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# The effect of arbuscular mycorrhiza fungal propagules on the growth of subterranean clover (*Trifolium subterraneum* L.) under Mn toxicity in *ex situ* experiments

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**Abstract**

The role of intact extraradical mycelium (ERM) as the most effective fungal propagule in the formation of the tripartite symbiosis between indigenous arbuscular mycorrhizal fungi (AMF), rhizobia and subterranean clover was investigated under conditions of Mn toxicity. ERM was previously developed in 8 L pots under greenhouse conditions by growing plants, which exhibited various levels of mycotrophicity and were tolerant to the levels of Mn in the soil used in the experiment (*Silene gallica* L, *Lolium rigidum* L, *Ornithopus compressus* L. and *Rumex bucephalophorus* L). Contrasting conditions of the integrity of the ERM at the planting of subterranean clover were created by soil disturbance (ERM fragmented – soil disturbed; ERM intact – soil undisturbed). Where an intact ERM was present at the time of planting, growth of subterranean clover was 2.5 times greater after 21 days and 3.9 times after 42 days relative to other forms of AMF propagule. This enhanced growth was associated with a reduction in the Mn concentration of roots due to a greater AMF colonization at 21 days after planting. The protection granted by an enhanced AMF root colonization allowed a greater root nodule development, leading to more N acquisition and plant growth. The ERM can be developed in the soil by mycotrophic plants tolerant to the stressing agent and kept intact at the seeding of the crop to be protected by adopting appropriate tillage techniques.

**Keywords:** Indigenous arbuscular mycorrhiza, extraradical mycelium, Mn toxicity, bioprotection, subterranean clover, rhizobia

## Introduction

Soil acidity constrains crop production on about 70% of the world's potentially arable land (Haug, 1984), mainly due to the associated effects of aluminium and manganese toxicity on plant growth (Mora *et al*., 2004), as the solubility of both ions increases under low soil pH. Legume crops depending on symbiotic N fixation are very sensitive to Mn toxicity due to a reduction in nodule number and activity (Dobereiner, 1966). For subterranean clover (*Trifolium subterraneum* L.), Evans *et al*. (1987) reported that the negative effect on plant growth of Mn was more evident on $N_2$-fixing plants than on plants supplied with mineral N. Mn concentrations of 45 and 90 mg/L in the solution culture affected symbiotic N fixation by reducing both nodule number and activity. Mn concentration in soil solution varying from 0.5 to 40 mg/kg consistently reduce nodule size (Vose & Jones, 1963), and both nodule number and size were negatively affected by Mn concentration in plant tissues for values varying from around 40 to 1600 mg/kg dry matter (DeHaan *et al*., 2002). Among pasture legumes, subterranean clover is considered relatively tolerant to soil acidity (Hayes, 2008) and the cultivars more tolerant to Mn excess seem to have the ability to avoid Mn in the shoots by retaining it in the roots (Evans *et al*., 1987), although this might aggravate the functioning of the symbiosis.

Arbuscular mycorrhiza (AM) confer several benefits to the host plant (Gupta *et al*., 2000), such as P acquisition (Clark & Zeto, 2000) and bioprotection against toxic metal ions, such as Mn (Hall, 2002; Yano & Takaki, 2005; Nogueira *et al*., 2007), particularly when a well-developed colonization of roots is achieved (Garg & Chandel, 2010). When arbuscular mycorrhiza fungi (AMF) colonization is preferentially initiated from an intact extraradical mycelium

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(ERM), that is the AM mycelium that grows outside the roots of the host plant, the infection develops faster and P acquisition is greater in earlier stages of plant growth (Fairchild & Miller, 1988; Brito *et al*., 2013). Goss & de Varennes (2002) used this strategy to enhance the tripartite symbiosis between AMF, rhizobia and soybean, reporting earlier formation of nodules, greater nodule mass and increased $N_2$ fixation. However, enhancing the formation of nodules did not require greater P acquisition and took place at an early stage of growth when plants were still dependent on seed reserves. Similar results were reported by Antunes *et al*. (2006) and were associated with a smaller concentration of flavonoids in the roots of the plants where AMF colonization was initiated by an intact ERM.

