properties of infective propagules at the sub-order level (Glominae versus Gigasporineae). INVAM Newsletter 3

Morton JB, Benny GL (1990) Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. Mycotaxon 37:471–491

Mulvaney RL (1993) Mass spectrometry. In: Knowles R, Blackburn TH (eds) Nitrogen isotope techniques. Academic, San Diego, pp 11–57

Oehl F, Sieverding E, Ineichen K, Mäder P, Boller T, Wiemken A (2003) Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of central Europe. Appl Environ Microbiol 69:2816–2824

Olsen SR, Summers LE (1982) Phosphorus. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil analysis, 2nd edn. Soil Science Society of America, Madison, pp 403–430

Saxena AK, Rathi SK, Tilak KVBR (1997) Differential effect of various endomycorrhizal fungi on nodulating ability of green gram by *Bradyrhizobium* sp. (*Vigna*) strain S24. Biol Fertil Soils 24:175–178

Scheublin TR, Karyn PR, Young JPW, van der Heijden MGA (2004) Nonlegumes, legumes, and root nodules harbour different arbuscular mycorrhizal fungal communities. Appl Environ Microbiol 70:6240–6246

Shapiro SS, Wilk MB, Chen HJ (1968) A comparative study of various tests of normality. J Am Stat Assoc 63:1343–1372

Smith FA, Jakobsen I, Smith SE (2000) Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula*. New Phytol 147:357–366

Smith SE, Smith FA, Jakobsen I (2004) Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New Phytol 162:511–524

Tommerup IC (1992) Methods for the study of the population biology of vesicular-arbuscular mycorrhizal fungi. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology, vol 24. Techniques for the study of mycorrhiza. Academic, New York, pp 23–51

Tukey JW (1949) Comparing individual means in the analysis of variance. Biometrics 5:99–114

Van der Heijden MGA, Boller T, Wiemken A, Sanders IR (1998) Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79:2082–2091

van Nuffelen M, Schenck NC (1984) Spore germination, and root colonization of six species of vesicular-arbuscular mycorrhizal fungi on soybean. Can J Bot 62:624–628

Vejsadová H, Siblíková D, Gryndler M, Simon T, Miksik I (1993) Influence of inoculation with *Bradyrhizobium japonicum* and *Glomus claroideum* on seed yield of soybean under greenhouse and field conditions. J Plant Nutr 16:619–629

Xavier LJC, Germida J (2003) Selective interactions between arbuscular mycorrhizal fungi and *Rhizobium leguminosarum* bv. *viceae* enhance pea yield and nutrition. Biol Fertil Soils 37:261–267