Mycorrhiza (1993) 3:25–29

Mycorrhiza

Soil disturbance and infection of *Trifolium repens* roots by vesicular-arbuscular mycorrhizal fungi

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Abstract. Removal and storage of the surface layers of soil is known to decrease the infectivity of vesicular-arbuscular mycorrhizal (VAM) fungi. Previous studies have mostly examined the effects of profound soil disturbance on the infectivity of VAM fungi. This study examined the effects of increasing degrees of topsoil disturbance on the infectivity of VAM fungi in two sites on sandstone soils in southeastern Australia. Intact soil blocks (20×20×15 cm) were taken from each of the two sites. Increasing degrees of topsoil disturbance were achieved by cutting the blocks longitudinally into four (dist. 1), nine (dist. 2), and 25 (dist. 3) equal portions. Seeds of *Trifolium repens* L. were sown into the blocks and harvested 14, 21, 28, 35 and 42 days after sowing. At each sampling date, total root length, root length colonised by VAM fungi and shoot dry mass were measured. VAM colonisation had commenced by 14 days in the roots of seedlings grown in intact, dist. 1, and dist. 2 soil blocks. The initiation of VAM colonisation was delayed by up to 6 weeks for seedlings grown in the dist. 3 soil blocks. The low (i.e. dist. 1) and intermediate (i.e. dist. 2) degrees of soil disturbance did not cause a delay in the initiation of VAM, bud did significantly reduce the proportion of root length colonised by VAM fungi after 21 days. After 21 days, shoot dry mass was significantly less in the seedlings grown in the dist. 3 soil blocks though not in the low and intermediate disturbance treatments. It is concluded that the most severe experimental disturbance probably disturbed the external hyphal network and root fragments (containing hyphae and vesicles), which in turn temporarily reduced the infective potential of the fungus to zero. The observed delay in the initiation of VAM in the most disturbed blocks can, therefore, be explained by the time required for hyphae to grow from other propagules in the soil which survived the disturbance event.

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Key words: Soil disturbance – Vesicular-arbuscular mycorrhizal fungi – Hyphal network – Shoot dry mass

Introduction

Plant roots infected with vesicular-arbuscular mycorrhizal (VAM) fungi carry a loose hyphal network extending into the surrounding soil, providing an extensive surface area for absorption of nutrients and a mechanism by which infection can be spread (Warner and Mosse 1983; Newman 1988). The external hyphal network can extend for several centimetres into the soil surrounding the plant roots they infect (Heap and Newman 1980). Sanders and Tinker (1973) found a total of about 80 cm of external hyphae for every centimetre of onion root infected by VAM fungi. The external hyphal network is considered to be particularly significant as a source of VAM infection in undisturbed soils containing few living spores (e.g. Read et al. 1976, 1985; Jasper et al. 1989a, b). Growing roots are sensitive to VAM colonisation only for a short time, and require rapid colonisation for an effective association (Brundrett 1991). The external hyphal network provides an extensive source of potentially infective propagules for actively growing roots to intercept.

Jasper et al. (1989a) argued that if the network of hyphae in undisturbed soils is an important inoculum source, then observed losses in infectivity after soil disturbance are likely to be because of damage to this network, rather than damage to the relatively robust structures of spores and root fragments colonised by VAM fungi. The external hyphal network could be disturbed in two ways: (1) the hyphae may be separated from their host roots, and/or (2) the hyphae may be physically broken up. VAM fungi are considered to be obligate symbionts in that they are dependent upon their host for an organic carbon supply (Harley and Smith 1983). However, it has been demonstrated that the external hyphae of VAM fungi can remain infective even after being separated from their host (e.g. Hepper and Warner 1983;

Jasper et al. 1989a). This suggests that physical disruption of the hyphae rather than separation from the host root, may be the cause of the loss of infectivity in disturbed soils. In a different study (Bellgard 1993) most of the propagules that initiate VAM were observed in topsoil. Additionally, very few living spores were found in the sandstone soils sampled.

