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Mycorrhiza

The propagules of vesicular-arbuscular mycorrhizal (VAM) fungi capable of initiating VAM infection after topsoil disturbance

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Summary. The removal and storage of topsoil decreases the infectivity of vesicular-arbuscular mycorrhizal (VAM) fungi. The propagules of VAM fungi include spores, root fragments containing hyphae and vesicles, and soil hyphae. The viability of each type of propagule after disturbance will determine the initiation of VAM associations with plants recolonizing the disturbed site. This study aimed to examine which of the propagules of VAM fungi are capable of initiating VAM infection after soil disturbance. Soil from an open woodland site of low soil fertility, in southeastern Australia was wet-sieved through a tier of three sieves (1 mm, 250 μm and 106 μm), and the following fractions were extracted: (i) root fragments, (ii) fungal hyphae, and (iii) VAM spores. Each fraction was tested to determine its potential to initiate VAM. Hyphae of VAM fungi grew from root fragments within 14 days. The VAM spore fraction initiated VAM infection after 28 days. VAM hyphal fragments did not produce any VAM infection even after 42 days.

Key words: Topsoil disturbance – VAM fungi – Colonized root fragments – VAM hyphae – Spores

Introduction

The removal and storage of topsoil decreases the infectivity of vesicular-arbuscular mycorrhizal (VAM) fungi (Moorman and Reeves 1979; Abbott and Robson 1991). The propagules of VAM fungi are blastospores, chlamydospores or azygospores (resting spores), soil-borne vesicles and mycelia or colonized root fragments containing hyphae and vesicles (Daniels and Skipper 1982). These propagules have been shown to have different susceptibilities both to the direct impacts of topsoil disturbance, and also to the associated changes in the soil environment (e.g. Stahl et al. 1988; Jasper et al. 1989a). The viability of each type of propagule after soil disturbance will determine in part the number of infective propagules available to initiate VAM with plants recolonizing the disturbed site.

Soil-borne spores have been considered to be the most important type of propagule of VAM fungi (Brundrett 1991). However, soils in a range of ecosystems often contain low numbers of living spores (e.g. Read et al. 1976; Janos 1980; Gay et al. 1982; Visser et al. 1984; Brundrett and Kendrick 1988; Bellgard, in preparation). Some species of VAM fungi apparently do not produce spores (e.g. Johnson 1977; McGee 1989). The successful germination of VAM spores is dependent upon interactions with a range of soil and environmental factors (e.g. Slankis 1974; Schenck et al. 1975; Black and Tinker 1979; Daniels and Trappe 1980; Tommerup 1983a, 1984; McGee 1989), but living spores of VAM fungi will not function as propagules if they are quiescent (Tommerup 1983b, 1985). Blastospores of VAM fungi, because of their lipid content and thick walls, are considered to be more resistant to adverse environmental conditions than other VAM propagules (Daniels Hetrick 1984; Abbott and Robson 1991). Tommerup and Kidby (1979) demonstrated that some species of *Glomus* and *Gigaspora* can remain infective after lyophilization. In comparison, *Scutellospora calospora* did not recover after one wet/dry cycle (McGee 1989). Consequently, it is reasonable to conclude that the importance of spores as a source of inoculum varies between sites and is dependent upon a range of variables, including the species of endophyte, the abundance of the endophyte, and the local soil and environmental conditions.

Non-spore propagules, such as roots colonized by VAM fungi (containing hyphae and vesicles), can initiate VAM, provided they are in close proximity to an actively growing root (e.g. Rives et al. 1980; McGee 1987). Tommerup and Abbott (1981) demonstrated that root pieces colonized by several species of VAM fungi retained their infective potential even when stored in dry soil at −50 MPa. It is not known whether storage under humidities more conducive to root decomposition or to root desiccation might result in loss of viability of hyphal fragments contained in root pieces (Daniels Hetrick 1984; Miller 1987). Thus, the ability of colonized root

fragments to function as propagules of infection may vary both between species of VAM fungi and the specific site and storage conditions (Nemec 1987; McGee 1989).