AMF colonization reduces Mn uptake by the plants (Nogueira *et al*., 2004, 2007), with differences in Mn concentration being more evident in the roots than in the shoots (Arines *et al*., 1989). Under field conditions, the toxic ions are continuously present, so bioprotection of susceptible crop plants is required immediately after germination. This can be achieved in wheat if colonization is initiated by an intact ERM of AMF, which provides greater protection against toxic levels of Mn than other AMF propagules (Brito *et al*., 2014).

All the available evidence suggests that AMF provide at least some protection to the tripartite interaction between legumes, rhizobia and AMF (Bethlenfalvay & Franson, 1989). When the leguminous host is subterranean clover, the effect of Mn could be mediated either through effects on the host plant or on its bacterial symbiotic partner.

We hypothesized that bioprotection of subterranean clover mediated by AMF would be more effective if root colonization was primarily initiated from an intact ERM rather than other types of propagules. Furthermore, the protection would be through a reduction in the amount of Mn entering the plant. To test these hypotheses, we chose to work on a soil containing a level of Mn already found toxic for wheat growth and where persistence of subterranean clover pastures sown in field conditions is very poor.

## Materials and methods

### *Soil, plant establishment and growth conditions*

The soil used in this study was collected from the 0 to 20 cm topsoil in a long-term natural pasture at Mitra Farm of the University of Evora, Alentejo, Portugal (38° 32′N; 08° 00′W). The soil was a sandy loam Eutric Cambisol (FAO) derived from granite and poor in Mg, in which Goss & Carvalho (1992) reported a significant reduction of wheat growth due to Mn toxicity, resulting from a reduction in soil pH promoted by root exudates and a low level of Mg, and despite the fact that the Mn concentration in this type of soil would not indicate a major problem. Basic fertility assessment showed that the air-dried and sieved (4 mm) soil contained 1.5 mg P/kg (Olsen), 28.2 mg K/kg, 0.4 mg N-$NO_3$/kg, 22.6 mg Mn/kg (DTPA), 0.1 mg B/kg, 0.4 mg Zn/kg (DTPA), 11 mg OM/g and had a pH (in water) of 6.0. The indigenous AMF population present consisted of 180 (most probable number – MPN) viable propagules per gram of dry soil, consistent with AM formation not being limited by available propagules (Al-Karaki & Clark, 1999).

The experiment consisted of two phases. In Phase 1, four plant species were grown to develop contrasting levels of ERM in the soil. The species selected are widespread throughout temperate regions and were chosen according to their different levels of mycotrophy, ranging from highly mycotrophic (*Lolium rigidum* L., and *Ornithopus compressus* L.) to very weakly (*Rumex bucephalophorus* L.) or nonmycotrophic (*Silene gallica* L.). In addition, they are components of the region's natural vegetation in the soil used for this experiment. Hereafter, these plants are referred as Developers. In Phase 2, different ERM integrity conditions were induced using soil disturbance as a tool (ERM disrupted – soil disturbed, ERM intact – soil undisturbed) before the planting of *Trifolium subterraneum* L. cv Nungarin. It must be stressed that no AMF inoculation was performed during the entire experiment, and therefore, only the native AMF population was present.

The complete experiment (phase 1 and 2) was repeated twice to confirm the results and was carried out in a greenhouse, where only the maximum temperature, which was set at 30 °C, could be regulated. Minimum and maximum air temperatures were recorded on a daily basis. Thirty-two pots were used to accommodate all the treatments (4 Developers × 2 Soil Disturbance × 4 Replicates), and an extra set of four pots per Developer was used to evaluate the parameters at the end of Phase 1.

*Experiment phase 1*. Two-day-old seedlings of Developers (3 per pot) were planted in 8 L pots and grew for 42 days to allow a good establishment of the plants and the development of an ERM on the mycotrophic hosts. Weeds that emerged subsequently were manually removed daily. Pots were watered approximately to maximum water-holding capacity (0.17 g/g) by weight according to plant needs.

At the end of Phase 1, Developers were killed by herbicide (6 mL per pot of a solution containing 1.3 g/L of glyphosate as Roundup® Supra™). The option for herbicide to control the Developers was to mimic possible field conditions where an intact ERM can be achieved by no or minimum tillage, in which weeds are controlled by a preseeding herbicide application. All the Developers were sprayed with herbicide irrespectively of the soil disturbance treatment in order to avoid glyphosate to be a factor in the experiment.

