Modelling/Site-specific Analysis/Biometrics/Technologies

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The Tripartite Symbiosis Formed by Indigenous Arbuscular Mycorrhizal Fungi, *Bradyrhizobium japonicum* and Soya Bean Under Field Conditions

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Abstract

The tripartite symbiosis formed by indigenous arbuscular mycorrhizal fungi (AMF), *Bradyrhizobium japonicum* (Kirchner) Jordan and soya bean (*Glycine max* L. Merr. cv. Evans) was investigated under field conditions to test the hypotheses that: (i) the tripartite symbiosis enhances nodulation and nodule activity; and (ii) its establishment does not rely on improved phosphorus (P) uptake through the fungal partner. Soil tillage was used to produce treatments with contrasting AMF colonization potentials while the amount of *B. japonicum* inoculum was kept constant. Nodulation, AMF colonization and the P and nitrogen (N) nutrition of plants were evaluated at 10 and 51 (full-bloom) days after emergence. N₂ fixation was estimated by the difference method and by the isotopic dilution method. At the early stage of plant growth, AMF hyphal colonization and nodulation were, respectively, 16 % and 33 % greater in plants from untilled than from rototilled soil. The establishment of the tripartite symbiosis was observed under field conditions, and factors other than P nutrition were critical to its formation. However, the tripartite symbiosis did not promote N₂ fixation under the high soil P conditions of this study.

Key words: indigenous arbuscular mycorrhizal fungi — N₂ fixation — P nutrition — plant–microbe interactions — soya bean — tripartite symbiosis

Introduction

Many leguminous plants have the ability to fix atmospheric nitrogen (N₂) through a symbiosis with rhizobia, and can also host arbuscular mycorrhizal fungi (AMF), which in return for carbon provide the plant with mineral nutrients (Smith and Read

(1997)). Each microbial symbiont has been shown to affect the activity of the other and their interaction can be detected on the host plant (Cluett and Boucher 1983, Barea et al. 1992, Vejsadová et al. 1993, Xie et al. 1995). This interaction among the three organisms usually results in a mutualistic tripartite symbiosis (Antunes and Goss 2005).

Generally, under controlled environment conditions, N₂ fixation in mycorrhizal soybean plants is greater than in non-mycorrhizal plants, with more nodules and greater nodule dry weight. Such effect of AMF on the nodulation of soya bean has been detected as early as 10 days after emergence and before any apparent acquisition of phosphorus (P) (Goss and de Varennes 2002, Antunes et al. 2006). These investigations have contributed to change the view that the tripartite symbiosis relies absolutely on the increased supply of P through the fungal partner. The AMF impose technical difficulties due to their obligate biotrophy, and the production of treatments with different potentials of indigenous AMF was achieved through the use of soil disturbance. This was based on the work of Miller et al. (1995), who found that the disruption of the AMF extra-radical hyphal network weakened the efficacy of AMF in corn (*Zea mays* L.). Here we investigate whether the tripartite symbiosis formed by an indigenous community of AMF can be detected under field conditions. Soil tillage was used to produce AMF treatments, thereby highlighting the influence of sustainable agricultural practices in relation to the tripartite symbiosis. In

addition, we further investigate the role of P during the establishment of the tripartite symbiosis, based on the hypothesis that this nutrient does not serve as a regulating agent in this process.

Materials and Methods

Experimental site

The experiment was conducted under field conditions at the Elora Research Station near Guelph, Ontario (43°39'N 80°25'W). The soil was a Conestoga silt loam, a gleyed Melanic Brunisol. The experimental design consisted of a two-factor factorial with tillage (no-till and rototill) and soya bean (*Glycine max* (L.) Merr. cv. Evans, nodulating (Nod) and non-nodulating (Nod⁻) isolines) treatments as factors arranged in a randomized complete block with four blocks. Each plot (experimental unit) was 3 × 10 m with 12 rows set 18 cm apart, and plants were 10 cm apart within a row.