Plant roots infected with VAM fungi carry a loose hyphal network extending into the surrounding soil which provides 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 is considered to be a particularly significant source of infection in undisturbed soils containing low densities of living spores (e.g. Read et al. 1976; Read et al. 1985; Jasper et al. 1989a). It is clear that the hyphal network can colonize host roots more efficiently than spore inoculum (e.g. Powell 1976; Hall 1976, 1979; Abbott and Robson 1981). However, the hyphal network may be more susceptible to the impact of soil disturbance than the more robust forms of inoculum, viz spores and colonized root fragments (Jasper et al. 1989a).

In a previous study which examined the impact of increasing degrees of topsoil disturbance on the infectivity of VAM fungi (Bellgard, in preparation), colonization of roots by VAM fungi was delayed by up to 6 weeks in the roots of seedlings grown in severely disturbed soil blocks. It was concluded that severe soil disturbance probably disrupted the external hyphal network and the colonized root fragments, thus delaying root infection. The delay observed in the initiation of VAM in the seedlings growing in the severely disturbed soil blocks was explained by the time required for hyphae to grow to the bioassay roots from other propagules remaining viable in the soil blocks. The aim of the present study was to identify which propagules of VAM fungi are capable of initiating VAM after topsoil disturbance.

Materials and methods

Description of the study site

The southern part of the Hawkesbury Sandstone plateau, located 90 km south of Sydney and to the west of Wollongong, Australia, covers approximately 1200 km^2. The average annual precipitation in this region is 1420 mm, with a slight summer predominance in distribution. The study site was a 100-ha area located within the Avon Catchment (34°22′S, 150°40′E) of the Sydney Water Board. The vegetation of the site was a woodland with an overstorey of *Eucalyptus haemostoma* Sm. and *E. racemosa* Cav. (Myrtaceae), and a shrub understorey comprising a variety of genera including *Acacia*, *Banksia*, *Grevillea* and *Isopogon*. The soil at the site was 30 cm of yellow clayey subsoil overlain by 20 cm of loamy sand topsoil [Yellow earths (Gn2.21)] (Hazelton and Tille 1990).

Experimental design

Five different plots within the site were selected at random and three intact soil blocks (20 × 20 × 15 cm) were taken from each plot. These blocks were placed in square, 6-l plastic containers. For each plot, two containers were divided longitudinally into 25 equal portions and the third was left undisturbed.

Experiment I – VAM formation in undisturbed versus disturbed soil blocks

Forty seeds of *Trifolium repens* L. were sown into one of the disturbed blocks and the undisturbed block from each plot. The remaining disturbed soil block from each plot was used in experiment II. The undisturbed block was assayed to ensure that the soil samples were potentially infective. To test for potential aerial contamination in the glasshouse by VAM fungi, five pots of washed river sand sown with 20 seeds of *T. repens* were used as a control. Ten seedlings were randomly extracted from each container 14, 28 and 42 days after sowing. The roots of each seedling were washed in a 0.4% sodium hexametaphosphate solution to remove any adhering soil. Roots were fixed in 50% ethanol, cleared and stained (Kormanick et al. 1980), and both the total root length and portion of root length colonized by VAM fungi were measured (Ambler and Young 1977). The length of root length colonized by VAM fungi (i.e. VAM length) is a composite index, i.e. the sum of the length of root colonized by vesicles, arbuscules and internal hyphae (N.B. % VAM = VAM length/total root length × 100).

Experiment II – isolation of inoculum fractions and examination of potential infectivity

The remaining disturbed-soil blocks from the five plots were separated into their constituent 25 soil columns. Five columns of the 25 were randomly selected from each block and individually washed through a tier of three sieves: (I) 1 mm, (II) 250 μm, and (III) 106 μm. The material caught on the 1-mm, 250-, and 106-μm sieves was sprayed with a strong jet of water in an attempt to remove any hyphae adhering to the root fragments. Cursory examination of a randomly selected sub-sample of root fragments confirmed that this technique successfully removed the majority of adhering soil hyphae from the root surface. The sieved fractions were examined under a dissecting microscope and divided into root fragments and fungal hyphae. The root fragments and the hyphae from each of the three sieve fractions were bulked.