*Experiment phase 2*. After 7 days, on half of the pots the shoots of the ERM Developers were excised and the soil was

removed from each pot as two layers of approximately 0.2 m depth and passed separately through a 4-mm sieve. All root material separated on the sieve was cut into 2-cm-long segments and mixed into the soil of the respective layer. Soil was repacked into the pots maintaining the relative position of the two layers. Shoot material was placed on the soil surface. This mechanical disruption of the soil constituted the disturbed treatment and was designed to ensure the fragmentation of the ERM after the growth of the mycotrophic Developers. In the remaining pots, the shoots were excised and left on the soil surface, but the soil was left undisturbed (undisturbed treatment). In this treatment, the ERM of the mycotrophic Developers is assumed to remain intact.

Immediately before planting *T. subterraneum* L. cv Nungarin, 2.5 mg Zn/kg and 1.2 mg B/kg were applied to the soil in each pot together with 12 mL per pot of an inoculum of an effective strain of *Rhizobium leguminosarum* bv. Trifolii as a dense suspension (ca. $10^9$ ufc/mL). Twelve subterranean clover seedlings (2-day old) were planted (14 days after terminating the Developers) and thinned to six plants after 10 days. After 21 days growth, 3 clover plants were harvest by gently pulling on the shoots to get a root sample for the assessment of mycorrhizal colonization and nodule formation. The remaining three plants were allowed to grow for a further 21 days. Live Developers were never present during the clover growth phase of these experiments, as they were completely susceptible to the herbicide. Pots were again watered to 0.17 g/g by weight. The purpose of watering the pots to weight was to eliminate the possibility of temporary waterlogging and hence further enhancing $Mn^{2+}$ ions in the soil solution.

### *Treatment evaluation*

For the Developers, the following parameters were measured in the extra set of 4 pots per species after 42 days growth, the end of Phase 1: biomass, P and Mn shoot concentrations. The roots were stained with trypan blue and AMF colonization assessed by the frequency of arbuscules (AC) according to the magnified intersections method (McGonigle *et al*., 1990). At the same time, the $NO_3$-N in the soil and Mn in the soil solution were determined. Soil solution was extracted from the pots by centrifugation according to the technique described in Goss *et al*. (1992) and the concentrations of Mn determined by Atomic Absorption Spectrometry. $NO_3$-N in the soil was determined using a nitrate ion-selective electrode (Dahnke, 1971).

After 21 days growth of subterranean clover, AC, shoot dry weight and nodule number were measured. Forty-two days after transplanting, in addition to the assessments made at 21 days, P and N concentrations in the shoots, and Mn concentrations in shoots and roots and nodule dry weight were also determined.

A composite plant sample of the four replicates of each treatment was ground and analysed for P and Mn content using an inductively coupled plasma-optical emission spectrometer. N concentration was measured by infrared spectroscopy.

To obtain a nitrogen balance, in the first experiment, the $NO_3$-N in the soil after the final harvest of the clover was measured as described above. The balance was based on the difference between the $NO_3$-N present in the soil at the end of phase 2 (after subterranean clover growth) and phase 1 (after Developer growth) with the addition of the N acquisition by shoots of the subterranean clover. Assuming that no gaseous nitrogen losses occurred during phase 2 of the experiment and N mineralization and nitrification rates were identical, differences in the N balance can be ascribed to biological N fixation. These calculations were restricted to the undisturbed treatments to avoid a possible bias introduced by soil disturbance in mineralization and nitrification rates.

### *Experimental design and statistical analyses*

The treatments were in factorial combination, and the experimental design was a complete randomized block with four replicates. ANOVAs were performed based on the two factors of the study, combined over the two experiments, using a generalized linear model. Developers present in the first phase of the experiments were considered as one factor (with four levels) and the integrity of the ERM (soil disturbance – two levels) as the second factor. Variances were equalized using a $\log_e$ transformation, whenever necessary. Student–Newman–Keuls multiple range test was used to separate the means. The results from the two experiments were consistent with one another and therefore are presented as the average of values from experiments 1 and 2.