It has been well documented that soil disturbance can reduce the infectivity of VAM fungi (e.g. Jasper et al. 1987, 1988; Evans and Miller 1988; Fairchild and Miller 1988; Jasper et al. 1989a, b, c). In these previous studies, only severe soil disturbance treatments were examined. In the present study, the relationship between the intensity of soil disturbance and the infectivity of VAM fungi (following McGonigle et al. 1990) was examined. Additionally, it has been proposed that if VAM infection is beneficial to plants, and if this benefit is derived from increased nutrient and water uptake, then removal or reduction of VAM infection should result in decreased nutrient uptake, especially in soils with low concentrations of essential plant nutrients (Fitter 1986). The reduction in nutrient uptake could in turn lead to a reduction in plant growth. Consequently, shoot dry mass was measured to assess the impact of any soil disturbance-induced reduction of VAM infection on the growth of the bioassay seedlings.

Materials and methods

Study sites

The study sites used in this experiment were the same as those described previously (Bellgard 1993).

Design of the experiment

At each of the two sites (Avon and O'Hares), five plots were selected at random and five intact soil blocks (20 × 20 × 15 cm) were taken at each plot. These intact blocks were placed in square plastic containers and removed to a glasshouse. For each plot, two of the containers were left undisturbed (No dist.), one was divided longitudinally into four equal portions (dist. 1), another into nine equal portions (dist. 2) and the last into 25 equal portions (dist. 3).

At least 30 seeds of *Trifolium repens* were sown into each container. All containers were tap-watered daily, and received no additional nutrients. To test for potential aerial contamination of containers by VAM fungi in the glasshouse, five containers of river sand sown with 30 seeds of *T. repens* were used as a control. All pots were placed in a naturally lit glasshouse, in which the mean daily temperatures ranged between 20.0 °C and 28.7 °C for the duration of the bioassay. At 14, 21, 28, 35, and 42 days after planting, five randomly chosen seedlings were carefully removed from each container. Although the removal of bioassay seedlings caused some soil disturbance, less than 5% of each soil block was disturbed at each sampling occasion. In addition, each soil block was treated in the same way and the disturbance attributed to the harvesting of the seedlings was not considered to be a confounding variable. The roots of each seedling were fixed, cleared and stained, and the amount of root colonised by VAM fungi assayed using the method described by Bellgard (1993). Shoot material was dried in an at 70 °C for 4 h and weighed.

Statistical analysis

The data were analysed by a "split-plot" analysis of variance (Cochran and Cox 1957) with seedlings nested within blocks, and blocks nested within plots. The analysis assumed a factorial relationship between the degree of disturbance and the elapsed time until harvesting of the seedlings, since each degree of disturbance occurred in conjunction with each harvest date. Comparisons within harvest dates are "within container" comparisons, so they are usually more precise than comparisons between different degrees of disturbance, which are "between container" comparisons (and involve more sources of variability). In addition, comparisons between degree of disturbance/harvest date combinations (e.g. No dist./21 days versus dist. 3/35 days) vary in precision depending on whether they have the same degree of disturbance (and are therefore "within container" comparisons) or have different degrees of disturbance (and are therefore "between container" comparisons). A consequence of this is that the analysis of variance involves more than one residual SS, and pairwise comparisons between two means will have different estimates of error depending on the particular comparison.

Results

Root growth and VAM formation

No VAM infection was found on the roots of any of the bioassay seedlings grown in the control containers to test for aerial contamination by VAM fungi. Root lengths was not affected by soil disturbance. The trends observed in the lengths of roots colonised by VAM fungi (i.e. VAM length) and the proportion of root length colonised by VAM fungi (i.e. % VAM) were identical. Consequently, only the proportion data are presented here. The trend observed in the two No dist. treatments were identical, and so the data were grouped. Consequently, the number of soil blocks for the No dist. treatment now equals 10.