Treatment establishment and sampling

Selected plots were rotary tilled on 17 May 2001, to a depth of 10 cm, one day prior to sowing whereas others were left undisturbed. To assess whether soil N levels could compromise the nodulation process, composite soil samples were collected from five plots for each tillage treatment 5 days after the tillage operations, and analysed for 2.0 M KCl extractable NH₄-N and NO₃-N (Keeney and Nelson 1982). As there was no record of a soybean crop in that site for the last 10 years, the seeds were inoculated. Inoculation consisted of mixing approximately 742 seeds with 1 g of peat-based inoculant of *Bradyrhizobium japonicum* strain 532C (HiStick +; MicroBio, Saskatoon, SK, Canada), a very effective strain for Ontario conditions. Seeds from the Nod⁻ isolate were mixed with the same amount of sterilized (autoclaved at 121 °C for 15 min) inoculant so that the edaphic conditions surrounding the newly formed radicle were kept similar for both treatments. A batch of seeds was analysed for P content (Thomas et al. 1967). In each plot a microplot with an area of 72 × 150 cm was marked and ¹⁵NH₄¹⁵NO₃ was applied (5 % ¹⁵N enrichment at a rate of 10 kg N ha⁻¹) on 23 May 2001. Samples were collected at 10 days after emergence and full-bloom growth stages. On the first sampling date, 16 plants were subsampled from each plot. The total plant biomass (oven-dried at 60 °C for 48 h) was determined for each experimental unit. Eight of these plants were used to quantify AMF colonization (McGonigle et al. 1990) and nodule numbers, and the eight remaining plants were used to determine P and N contents of the shoots and roots (Thomas et al. 1967). Composite soil samples were taken from around the roots; this soil was ground to pass a 2-mm sieve and the NaHCO₃-extractable P (Schoenau and Karamanos 1993) and the acid and alkaline phosphatase activity (Tabatabai and Bremner 1969, Eivazi and Tabatabai 1977) were determined. Eight soya bean plants were collected from each plot at the full-bloom stage; the shoots were oven dried at 60 °C for 48 h,

weighed and then ground for analysis of N and P (Thomas et al. 1967). The AMF colonization of the roots was assessed (McGonigle et al. 1990). A further six plants were collected from each ¹⁵N microplot and the shoots were oven dried (60 °C for 48 h), pulverized in a Brinkman Retsch mill MM300 (Retsch, Haan, Germany), and analysed for the ¹⁵N:¹⁴N ratio by mass spectrometry (Mulvaney (1993)). The N₂ fixation was estimated by the difference method and by the isotopic dilution method. For the difference method, the total N content of Nod⁻ control plants (derived from soil) was subtracted from the total N content of Nod plants (derived from soil and atmosphere). For the isotopic dilution method N fixation was assessed based on the percentage of N derived from atmosphere (%Ndfa), which was calculated according to the equation reported by Fried and Middelboe (1977).

Statistical analysis

Statistics were performed using the 'Proc mixed' procedure of the SAS system for Windows, Release 8.02 TS Level 02MO (SAS Inc., Cary, NC, USA). 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). Unless otherwise specified, when the F-test of the treatment mean square indicated that there were significant variances due to treatment effects, mean values were compared using the Tukey's honest significance difference test (*P* < 0.05) (Tukey 1949).

Results

Plant growth and nutrition

The P content in the shoots of soya bean plants was not affected by tillage for any of the stages of plant growth analysed (Fig. 1). There was no uptake of P by plants in the initial 10 days after emergence. At

Fig. 1: Effect of tillage (rototill, black bullets and no-till, white bullets) on the P concentration and content of soya bean plants (error bars are S.E.M.)

Table 1: Effect of tillage on the soil (NaHCO₃)-extractable P and acid and alkaline phosphatase activity in the rhizosphere of soya beans 10 days after emergence (values in parentheses represent S.E.M.)