## Results

### *Mycorrhizal colonization of roots and Mn and P concentrations in shoots of developers*

Mycorrhizal colonization (AC) was significantly different ($P \leq 0.05$) between Developer species, with *Ornithopus* having the largest value followed by *Lolium*. There were no significant differences ($P > 0.05$) between *Silene* and *Rumex*, consistent with the former being nonmycotrophic plant and the latter having only residual colonization (Table 1). The accumulation of P in the shoots of the Developers was significantly different, and Mn uptake by *Silene* was significantly greater than for other plants (Table 1).

### *Nutrient content in soil after growth of developers*

Neither the concentration of Mn or $NO_3$-N in the soil solution at the end of Phase 1 was significantly affected by the growth of the ERM Developers (Table 1).

**Table 1** Arbuscular mycorrhizal fungi colonization (AC), plant Mn and P content, $NO_3$-N in the soil and Mn in soil solution, at the end of Developers growing period. Results are the average for the two experiments

| | | Shoot content | | Soil or soil solution | |
|---|---|---|---|---|---|
| Developer | AC | Mn ($\mu$g/plant) | P (mg/plant) | $NO_3$-N (mg/kg) | Mn (mg/L) |
| *Silene* | 0.00 c | 482 a | 3.6 | 14.5 | 0.76 |
| *Rumex* | 0.01 c | 243 b | 2.6 | 19.4 | 0.22 |
| *Lolium* | 0.54 b | 173 b | 1.3 | 8.6 | 0.24 |
| *Ornithopus* | 0.73 a | 170 b | 2.3 | 22.2 | 0.83 |

Values in the same column followed by different letters are significantly different from each other ($P \leq 0.05$). Values in columns without letters are not significantly different ($P > 0.05$).

### *Growth and colonization of subterranean clover by AMF and Rhizobium leguminosarum* bv. Trifolii

In Phase 2, AC of subterranean clover roots after 21 days was affected by the preceding Developer and the level of soil disturbance that is the integrity of ERM present in the soil at subterranean clover planting. Colonization was much greater after the mycotrophic Developers, *Lolium* and *Ornithopus*, than after the nonmycotrophic *Silene* and residual *Rumex* in the undisturbed soil. However, in the disturbed soil the colonization after *Lolium* was greater than after other Developers but all values were less than those in undisturbed soil (Table 2). After 42 days, all these differences had disappeared (Table 2).

Nodule dry weight after 42 days in intact ERM treatment (undisturbed soil) was significantly greater after *Lolium* than after *Ornithopus*, which in turn was greater than after *Silene* and *Rumex*. When the ERM was disrupted (disturbed soil), there was no effect of Developer species on nodule dry weight but the values after *Lolium* and *Ornithopus* were significantly smaller than those from the undisturbed soil, that is ERM kept intact (Table 2). The number of nodules per plant was not affected by the treatments at either of the clover sampling occasions indicating that there was no effect of ERM integrity on root colonization by rhizobia (data not shown).

There was significant interaction between the preceding Developer species and the integrity of ERM on shoot growth of subterranean clover after 21 days, with the disruption of ERM having a large negative effect only on the two-mycotrophic Developer species (Table 2). The largest dry matter accumulation occurred after *Ornithopus* in undisturbed soil. The dry weight of shoots was also greater after *Lolium* than after the two nonmycotrophic or residually mycotrophic plants, *Silene* and *Rumex*, in this soil treatment. In the disrupted ERM treatment, there was no effect of preceding Developer species on growth of subterranean clover (Table 2). These same results were also found after 42 days of subterranean clover growth, but the difference between the shoot dry matter after *Ornithopus* and *Lolium* in

