The tripartite symbiosis between legumes, rhizobia and indigenous mycorrhizal fungi is more efficient in undisturbed soil

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Abstract

We investigated how the rate of colonization by indigenous arbuscular mycorrhizal fungi (AMF) affects the interaction between AMF, *Sinorhizobium meliloti* and *Medicago truncatula* Gaertn. To generate a differential inoculum potential of indigenous AMF, five cycles of wheat, each of 1 month, were grown in sieved or undisturbed soil before *M. truncatula* was sown. The early colonization of *M. truncatula* roots by indigenous AMF was faster in undisturbed soil compared with sieved soil, but by pod-fill the frequency of hyphae, arbuscules and vesicles was similar in both treatments. At this latter stage, *M. truncatula* grown in undisturbed soil had accumulated a greater biomass in aboveground tissues, had a greater P concentration and derived more N from the atmosphere than plants grown in disturbed soil, although soil compaction resulted in plants having a smaller root system than those from disturbed soil. The difference in plant P content could not be explained by modifications in hydrolytic soil enzymes related to the P cycle as the activity of acid phosphatase was greater in sieved than in undisturbed soil, and the activity of alkaline phosphatase was unaffected by the treatment. Thus, the results observed were a consequence of the different rates of AMF colonization caused by soil disturbance. Together with earlier results for soybean, this study confirms that soil disturbance modifies the interaction between indigenous AMF, rhizobia and legumes leading to a reduced efficacy of the bacterial symbiont.

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1. Introduction

Many legumes form symbioses with both rhizobia and arbuscular mycorrhizal fungi (AMF). Dual inoculation with both microorganisms results in a tripartite mutualistic symbiosis and generally increases plant growth to a greater extent than inoculation with only one (for a review see Chalk et al., 2006). There is now evidence that both enhanced acquisition of P by the host and effects on molecular signalling between the three symbionts may explain the synergism of AMF and rhizobia. Flavonoids are thought to be key signal compounds associated with the establishment of the tripartite symbiosis and Antunes et al. (2006a, b) reported that the presence of both symbionts

changed the accumulation of flavonoids in soybean roots. Overall, the root content of daidzein was reduced when *Glomus clarum* was present, whereas genistein and coumestrol only decreased when soybean plants (*Glycine max* (L.) Merr.) were inoculated with both *G. clarum* and *Bradyrhizobium japonicum*.

Goss and de Varennes (2002) studied the interaction of soybean, *B. japonicum* and indigenous AMF. They generated a differential inoculum potential for AMF by growing maize (*Zea mays* L.) in sieved or undisturbed soil before soybeans were sown. The soybean plants grown in undisturbed soil developed faster and accumulated a greater biomass than plants from disturbed soil. Moreover, there was a positive interaction between the two microbial symbionts in undisturbed soil that resulted in more abundant colonization of soybean roots by both microbes. By pod-fill, the frequency of hyphal, arbuscular

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and vesicular colonization was greater in undisturbed than in sieved soil. At this stage, nodule weight and the percentage of N derived from the atmosphere was also greater in undisturbed than disturbed soil.

The effect of the extent of AMF colonization on the efficacy of the tripartite symbiosis has only been shown for one crop (soybean), one soil (a gleyed Melanic Brunisol from the Elora Research Station near Guelph—43°39'N, 80°25'W) and one bacterial symbiont (*B. japonicum*) (Goss and de Varennes, 2002).

The hypothesis we tested was that this is a common phenomenon, and should therefore be observed using a very different combination of soil, plant and microbial symbionts, especially the bacteria.

2. Materials and methods

The experiment took place under greenhouse conditions (minimum temperature: 8 °C; maximum temperature: 25 °C) and comprised two phases. In the first phase, the objective was to promote indigenous mycorrhizal development within the soil and establish a differential potential through contrasting soil disturbance. Eighteen pre-germinated seeds of wheat (*Triticum aestivum* L. cv Anza) were sown into each of eight pots containing 7 kg topsoil of a clay soil (Chromic Luvisol) from Revilheira, in southern Portugal (38°28'N, 7°28'W). The soil, which had been passed through a 4 mm sieve before being packed into the pots to a bulk density of approximately 1.2 cm⁻³, contained 21 mg N kg⁻¹ as mineral N (extracted by 2 M KCl), 8 mg P kg⁻¹ and 58 mg K kg⁻¹ (Riehm, 1958); the pH was 6.2 (1:2.5 in water). The soil received a basal dressing of 13 mg P and 30 mg K kg⁻¹ as calcium dehydrogenophosphate and potassium chloride, respectively.