Tillage treatment	(NaHCO ₃)-extractable P (mg kg ⁻¹)	Acid phosphatase activity (μmol <i>p</i> -nitrophenol h ⁻¹)	Alkaline phosphatase activity (μmol <i>p</i> -nitrophenol h ⁻¹)
Rototill	43.7 (2.80) a	14.4 (1.20) a	23.2 (0.53) a
No-till	50.1 (3.92) a	14.1 (0.57) a	23.8 (0.93) a

For each column, mean values followed by the same letter are not significantly different ($P < 0.05$).

Table 2: Effect of tillage on AMF colonization and nodule formation (values in parentheses represent S.E.M.)

Days after emergence	Tillage treatment	AMF colonization parameters			Nodules	
		Hyphal colonization (×100)	Arbuscular colonization (×100)	Vesicular colonization (×100)	Number (nodules per plant)	Dry weight (mg per plant)
10	Rototill	51 (3.2) b	25 (2.9) a	0.3 (0.13) a	6.1 (0.61) b*	n.d.
	No-till	62 (3.4) a	31 (3.5) a	0.1 (0.06) a	9.1 (1.37) a*	n.d.
Full-bloom 51	Rototill	80 (2.1) a	36 (2.2) a	2.1 (0.40) a	109.3 (11.68) a	474.8 (59.55) a
	No-till	84 (1.6) a	39 (2.2) a	2.1 (0.44) a	96.2 (6.28) a	477.2 (30.89) a

Statistical comparisons apply to rototill vs. no-till within sampling time – mean values followed by the same letter are not significantly different ($P < 0.05$, * $P < 0.1$).

n.d., not detected.

10 days after emergence, the P content of the shoots was similar to the P content initially present in the seed, and the result is the same when the total plant P (shoot + root) is considered.

The P content of soya bean roots was similar in the different soil tillage treatments (0.52 ± 0.038 mg per plant for rototill and 0.44 ± 0.040 mg per plant for no-till). Furthermore, there was no significant difference in the soil (NaHCO₃)-extractable P and acid and alkaline phosphatase activity between tillage treatments (Table 1).

Root colonization by indigenous AMF and *B. japonicum*

No differences in AMF colonization were found between the Nod and Nod⁻ plants, indicating that both had identical capability of hosting AMF, and that the presence of *B. japonicum* did not affect the establishment of the fungal symbiont (data not shown).

Tilling the soil significantly affected AMF colonization at 10 days after plant emergence (Table 2). Hyphal colonization was significantly greater in plants under no-till than in plants under rotary tillage, suggesting that there were more effective AMF propagules in untilled soil.

In agreement with the observation for AMF colonization, nodulation was found to be greater in

plants under no-till than under rototill at 10 days after emergence, but such a difference disappeared 41 days later for both symbioses. As emerging roots passed through the same amount of inoculum (i.e. *B. japonicum* potential in the soil was considered to be identical for all treatments), the data suggest that nodule development was enhanced by greater amounts of AMF root colonization (i.e. the tripartite symbiosis was established). Importantly, the soil N levels were similar between tillage treatments 5 days after the tillage operations were completed and were unlikely to have compromised nodulation. The soil contained 5.25 ± 0.30 mg NO₃-N kg⁻¹ and 2.1 ± 0.26 mg NH₄-N kg⁻¹ in untilled plots and 5.0 ± 0.31 mg NO₃-N kg⁻¹ and 2.27 ± 0.23 mg NH₄-N kg⁻¹ in rototilled plots.

Biological N₂ fixation

The amount of N₂ fixation at full-bloom did not differ between tillage treatments (Fig. 2), which was consistent with the data on P uptake as well as AMF colonization and nodulation reported above.

Discussion

The colonization potential of AMF was found to vary between tillage treatments, while the inoculum of *B. japonicum* was maintained constant. Nevertheless, nodulation was greater in untilled soil than