**Table 2** Effect of the Developer and the ERM integrity on arbuscular mycorrhizal fungi colonization (AC), shoot dry weight (SDW) at 21 and 42 days, nodule dry weight (NDW), P and N content in the shoots and Mn concentration in the roots at 42 days of subterranean clover growth. Results are the average for the two experiments

| | | | | | | | | Shoot content | |
|---|---|---|---|---|---|---|---|---|---|
| Developer | ERM Integrity | AC 21 days | AC 42 days | SDW 21 days (mg/plant) | SDW 42 days (mg/plant) | NDW 42 days ($\mu$g/nod) | Mn in the Roots (mg/kg) | P (mg/plant) | N (mg/plant) |
| *Sil* | Int. | 0.36 bc | 0.69 | 35 c | 457 c | 24 c | 279.5 a | 1.05 b | 21.8 b |
| | Dis. | 0.15 e | 0.66 | 31 c | 240 c | 42 c | 273.1 a | 0.50 b | 11.8 b |
| *Rum* | Int. | 0.33 bd | 0.64 | 36 c | 415 c | 28 c | 210.0 a | 0.95 b | 20.2 b |
| | Dis. | 0.22 de | 0.64 | 34 c | 279 c | 61 c | 339.3 a | 0.65 b | 13.5 b |
| *Lol* | Int. | 0.62 a | 0.78 | 64 b | 1223 b | 418 a | 126.1 b | 2.45 a | 39.8 a |
| | Dis. | 0.40 b | 0.66 | 38 c | 447 c | 105 c | 223.0 a | 1.02 b | 19.5 b |
| *Orni* | Int. | 0.68 a | 0.72 | 104 a | 1458 a | 160 b | 123.6 b | 2.83 a | 51.7 a |
| | Dis. | 0.24 ce | 0.64 | 32 c | 236 c | 25 c | 356.9 a | 0.55 b | 10.8 b |

Values in the same column followed by different letters are significantly different from each other ($P \leq 0.05$). Values in columns without letters are not significantly different ($P > 0.05$). *Sil – Silene*, *Rum – Rumex*, *Lol – Lolium*, *Orni – Ornithopus*. Int.- Intact, grown in undisturbed soil, Dis. – Disrupted, grown in disturbed soil.

the intact ERM treatment, in comparison with the all the others, increased over time (2.5 times and 3.9 times on shoot growth of subterranean clover after 21 days and 42 days, respectively) (Table 2).

Growth of subterranean clover at 42 days increased significantly ($P < 0.001$) with AMF colonization in the roots at 21 days (Figure 1). There was also a significant linear relationship ($P < 0.05$) between AC at 21 days and the dry weight of root nodules after 42 days (Figure 2a).

### *Nutrient accumulation in subterranean clover after 42 days growth*

The accumulation of P in shoots of subterranean clover was greatest after the growth of *Ornithopus* and *Lolium*, in undisturbed soil (Table 2). The content of P in all other treatments was not statistically different from each other ($P > 0.05$) (Table 2). The concentration of P either in the shoots or in the roots was not affected by the treatments (data not shown).

When the ERM was kept intact, N acquisition by subterranean clover was greatest after the mycotrophic Developer (*Ornithopus*) than after *Silene* or *Rumex*. The value for *Lolium* was significantly greater than those for the nonmycotrophic or poor mycotrophic Developers but was smaller than that for Ornithopus (Table 2). There was no effect of preceding Developer on N acquisition when ERM was disrupted. There was a significant but negative effect of ERM disruption in subterranean clover following the mycotrophic Developers but not after other plants (Table 2). N accumulation in shoots of subterranean clover after 42 days increased linearly with root colonization at 21 days (Figure 2b).

There were no significant differences ($P > 0.05$) in the accumulation of Mn in the shoots of subterranean clover, nor was there any relationship between this parameter and shoot dry matter (data not shown). However, the concentration of Mn in the roots of subterranean clover after 42 days growth following the mycotrophic Developers, *Lolium* and *Ornithopus*, with an Intact ERM was significantly less than in the other treatments, where no ERM was present or it was disrupted (Table 2). The Mn concentration in the roots after 42 days growth was inversely related to AC of roots at 21 days (Figure 2c).