In the undisturbed (No dist.), dist. 1 and dist. 2 treatments, VAM formation had commenced by 14 days (Fig. 1). In the most disturbed of the soil blocks (dist. 3), the onset of VAM formation was delayed by between 28 and 35 days in the Avon soil and between 35 and 42 days in the O'Hares soil (Fig. 1). Furthermore, even when colonisation commenced in the seedlings growing in the most disturbed blocks, the levels of VAM were significantly lower than the No dist. treatment at all stages of the experiment (Fig. 1; Table 1).

The low (i.e. dist. 1) and intermediate (i.e. dist. 2) degrees of soil disturbance had no effect on the proportion of root colonised by VAM fungi up to day 14 (Fig. 1). However, at 21 days, the low and intermediate degrees of soil disturbance significantly reduced the proportions of root length colonised by VAM fungi in both the Avon and O'Hares soils (Fig. 1; Table 1).

Shoot dry mass

The low and intermediate degrees of soil disturbance had no impact upon shoot dry mass. In the Avon soil, dist. 3 had no affect on shoot mass for the first 3 weeks. After 21 days, shoot mass was significantly lower in the

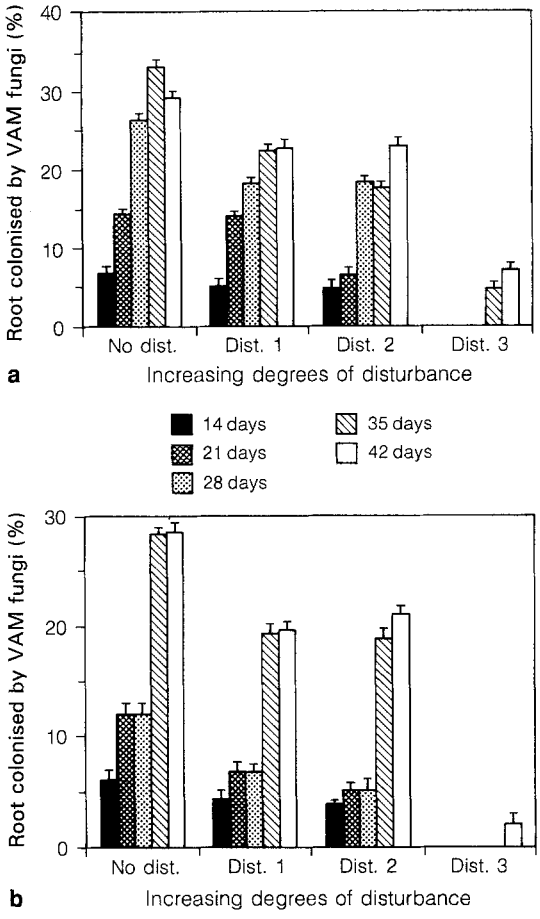

Fig. 1. Proportion of root length colonised by VAM fungi in relation to increasing degrees of soil disturbance using intact soil blocks removed from **a** the Avon and **b** the O'Hares study sites. *Bars* are the means (± sem) of five seedlings from each of five replicates except the No dist. treatment, which is the mean (± sem) of five seedlings from each of 10 replicates