One month after emergence, the shoots were measured and then excised, dried at 65 °C for 48 h, and weighed. The plant material from each pot was ground and analyzed for N and P contents. Four of the pots were taken and the soil passed through a 4 mm sieve. All root material collected on the sieve was cut into 2 cm long fragments and mixed with the soil. The soil was repacked in the pots to the same bulk density. Eighteen more pre-germinated wheat seeds were then sown (between the root systems left on the soil in the case of undisturbed soil) in all pots and again plants were excised 1 month after emergence. The process was repeated so that in total six cycles of wheat were carried out, five of which took place after the soil treatment was established.

In the second phase, the objective was to evaluate the growth, mycorrhizal development, nitrogen fixation and P content in a non-grain legume. Soil in the four pots was sieved again as described earlier and 19 pre-germinated seeds of an annual medic (*Medicago truncatula* Gaertn) were sown in each pot, four containing sieved soil and in the other four the soil remained undisturbed. Approximately 0.9 g of peat-based inoculum of *Sinorhizobium meliloti* was placed at the bottom of the holes into which the seeds were placed, arranged into three rows. This

ensured that the inoculum potential for *S. meliloti* was the same in the two disturbance treatments. The pots received 40 mg N kg⁻¹ supplied as a solution of ammonium sulphate enriched with 99.8% ¹⁵N so that the source of N in the plants (from the soil or from the atmosphere) could be assessed. *M. truncatula* was harvested at 14 (first row), 29 (second row) and 56 days (third row) after emergence. The height and dry weight of the shoots were determined, and then these were ground for analysis of their N concentration (Dumas combustion) and P concentration (colorimetry after ashing the samples at 500 °C and dissolving in 0.3 M HCl), and for the ratio of ¹⁴N/¹⁵N by mass spectrometry. Roots were examined for nodules, and those visible with the naked eye were enumerated. Roots were washed, weighed and then fixed in formyl acetic alcohol, cleared in KOH, and stained with chlorazol-black E (Brundrett et al., 1984) before being examined for AMF colonization by the intersections method described by McGonigle et al. (1990).

At each sampling date, soil samples were taken from the pots, passed through a 2 mm sieve and analyzed for acid (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) according to Eivazi and Tabatabai (1977).

Data were analyzed for variance by the General Linear Model (GLM) and mean separation was performed using the Newman–Keuls test at $p \leq 0.05$.

3. Results

The soil disturbance treatment had no significant effect on wheat. In fact, plant height, dry matter and N and P contents were not significantly different when overall averages for the five cycles were compared (data not shown).

Although the growth of the *M. truncatula* was not affected by soil disturbance over the first 14 days after emergence, it then accelerated in the undisturbed soil so that at flowering (29 days after emergence) and at pod-fill (56 days after emergence), the plants were taller than those grown in sieved soil (Table 1). By pod-fill, dry matter of plants from undisturbed soil was also significantly greater in undisturbed soil than in sieved soil.

Soil disturbance affected the time-course of AMF colonization of roots from *M. truncatula*. At 14 days after emergence, there was a greater frequency of fungal hyphae and arbuscules (65% and 42%, respectively) in the cortex of roots from undisturbed soil than in those from sieved soil (7% and 4% for hyphae and arbuscules, respectively). However, by pod-fill, there were no longer differences between disturbance treatments in the parameters of AMF colonization (45% for hyphae, 22% for arbuscules and 11% for vesicles).

The concentration of P was always greatest in plants from undisturbed soil (Table 1), although the difference between treatments became smaller as plants aged, being more than 100% at 14 days after emergence but only about 10% at pod-fill.