Fig. 2: Effect of tillage (black bars, no-till; white bars, rototill) on N_2 fixation at full-bloom (*calculated by the difference method; error bars are S.E.M.); Ndfa, N derived from atmosphere; Ndff, N derived from fertilizer; Ndfs, N derived from soil

in rototilled soil, the difference dependent on AMF colonization, which strongly supports that the early establishment of the tripartite symbiosis enhances nodulation. In contrast, the data for Nod and Nod⁻ soya bean plants indicate that AMF colonization was not affected by the presence of the bacterial symbiont, which was consistent with the research by Bagyaraj et al. (1979) and Kucey and Paul (1982) that showed no significant change in AMF colonization after inoculation with rhizobia. Conversely, Pacovsky et al. (1986) reported *Rhizobium* strain effects on AMF development, and Xie et al. (1995) showed that rhizobial Nod factors stimulated AMF colonization. Such contrasting findings indicate that the idea of a reciprocal benefit between the microsymbionts needs to be further tested. In our study, perhaps the high infectivity of indigenous AMF, which are well adapted to their field environment, in combination with the high degree of mycotrophy of soya bean, overshadowed any mycorrhizal promotion effect induced by *B. japonicum*.

Consistent with previous reports (Goss and de Varennes 2002; Antunes et al. 2006) our results show that the formation of the tripartite symbiosis was established in the first 10 days after emergence, before any differences in plant weight or P shoot and root content were observed. The results for P content are consistent with the findings of Pearson and Jakobsen (1993) relative to contributions of roots and mycorrhizal hyphae to P uptake at an early phase of growth. Moreover, the acid and alkaline phosphatase activity, which has been

reported to increase in untilled soil (e.g. Deng and Tabatabai 1997) as a consequence of increased microbial activity (Doran 1980), was unaffected by the soil tillage treatment at that early stage. The amount of photoassimilates required by the AMF can be considerable; e.g. 4 % of net photoassimilates in a young faba bean (*Vicia faba* L.) (Kucey and Paul 1982). Where necessary, the plant host may increase the rate of photosynthesis (e.g. through increased leaf area) to prevent the diversion of assimilates from causing a reduction in overall growth (Killham 1995). Kadir (1994) observed no significant differences in leaf area between young soya bean plants grown in an undisturbed soil vs. plants grown in a disturbed soil. At 10 days after emergence, the cotyledons still provide the plant with carbon compounds (Ritchie et al. 1994), so it is not likely that the greater nodulation could be due to an increase in photoassimilates in the roots.

Contrary to many experiments conducted under controlled environment conditions, the results reported here showed that the early effect of the tripartite symbiosis on nodulation did not lead to improved N_2 fixation. As the soil P content was very high, the possibility that the efficacy of the AMF was reduced and the benefit from the tripartite symbiosis lost, requires consideration. One could argue that this possibility is not supported for three main reasons: (i) the proportion of AMF root colonization was large; (ii) we have observed improved N_2 fixation in controlled environment experiments for similar levels of P (Antunes et al. 2006); and (iii) the literature on the effectiveness of AMF under different levels of soil P indicates that only when the soil level of bicarbonate-extractable P exceeded 140 mg kg^{-1} the rate of AMF colonization was found to decrease (Amijee et al. 1989). Schubert and Hayman (1986) found that AMF were no longer effective when 100 mg P kg^{-1} or more was added. Furthermore, they observed that AMF colonization was most favoured for soil P levels of 50 mg kg^{-1} . Despite all this, we need to consider that there is little comparability of P status for plants grown in controlled vs. field environment conditions. Given the greater amount of growth limiting factors present in field vs. controlled environment conditions, it is reasonable to expect greater plant P responses under the latter. Furthermore, Schubert and Hayman (1986) showed that larger AMF colonization levels did not consistently translate into greater plant growth compared with the

control. Given the unfeasibility to produce a valid AMF-negative control under field conditions, we do not have any evidence that the AMF present in the field effectively increased growth regardless of tillage treatment.

All in all, we predict that if this study had been conducted under lower soil P conditions the amount of AMF colonization would have been just as high and the AMF would have also overcome the effect of tillage. Nevertheless, the possibility exists that under those conditions the efficacy of the AMF could have been greater during their recovery period after tillage. We conclude that the infectivity of the indigenous AMF in field grown soya bean far outweighed the effect of tillage on the AMF colonization potential at later stages, but perhaps N₂ fixation could have been improved had the soil P level been lower.

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