The spores on the 106-μm sieve were not separated from the fine soil and this was termed the "spore/soil" fraction. The fraction caught in a collecting vessel below the tier of three sieves (i.e. < 106 μm) was termed the "fines" fraction. The four fractions – roots, hyphae, spore/soil and fines – were each examined for potential infectivity.

Root fragments

The ability of hyphae to grow from root fragments was tested using a "membrane filter" technique (Tommerup and Kidby 1979). This involved sandwiching at least five root pieces between a pair of 0.45-μm membrane filters (Millipore Corp.) which are inserted between 1-cm layers of steamed river sand. The steamed sand was wetted to field capacity and incubated at 22° C. Three "blanks" containing a clean pair of membrane filters sandwiched between steamed sand were used as a control to monitor potential contamination. Twelve randomly chosen samples of root fragments (each sample containing five root fragments between 1 and 6 mm in length) were selected from each of the soil columns. Four randomly selected filter-sandwiches from soil column were examined after 14, 28 and 42 days. Each pair of filters was recovered and the root fragments stained in place on the filters (Tommerup and Kidby 1979) with 0.01% acid fuchsin (Kormanick et al. 1980).

Hyphal fragments

The viability of hyphal fragments was tested using the "soil-funnel" technique (Menge and Timmer 1982). Here, seedlings are

forced to grow in close proximity to VAM fungal inocula, thus optimizing the chance of initiating an association. At least ten hyphal fragments were used as inocula in each apparatus. Six randomly chosen samples of VAM fungal hyphae (each sample containing at least 10 hyphal pieces, 3–6 mm long) were chosen from each soil column. Ten seeds of *T. repens* were sown into each funnel setup, which were placed in a glasshouse with natural light and watered daily without nutrients. The diurnal temperature range in the glasshouse for the duration of this experiment between 17 June and 28 July 1991 was 18.4–25.4° C. To monitor for potential aerial contamination in the glasshouse by VAM fungi, five funnel setups with 10 seeds of *T. repens* and no VAM hyphae were used as a control. Plants from two entire funnel setups from each soil column were harvested 14, 28 and 42 days after sowing. Plants were removed from the funnels and their roots washed in a 0.4% sodium hexametaphosphate solution to remove any adhering soil. The roots were fixed in 50% ethanol, cleared and stained and the proportion of root length colonized by VAM fungi was quantified.

Spore/soil and fines fraction

The ability of the spore/soil and the fines fraction to initiate VAM infection was tested by bioassay. The two fractions were put into 10-cm plastic pots placed on gravel to prevent the fine soil from washing through. Twenty *T. repens* seeds were sown into the fractions. The pots were placed in a naturally lit glasshouse and watered daily without nutrients. The diurnal temperature range in the glasshouse for the duration of this experiment between 17 June and 28 July 1991 was 18.4–25.4° C. To monitor for potential aerial contamination in the glasshouse by VAM fungi a further five pots of river sand sown with 20 seeds of *T. repens* were used as a control. Five plants were harvested from each pot after 14, 28 and 42 days. The plants were removed from the pots and their roots washed in a 0.4% sodium hexametaphosphate solution to remove any adhering soil. The roots were fixed in 50% ethanol, cleared and stained and the proportion of root length colonized by VAM fungi was quantified.

Statistical analysis

A chi-square statistic (Zar 1984) was used to determine if the numbers of seedlings colonized by VAM at day 42 in the intact and disturbed soil treatments were independent. An unpaired, two-tailed *t*-test (Zar 1984) was used to determine if the mean proportion of root length colonized by VAM fungi after 42 days differed in intact- and disturbed-soil blocks. The growth of hyphae from root fragments was designated either "success" (i.e. hyphae grew from the root fragments) or "failure" (i.e. no hyphae grew from the root fragments). Chi-square contingency tables were used to determine whether the frequencies of "successes" versus "failures" were independent of sampling dates.