Table 1

Effect of differential soil disturbance (sieved or undisturbed) on biomass, P and N concentrations and contents and % ^{15}N atom excess in shoots of *Medicago truncatula*, and results from the analysis of variance using the General Linear Model

Days after emergence	Treatment	Shoot height (cm)	Shoot dry weight (g per pot)	Root fresh weight (g per pot)	P (g kg ⁻¹)	P content (mg per pot)	N (g kg ⁻¹)	N content (mg per pot)	^{15}N atom excess (%)
14	+	5.1 a	0.23 a	0.35 a	0.90 b	0.2 b	nd	nd	nd
14	–	5.3 a	0.25 a	0.35 a	1.94 a	0.5 a	nd	nd	nd
29	+	9.6 b	1.10 a	1.1 a	2.01 b	2.2 b	21 a	27 a	nd
29	–	11.1 a	1.28 a	0.9 a	3.04 a	3.9 a	21 a	31 a	nd
56	+	17.0 b	2.33 b	1.9 a	3.41 b	7.9 b	25 a	49 a	1.34 a
56	–	18.3 a	2.58 a	1.4 b	3.78 a	9.7 a	24 a	53 a	0.89 b
Effect of treatment									
Date		264.95***	633.56***	56.65***	49.90***	677.95***	25.53***	235.99***	–
Disturbance		4.91*	8.68**	5.37*	21.17***	42.04***	ns	6.71*	57.09***
Date × disturbance		ns	ns	ns	ns	6.44**	ns	ns	–

For each date, values in a column followed by the same letter are not significantly different as estimated by the Newman–Keuls test at $p < 0.05$.

Treatments: +: sieved soil; –: undisturbed soil; nd: not determined; ns, *, **, ***: F -values non-significant and significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

Fig. 1. Activities of acid (left) and alkaline phosphatases (right) in the soil. The vertical bars represent the 95% confidence intervals.

While the concentration of N was similar in both treatments, the N content was marginally greater in plants from undisturbed soil (significantly different according to the GLM, but not according to the Newman–Keuls test, Table 1).

The number of root nodules was similar for the disturbance treatments at 14 and 29 days after emergence (25 and 60 per plant, respectively). By pod-fill, the nodules from plants grown in undisturbed soil were considerably larger than those from sieved soil. However, because they were also more senescent, it was not possible to evaluate their number and weight, as they became easily detached from the roots when these were removed from the soil. This greater biomass of nodules was consistent with the ^{15}N atom excess in shoots from plants grown in undisturbed soil being considerably smaller than that of plants grown in the sieved soil (Table 1). These confirmed that less N was

derived from the soil in the first case, i.e. that biological N_2 fixation was impaired in the sieved soil.

The activity of acid phosphatase was greater in sieved soil compared with undisturbed soil, but the activity of alkaline phosphatase was not affected by treatment (Fig. 1).

4. Discussion

Most of the previous research on the interaction of AMF and rhizobia concerning growth and acquisition of P and N by legumes took place with and without inoculation with AMF, often in sterilized media to eliminate the influence of indigenous AMF. In general, results from these studies have shown that dual inoculation with AMF and rhizobia increase plant growth and N_2 fixation to a greater extent than inoculation with only one microorganism

(Vejsadova et al., 1992, 1993; George et al., 1995; Ibjibijen et al., 1992; Antunes et al., 2006a). As a consequence of the large number of studies already available, it is commonly accepted that there is a mutualistic tripartite symbiosis between AMF, rhizobia and legumes.

The study of Goss and de Varennes (2002) with soybean suggested that not only the presence but also the extent of AMF colonization would determine the efficacy of the tripartite symbiosis. Their work relied entirely on creating a differential inoculum of indigenous AMF. In part, this approach was encouraged by the need for a greater understanding of the basic biology and diversity of AMF (Abbott et al., 1995) and the recognition of practical limitations to the use of introduced inoculum (Saito and Marumoto, 2002).

Unlike the studies using dual inoculation, the results of Goss and de Varennes (2002) were never confirmed with other genera of plants or microbes. The primary objective of this study was to establish the general applicability of the original study by testing another combination of soil, legume plant, rhizobium and indigenous AMF.

Goss and de Varennes (2002) used a silt loam from Canada—a soil formed under climatic conditions of low temperatures during winter (with a snow cover during several months) and hot wet summers. The soil used in the present experiment was a clay soil from Portugal—formed under Mediterranean climate with mild wet winters (no snow) and hot dry summers. Additionally, in the present experiment the soil was supplied with P whereas in Goss and de Varennes (2002) no fertilizer was added although the soil also had a small content of plant available P. This modification in the experimental design would bring the conditions closer to those expected under field conditions. It might have been the reason for the lack of response between treatments in the first phase, when the differential inoculum potential for AMF was being built. The availability of P in the soil may not have limited wheat growth and the plants would therefore be less dependent on AMF. However, that variation may also arise from differences between the plant species used in the two experiments (maize and wheat) in the response to and stimulation of AMF. Mozafar et al. (2000) found more AMF colonization of maize in less disturbed soil but in wheat the level of disturbance did not affect the colonization. Although controlled environment studies (e.g. Hart and Reader, 2004) and some field experiments (e.g. Oehl et al., 2003) have suggested that some groups of AMF are more tolerant of soil disturbance than others, others have found no significant differences in diversity of AMF between tillage treatments (Franke-Snyder et al., 2001; Jansa et al., 2002).