Nodule dry weight for subterranean clover declined exponentially with the concentration of Mn in the roots (Figure 3a), but no significant relationship between nodule

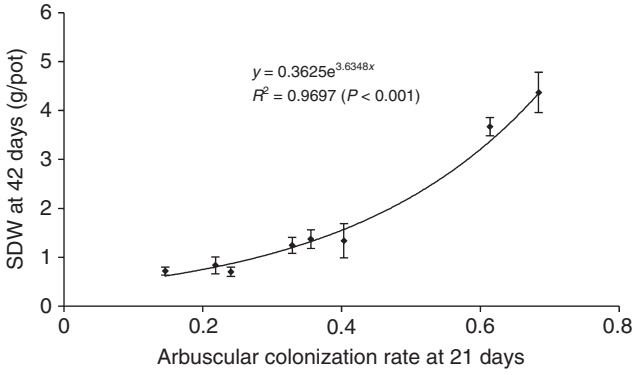


**Figure 1** Relationship between mycorrhizal colonization at 21 days and shoot dry weight (SDW) of subterranean clover at 42 days after planting. Bars indicate the standard error of the mean.

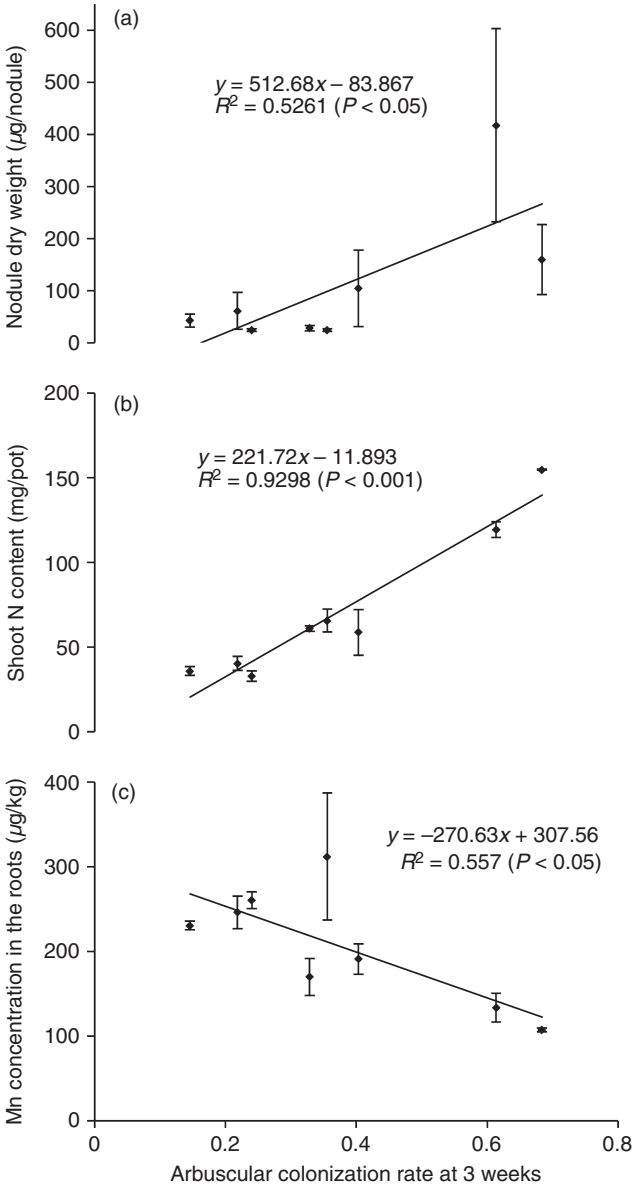


**Figure 2** Relationship between mycorrhizal colonization (at 21 days) and (a) nodule dry weight, (b) N acquisition by the shoots and (c) Mn concentration in the roots of subterranean clover at 42 days of growth. Bars indicate the standard error of the mean.