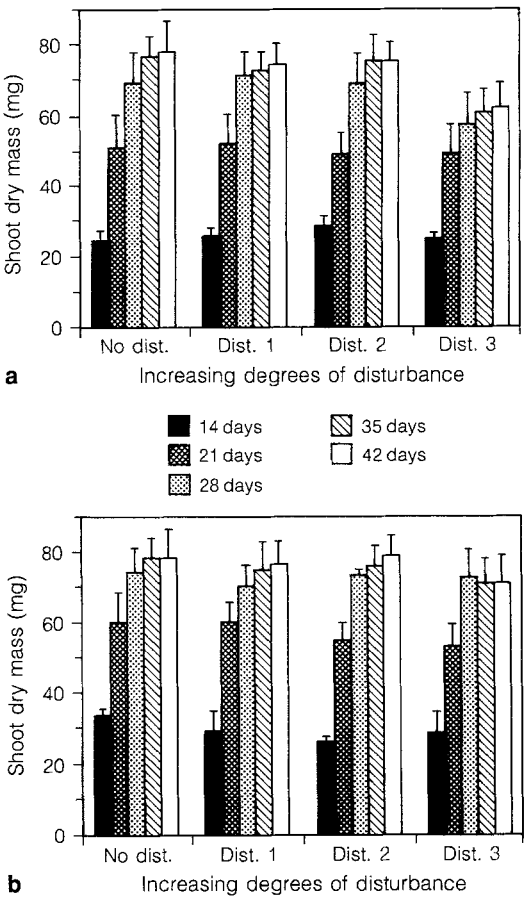

Fig. 2. Shoot dry mass accumulation in relation to increasing degrees of soil disturbance using intact soil blocks removed from **a** the Avon and **b** the O'Hares study sites. *Bars* are the means (± sem) of five seedlings from each of five replicates except the No dist. treatment, which is the mean (± sem) of five seedlings from each of 10 replicates

Table 1. Details of the analysis carried out on % VAM data from the Avon and O'Hares sites using a two-factor "split-plot" ANOVA. Significant "treatment × time" interactions occurred in all cells. *A posteriori* comparisons using the appropriate residual SS were carried out to identify differences between means. Data presented are mean % VAM ($n = 25$ in all cases except for No dist. where $n = 50$)

	14 days	21 days	28 days	35 days	42 days
Avon[a]					
No dist.	6.80^{1}_{a}	14.29^{1}_{b}	26.46^{1}_{c}	32.99^{1}_{d}	29.22^{1}_{e}
Dist. 1	5.24^{1}_{a}	$13.98^{1,3}_{b}$	$18.24^{2,3}_{c}$	$22.65^{2,3}_{d}$	$22.78^{2,3}_{d}$
Dist. 2	4.88^{1}_{a}	$6.55^{2,4}_{a}$	$18.44^{2,3}_{b}$	$17.60^{2,4}_{b}$	$23.15^{2,3}_{c}$
Dist. 3	0.00^{2}_{a}	$0.00^{2,5}_{a}$	$0.00^{2,4}_{a}$	$4.85^{2,5}_{b}$	$7.16^{2,4}_{b}$
O'Hares[b]					
No dist.	5.98^{1}_{a}	12.09^{1}_{b}	27.77^{1}_{c}	28.29^{1}_{c}	28.55^{1}_{c}
Dist. 1	$4.39^{1,3}_{a}$	$6.78^{2,3}_{a}$	$22.33^{2,3}_{b}$	$19.42^{2,3}_{b}$	$19.57^{2,3}_{b}$
Dist. 2	$3.80^{1,3}_{a}$	$5.17^{2,3}_{a}$	$19.49^{2,3}_{b}$	$18.79^{2,3}_{b}$	$21.08^{2,3}_{b}$
Dist. 3	$0.00^{2,4}_{a}$	$0.00^{2,4}_{a}$	$0.00^{2,4}_{a}$	$0.00^{2,4}_{a}$	$2.07^{2,4}_{a}$

[a] Entries in the horizontal row "No dist." followed by the same letter are not significantly different as indicated by analysis of variance (LSD = 2.67, $P = 0.01$. Entries in the other horizontal rows followed by the same letter are not significantly different as indicated by analysis of variance (LSD = 3.78, $P = 0.01$). Entries in vertical columns followed by same number are not significantly different as indicated by analysis of variance ($LSD_{\text{No dist. vs dist. }1,2,3} = 3.93$, $LSD_{\text{Dist. 1 vs dist. 2 vs dist. 3}} = 4.54$, $P = 0.01$)