Results

VAM formation in intact versus disturbed soil blocks

No VAM were found in any of the controls monitoring for glasshouse contamination at any of the harvests. VAM formation had commenced by 14 days in the intact-soil blocks and in the roots of seedlings growing in the disturbed-soil blocks after 42 days. At 42 days, the number of seedlings colonized by VAM was significantly higher in the intact-soil blocks than in the disturbed-soil blocks ($\chi^2 = 36.97$, $P \leq 0.0001$). Additionally, a higher proportion of the total root length of plants was mycorrhizal from undisturbed soil (20%) than disturbed soil (7%) ($t = 9.86$, $P \leq 0.001$).

Table 1. Chi-square analysis of root fragments extracted from soil blocks taken from the Avon study site in southeastern Australia. Data represent the number of root fragments out of 100 from which hyphae were produced

	Time since start of assay (day)		
	14	28	42
Block 1			
Hyphae produced	56	47	68
No hyphae produced	44	53	32
$\chi^2 = 9.06$, $P = 0.011$[a]			
Block 2			
Hyphae produced	46	54	63
No hyphae produced	54	46	37
$\chi^2 = 5.83$, $P = 0.054$[b]			
Block 3			
Hyphae produced	52	51	60
No hyphae produced	48	49	40
$\chi^2 = 1.96$, $P = 0.375$[b]			
Block 4			
Hyphae produced	51	48	53
No hyphae produced	49	52	47
$\chi^2 = 0.51$, $P = 0.776$[b]			
Block 5			
Hyphae produced	52	48	56
No hyphae produced	48	52	44
$\chi^2 = 1.28$, $P = 0.527$[b]			

[a] Reject null hypothesis
[b] Accept null hypothesis

Root fragments

Hyphae had grown from the root fragments after 14 days. In all cases, the hyphae produced were straight, thick-walled, aseptate, 4–8 µm in length and did not branch. In four out of the five soil blocks sampled, hyphae had grown from a similar number of root fragments at each harvest (Table 1).

Hyphal fragments

Soil collected from the Avon site contained 13.0 ± 1.5 m of fungal hyphae per gram of soil at field capacity (mean ± SEM, $n = 25$). This estimate does not include hyphal fragments smaller than 106 µm which went through the sieve.

No VAM was found in the control funnels used to monitor for aerial contamination. At each of the three sampling dates, the hyphal fragments failed to produce any VAM in the roots of the bioassay plants.

Spore/soil and fines fraction

No VAM was found in the controls at any of the sampling times. The spore fraction produced VAM infection at the 42-day harvest. At 42 days, 53 of the 150 seedlings sampled were colonized by VAM. The mean proportion of root length colonized by VAM fungi was $4.85 \pm 0.62\%$ (mean $\pm$ SEM, $n = 150$). VAM were not formed in the fines fraction from any of the three sampling occasions.

Discussion

Soil disturbance can reduce the infective potential of VAM fungi in several ways: (I) propagules may be physically damaged, i.e. spores may be crushed and/or the soil hyphal network and colonized root fragments may be disrupted (Jasper et al. 1989a; Evans and Miller 1990); (II) disturbance may alter the physical, chemical, or biological environment of the soil, which in turn prevents the colonization by or germination of VAM propagules (Warner 1983; Stahl et al. 1988); (III) disturbance may eliminate host plants, leading to changes in the carbon supply available to the fungus (Abbott and Robson 1991). Brundrett (1991) commented that the relative importance of these mechanisms has not yet been fully established. Additionally, individual fungal species may exhibit different responses to both the direct and indirect impacts of soil disturbance, depending upon their own specific host and environmental requirements.