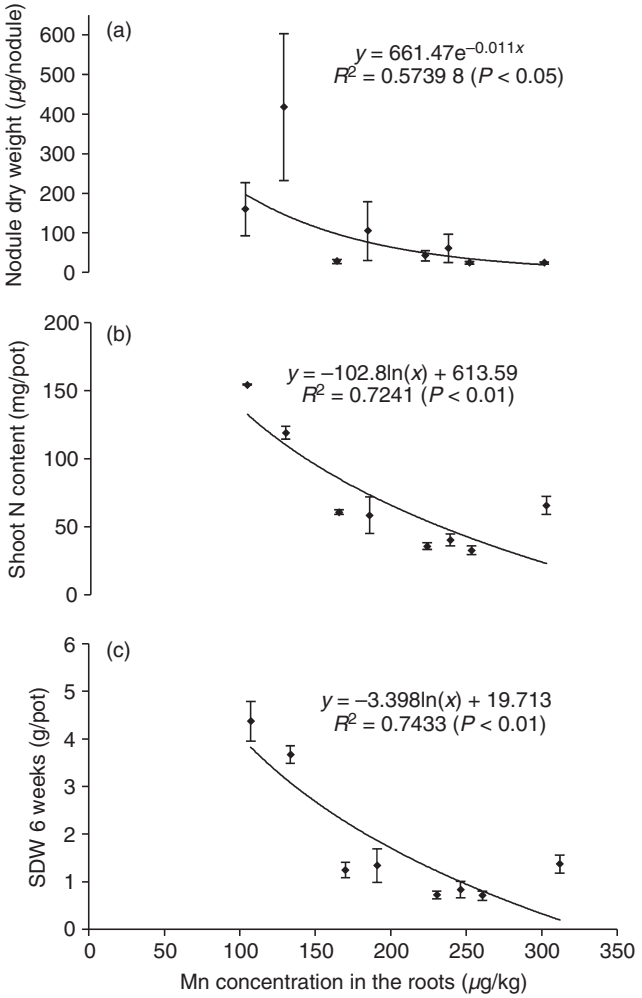


**Figure 3** Relationship between Mn concentration in the roots of subterranean clover and (a) the nodule dry weight, (b) N acquisition by the shoots and (c) the shoot dry weight at 42 days of growth. Bars indicate the standard error of the mean.

dry weight and P concentration either in shoots or roots of subterranean clover was found. N acquisition also declined logarithmically with the concentration of Mn in the roots (Figure 3b) as did shoot dry matter (Figure 3c).

### *N Balance of phase 2 of the experiment*

The N balance was greater after the mycotrophic Developers than after *Rumex* or *Silene*, when an intact ERM was present at the planting of the subterranean clover. Furthermore, the balance was approximately 50% greater following *Ornithopus* than after *Lolium* (Table 3).

## Discussion

The presence of an intact ERM in the undisturbed soil at the planting of the subterranean clover, promoted faster AC of its roots by 21 days after planting, confirming the results of other authors (Fairchild & Miller, 1988 and Goss & de Varennes, 2002; Brito *et al*., 2013, 2014). Although the level of AC in subterranean clover roots for the other treatments caught up during the subsequent period of plant development, the initial differences in AC were crucial for a reduction of Mn concentration, especially in the roots, and the enhancement of growth. This effect of AMF on the reduction of Mn uptake has already been observed (Arines *et al*., 1989). However, in our study, the bioprotection against Mn was only achieved after a mycotrophic Developer when the soil was not disturbed, confirming the importance of AMF colonization starting from an intact ERM in the bioprotection against Mn toxicity (Brito *et al*., 2014). The benefit of the mycotrophic Developers cannot be attributed to a differential Mn soil depletion considering that this effect disappeared with soil disturbance and *Silene* (the nonmycotrophic plant) showed the greatest uptake of Mn. It also cannot be attributed to an increase of Mn availability in the disturbed treatments because there was only a significant benefit of the undisturbed treatments after the mycotrophic Developers. Moreover, given the sieving of soil in the disturbed treatment, the undisturbed treatment was the more likely to be impacted by poor aeration, especially in a sandy soil, and this would tend to increase any difference in Mn availability between these two treatments but likely reduce any benefit to clover from an intact ERM. Although the difference in AMF colonization between disturbance treatments for the subterranean clover disappeared over time, the contrast in plant growth increased between the treatments where AMF colonization was initiated by an intact ERM and all other treatments, indicating that initial bioprotection was decisive for successful growth of the crop.