[b] Entries in the horizontal row "No dist." followed by the same letter are not significantly different as indicated by analysis of variance (LSD = 2.46, $P = 0.01$. Entries in the other horizontal rows followed by the same letter are not significantly different as indicated by analysis of variance (LSD = 3.48, $P = 0.01$). Entries in vertical columns followed by same number are not significantly different as indicated by analysis of variance ($LSD_{\text{No dist. vs dist. }1,2,3} = 3.27$, $LSD_{\text{dist. 1 vs dist. 2 vs dist. 3}} = 3.77$, $P = 0.01$)

Table 2. Details of the analysis carried out on shoot dry mass from the Avon and O'Hares sites using a two-factor "split-plot" ANOVA. Significant "treatment × time" interactions occurred in all cells. *A posteriori* comparisons using the appropriate residual SS were carried out to identify differences between means. Data presented are mean shoot dry mass (mg) ($n = 25$ in all cases except for No dist. where $n = 50$)

	14 days	21 days	28 days	35 days	42 days
Avon[a]					
No dist.	24^{1}_{a}	51^{1}_{b}	69^{1}_{c}	76^{1}_{d}	77^{1}_{d}
Dist. 1	25^{1}_{a}	52^{1}_{b}	$71^{1,3}_{c}$	$72^{1,3}_{c}$	$74^{1,3}_{c}$
Dist. 2	29^{1}_{a}	49^{1}_{b}	$69^{1,3}_{c}$	$75^{1,3}_{d}$	$75^{1,3}_{d}$
Dist. 3	25^{1}_{a}	49^{1}_{b}	$57^{2,4}_{c}$	$61^{2,4}_{d}$	$61^{2,4}_{d}$
O'Hares[b]					
No dist.	35^{1}_{a}	60^{1}_{b}	74^{1}_{c}	78^{1}_{c}	79^{1}_{c}
Dist. 1	29^{1}_{a}	60^{1}_{b}	70^{1}_{c}	75^{1}_{c}	77^{1}_{c}
Dist. 2	26^{1}_{a}	57^{1}_{b}	73^{1}_{c}	76^{1}_{c}	79^{1}_{c}
Dist. 3	28^{1}_{a}	53^{1}_{b}	73^{1}_{c}	70^{2}_{c}	70^{2}_{c}

[a] Entries in the horizontal row (No dist.) followed by the same letter are not significantly different as indicated by analysis of variance (LSD = 7, $P = 0.01$). Entries in the other horizontal rows followed by the same letter are not significantly different as indicated by analysis of variance (LSD = 9, $P = 0.01$). Entries in vertical columns followed by same number are not significantly different as indicated by analysis of variance ($LSD_{\text{No dist. vs dist. 1,2,3}} = 10$, $LSD_{\text{Dist. 1 vs dist. 2 vs dist. 3}} = 10$, $P = 0.01$)

[b] Entries in horizontal row (No dist.) followed by the same letter are not significantly different as indicated by analysis of variance (LSD = 6, $P = 0.01$). Entries in the other horizontal rows followed by the same letter are not significantly different as indicated by analysis of variance (LSD = 9, $P = 0.01$). Entries in vertical columns followed by same number are not significantly different as indicated by analysis of variance ($LSD_{\text{No dist. vs dist. 1,2,3}} = 8$, $LSD_{\text{Dist. 1 vs dist. 2 vs dist. 3}} = 9$ $P = 0.01$)

seedlings growing in the most disturbed soil blocks (dist. 3) (Fig. 2; Table 2). In the O'Hares soil, this level of disturbance had no impact on shoot mass until 5 weeks after sowing. After 28 days, shot mass was significantly lower in the seedlings growing in the most disturbed soil blocks (dist. 3) than the less-disturbed soil blocks (i.e. No dist., dist. 1, dist. 2) (Fig. 2; Table 2).