Seedlings growing in the undisturbed topsoil blocks of experiment I were rapidly colonized by VAM fungi. This implies that VAM fungi inoculum levels in this soil were high. Read et al. (1976) observed rapid VAM development in various grassland, shrub and woodland species. The rapid colonization of roots was attributed to seedling roots intercepting colonized roots of established plants rather than infection from spores, which were found to occur in low numbers. In a similar way, the infections which were observed in 2-week-old seedlings in the present study are believed to be indicative of infection from roots colonized by VAM fungi and their associated hyphal network.

Cutting the soil blocks longitudinally into 25 equal-sized portions temporarily reduced the infective potential of VAM fungi to nil. These results reinforce the findings of my earlier study (Bellgard, in preparation), in which colonization of roots by VAM fungi was delayed by up to 6 weeks for seedlings growing in soil blocks which were cut longitudinally into 25 equal portions. Colonization of roots by VAM fungi had commenced by the 42-day harvest. The delay in the initiation of infection may be due to the time required for hyphae to grow from propagules in the soil which survived both the direct impact of the disturbance and the associated changes to the soil environment.

Root fragments produced hyphae after 14 days. Similarly, McGee (1987) demonstrated that outgrowth of VAM fungi from dried root pieces occurred by 14 days. The studies of Powell (1976), Warner and Mosse (1980), Tommerup and Abbott (1981) and Biermann and Linderman (1983) did not determine when hyphae first emerged from colonized root fragments. However, the hyphae produced from the colonized root fragments initiated VAM infection in bioassay plants within 28–60 days. It is reasonable to conclude that outgrowths of hyphae from colonized root pieces may have been responsible in part for the initiation of infection in the disturbed soil blocks of experiment I.

Hyphal fragments failed to initiate VAM infection even after 42 days. A number of authors have demonstrated that disruption of the soil hyphal network results in a decrease in the infectivity of VAM fungi (e.g. Fairchild and Miller 1988; Jasper et al. 1989a, b; Evans and Miller 1990). However, these experimental protocols failed to differentiate between physical damage to the hyphal network and indirect changes to the soil environment as a result of the disturbance. A number of studies have shown that soil conditions play a critical role in the growth of the hyphae of VAM fungi (e.g. Graham et al. 1982; Abbott et al. 1984; Abbott and Robson 1985). Consequently, the post-disturbance reduction in infectivity of the external hyphal network may be due in part to exposure of hyphal fragments to unfavourable conditions for germination and/or colonization caused by redistribution of soil.

Another factor which may have contributed to the failure of hyphal fragments to produce infection is inoculum density. It has been demonstrated that increased inoculum dosage results in increased percentage root colonization (e.g. Daft and Nicolson 1969; Sanders and Sheikh 1983; Wilson and Trinick 1983; Wilson 1984). The main effect of increasing the inoculum density appears to be to increase the rate of development of infection. It has been proposed that the increase in the rate of infection results from an increase in the rate of formation of primary points of infection (Wilson and Trinick 1983). It was not known how much hyphal inoculum was required to initiate VAM. Additionally, the hyphae may have included species other than VAM fungi, the hyphae may have been severely battered by the extraction and inoculation process and, even if the hyphae were extracted satisfactorily, not enough hyphae may have been used to initiate infection.

The spore fraction initiated VAM infection at the 42-day harvest. This coincided with the observed onset of VAM infection in the roots of bioassay seedlings growing in the disturbed-soil blocks of experiment I. It must be noted that experiments I and II were not running concurrently. However, the observed coincidental onset of VAM infection suggests that spores may have been responsible in part for the initiation of VAM infection in the disturbed-soil blocks of experiment I.

Both colonized root pieces and spores can be effective propagules initiating VAM in host plants after topsoil disturbance. Thus, the ability of VAM fungi to persist in soil after disturbance may depend partly on the type of propagules formed. Studies investigating the relationship between soil disturbance and the colonization and sporulation of VAM fungi species are required be-

fore we can fully understand the processes by which VAM fungi persist after topsoil disturbance and storage.

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