The reduction of Mn concentration in the subterranean clover roots was associated with an increase of the size of the nodules and a more favourable N balance. The growth of the nodules was related with the plant growth and nitrogen acquisition, indicating that the AMF colonization of the clover initiated by an intact ERM protected the nodules from an excess of Mn, allowing a better growth of the nodules and more $N_2$ fixation. The negative effect of Mn in the growth and activity of the nodules has been reported (Vose & Jones, 1963; DeHaan *et al*., 2002) and that explains why subterranean clover growing on symbiotic nitrogen is more sensitive to this toxicity than growing on mineral N (Evans *et al*., 1987). Moreover, more available soil N would be expected from mineralization, when the soil was disturbed, although the N acquisition by subterranean clover and the N balance in the pots were much greater in the undisturbed soil after *Lolium* and *Ornithopus*.

AMF colorization of subterranean clover initiated by an intact ERM, enhanced P acquisition, which can also explain the better shoot growth of subterranean clover after 21 and 42 days. Goss & de Varennes (2002) investigated the

**Table 3** The effect of the Developer on the N balance in the pots, in the undisturbed soil treatments. Results are only for the first experiment

| | Soil after Developer | Soil after subterranean clover | Subterranean clover shoots | N balance |
|---|---|---|---|---|
| Developer | $NO_3$-N (mg N/pot) | | (mg N/pot) | |
| *Silene* | 7.7 | 26.1 | 47.8 c | 66.3 c |
| *Rumex* | 12.9 | 23.8 | 56.3 c | 67.2 c |
| *Lolium* | 10.6 | 8.3 | 106.8 b | 104.5 b |
| *Ornithopus* | 12.9 | 12.0 | 155.7 a | 154.9 a |

Values in the same column followed by different letters are significantly different from each other ($P \leq 0.05$).

tripartite symbiosis of soyabean in the absence of Mn toxicity and found the same long-term effect on P acquisition, which contributed to better N fixation. However, the increase in nodule number in the initial stage of plant growth found by these authors was not detected in our study. A possible explanation is the negative effect on subterranean clover of the excess Mn in the soil solution on nodule formation (Evans *et al*., 1987) that was present in our experiment. Therefore, the AMF conferred protection to rhizobia only during its symbiotic stage, but not during the initial colonization process.

Our original hypotheses were supported by these results. In the presence of Mn toxicity, the AMF enhanced the tripartite symbiosis with rhizobia and subterranean clover, by reducing the amount of Mn passing into the roots, allowing an increase in nodule size, a greater N acquisition by the plant and a better N balance. This effect seems to be more important than the acquisition of P, as its concentration, both in the shoots and roots, was not affected by the treatments and no significant relationship with the nodules dry weight was found. However, an adequate level of protection was only achieved when AMF colonization was initiated by an intact ERM. Although differences in AMF colonization between treatments disappeared over time, those in plant growth did the opposite, confirming the importance of colonization in early stages of plant growth, for its protection. The amount of colonization of the mycotrophic Developers was not critical to guarantee earlier and faster colonization of the crop to be protected. However, there was a significant difference in the efficacy of the protection from the Developer, both in terms of subterranean clover growth and N balance indicating that other criteria for the selection of Developers should be considered. Brito *et al*. (2014) suggested that may be functional diversity within the ERM developed by different mycotrophic plants.

Soil acidity and associated problems like Mn toxicity can be attenuated by the application of lime, which is not always available. Lime is difficult to apply to depth and can be too costly for pasture production. The results suggest that protection through AMF colonization initiated by an intact ERM from indigenous mycorrhiza species could be achieved under field conditions by adopting proper production techniques. The establishment of the ERM in the soil could be achieved by growing mycotrophic Mn-tolerant plants to act as Developers. If appropriate tillage techniques are adopted, the ERM could be kept intact. Furthermore, this approach would not require inoculation, could have additional advantages, such as the enhancement of nutrients acquisition, and could act in deep layers of the soil, following root development.

## Conclusions

The growth of subterranean clover was impaired by high levels of Mn in its roots. AMF colonization of subterranean clover initiated by an intact ERM reduced the Mn concentration in the roots providing protection of the nodules, leading to a better growth of the nodules, N acquisition, N balance and greater plant dry matter. An adequate degree of protection requires good AMF colonization during the early stages of plant growth, which was only achieved when an intact ERM was the source of AMF propagules. Under field conditions, an ERM might be developed by growing mycotrophic plants tolerant to Mn and kept intact by adopting no-till for the seeding of subterranean clover.

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