Discussion

A number of authors have suggested that propagules of VAM fungi including infected root fragments (Rives et al. 1980), spores (Gould and Liberta 1981; Jasper et al. 1987, 1988), or hyphal fragments (Jasper et al. 1989a) lose their capacity to initiate infection during soil disturbances because they are (1) physically damaged, and/or (2) exposed to post-disturbance soil conditions unfavourable for germination or colonisation (Stahl et al. 1988). The relative importance of these two mechanisms has not been fully established and may vary in different situations (Brundrett 1991). Cutting the soil blocks into 1/25 fractions in the experimental disturbance here is likely to have disrupted the external hyphal network and the root fragments (containing hyphae and vesicles). This would in turn temporarily reduce the infective potential of the fungus to zero. This explanation is supported to some extent by the findings of Fairchild and Miller (1988) and Jasper et al. (1989a, b). The observed delay in the initiation of VAM infection in seedlings growing in the most disturbed blocks may be explained by the time required for hyphae to grow from propagules in the soil, such as spores and root fragments colonised by VAM fungi.

Evans and Miller (1990) and McGonigle et al. (1990) concluded that disruption of the external hyphal network in agricultural soils can reduce nutrient uptake by VAM in a way that is independent of the amount of root length colonised by VAM fungi. This conclusion contrasts with the results of the current study, where the most severe experimental disturbance was associated with a marked decrease in the amount of root length colonised by VAM fungi. However, the seeming disparity between the studies may be explained by differences in the number of surviving propagules. Jasper et al. (1991) demonstrated that colonisation of roots by VAM fungi was not decreased after disturbance of soil from an annual pasture. In contrast, in both a forest soil and a heathland soil, the percentage root length colonised by VAM fungi was almost halved if the soils were disturbed. In the pasture soil, up to 25 times more propagules (i.e. spores and colonised root fragments) survived disturbance than in the forest or heathland soil. They concluded that the larger number of surviving propagules in the pasture soil was responsible for the maintenance of the high level of infectivity. The sandstone soils in the current study are characterised by very low densities of viable spores (Bellgard 1993). This suggests that in these intact soils the external hyphal network may be the main source of VAM infection. Furthermore, it would appear that the infectivity of VAM fungi decreases dramatically if the hyphal network is fragmented in these soils.

The low and intermediate degrees of soil disturbance did not delay the onset of VAM, and had no affect on either the length or proportion of roots colonised by VAM for the first 14 days of the bioassay. After this time, both the length and the proportion of root colonised by VAM were significantly lower than for the seedlings growing in the intact soil blocks. However, the depression in VAM colonisation at 21–28 days observed in these soil block treatments may not have any biological

significance, because the reduction in VAM colonisation was not associated with a decrease in shoot dry mass for seedlings growing in these soil disturbance treatments.

In addition to delaying the initiation of VAM, the most severe disturbance was also associated with a reduction in shoot biomass of 21- to 28-day-old seedlings of *T. repens*. The results of the present study reinforce the findings of earlier studies which demonstrated that seedlings with a higher proportion of root length colonised by VAM fungi have greater shoot dry mass than seedlings with less VAM colonisation (e.g. Evans and Miller 1988; Fairchild and Miller 1988; Jasper et al. 1989c).

Overall, cutting the intact soil blocks longitudinally into four and nine equal portions did not affect the infectivity of VAM fungi to any great extent. However, cutting the soil block into 25 equal portions temporarily reduced the infective potential of VAM fungi to zero. Additionally, the observed reduction in the proportion of root colonised by VAM was associated with a significant decrease in shoot dry mass after 21 days. Long-term studies are required to assess the impacts of different levels of VAM colonisation on overall plant performance, especially for seedlings re-colonising disturbed sites.

Acknowledgements. This research was supported by a University of Wollongong Special Research Studentship and the Sydney Water Board. My thanks go to Associate Professor R. J. Whelan, Drs. P. A. McGee, D. A. Jasper and M. C. Brundrett for constructive comments which improved the manuscript, Dr. K. G. Russell for carrying out the statistical analysis, and Associate Professor Zeng Liangzhong for technical assistance. This is contribution no. 101 from the Ecology and Genetics Group at the University of Wollongong.

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