Goss and de Varennes (2002) used soybean as the experimental plant, a legume native to Eastern Asia grown for its seed which has a very high protein and oil content. Soybean establishes a symbiosis with *B. japonicum* and can derive from 25% to 85% of its N from atmosphere (Jefing et al., 1992; Vasilas et al., 1995), although the average value

in central Ontario is only approximately 40% (Ravuri and Hume, 1992). Annual medics (*Medicago* spp.) are small seeded legumes native to the Mediterranean basin. They are used as pastures suited to grazing in semiarid dryland farming (Crawford et al., 1989). They establish a very effective symbiosis with *S. meliloti* and can derive 60–90% of their N from the atmosphere (Elabbadi et al., 1996; de Varennes et al., 2001).

Our results partially agreed with the study of Goss and de Varennes (2002). In undisturbed soil, *M. truncatula* developed faster, had a greater P content (and possibly a greater N content) and derived more N from atmosphere than did plants grown in sieved soil. However, there were some major differences between the two experiments.

Root biomass of *M. truncatula* was affected by treatment. Roots were smaller in undisturbed soil (a decrease in fresh weight of 26% compared with roots from sieved soil) because the clay soil became very compact. This would compromise the capacity of the plant to extract nutrients from the soil, in particular less mobile nutrients such as P, if it was not compensated by AMF colonization. Furthermore, sieving the soil led to increased acid phosphatase activity, while Goss and de Varennes (2002) found no differences in the activity of this soil enzyme between both treatments.

In the present experiment, the upper layers of the undisturbed soil became very compact, and although the sieved soil was packed to the sample bulk density, the pore distribution was probably very different in the two treatments. The difference in acid phosphatase activity between treatments could reflect different environments, especially the air permeability and oxygen availability. Alkaline phosphatase seems to be derived largely from microbial sources such as *Bacillus subtilis* (Cashel and Freese, 1964), while acid phosphatase is also released by roots (Juma and Tabatabai, 1988). As already stated, soil compaction in undisturbed soil led to a smaller root system that may have released fewer enzyme molecules.

Acid and alkaline phosphatases are important in soil organic P mineralization and plant nutrition. The greater root biomass and activity of acid phosphatase in sieved soil would be expected to lead to a greater P content of plants grown in this soil. As the opposite was observed, the greater P content in plants from undisturbed soil had to derive from the arbuscular mycorrhiza formation. However, by pod-fill the extent of AMF colonization in *M. truncatula*, as determined for hyphae, arbuscules and vesicles, was the same under both disturbance treatments. This contrasts with results for soybean (Goss and de Varennes, 2002), when disturbing the soil impaired the rate and extent of colonization, with hyphal, arbuscular and vesicular frequencies remaining greater in undisturbed soil throughout. From the two investigations we conclude that it was the rate of colonization, and not the extent, that modulated the tripartite interaction between the plant host and the microbial symbionts.

The results obtained may have implications for field-grown legumes. Although soybean yields are commonly as good under no-till as under conventional tillage, AMF hyphal colonization and number of nodules were greater in young plants grown in the field under no-till compared with soil that was rotary tilled (Antunes et al., 2006c), in agreement with the results of Goss and de Varennes (2002) obtained with soybean grown in pots. This means that the need for starter N may be reduced or eliminated in field-grown legumes following the implementation of no-till, as the greater rate of early AMF colonization should stimulate the onset of N_2 fixation.

5. Conclusions

This experiment is the first report on the effect of soil disturbance on the interaction of *Sinorhizobium*, indigenous AMF and legumes. Together with previous work with soybean and *Bradyrhizobium*, this study suggests that the positive interaction of rhizobia, AMF and legumes is modulated by the rate of early AMF colonization, and that soil disturbance impairs this interaction by delaying the colonization of roots by the fungal partner.

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