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Survival of the external mycelium of a VAM fungus in frozen soil over winter

Abstract The present investigation examines (1) whether the external VAM mycelium survives winter freezing to act as a source of inoculum in the spring, and (2) whether soil disturbance reduces the infectivity of the external VAM mycelium following freezing of the soil. Sealed pouches of fine nylon mesh were placed in pots containing soil inoculated with a *Glomus* species. The mesh was impervious to roots but not to hyphae. Following two 3-week growth cycles of maize in the pots, the pouches were transplanted to the field. Pouches were removed from the field once during the 4 months when the soil was frozen, and once after spring thaw. Measurements were made of VAM spore density, hyphal length and viability in the pouches. Bioassays for infectivity were conducted on all pouches. Some VAM hyphae survived freezing and remained infective following winter freezing, in the absence of plant roots. Soil disturbance did not reduce the infectivity of hyphae following exposure to freezing temperatures. We observed a change in the distribution of viable cytoplasm within hyphae over winter, which we hypothesize represents an adaptation allowing hyphae to survive freezing temperatures. We suggest that the effect of disturbance on hyphal infectivity may be related to this seasonal change in the distribution of hyphal viability.

Key words Vesicular-arbuscular mycorrhizae (VAM) Overwinter survival · Extraradical hyphae Fluorescein diacetate · Disturbance

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Introduction

The importance of the intact mycelial network of VAM fungi in both root colonization and nutrient uptake has been demonstrated in a series of recent studies. The adverse effects of soil disturbance on the functioning of the VAM symbiosis in long-term zero-till maize plots were initially shown in an experiment by O'Halloran et al. (1986). In that study, disturbance of zero-tilled soil decreased both P absorption by young maize plants and VAM colonization of maize roots. Subsequent experiments by Evans and Miller (1988) and Fairchild and Miller (1988) further explored the effects of soil disturbance on VAM symbiosis. Their results revealed a relationship between the reduced P absorption and decreased VAM colonization following soil disturbance. It was hypothesized that there was a cause-and-effect relationship between the two processes, i.e. soil disturbance reduced colonization, which then reduced P uptake by young maize plants. Further research by Evans and Miller (1990) demonstrated that the external mycelial network present in undisturbed soil is an important component of the inoculum potential. Destruction of this network may be directly responsible for most of the effect of soil disturbance upon mycorrhizal colonization. Independent research conducted in Australia supported this hypothesis (Jasper et al. 1989a, b).

However, field and growth chamber studies by McGonigle et al. (1990b) showed no reduction in VAM colonization following soil disturbance, although P absorption was reduced. The authors hypothesized that soil disturbance destroys the integrity of the external mycelial network of the VAM fungi, which is the network capable of providing P for a newly connected developing root system. Colonization may also be reduced following destruction of this network, but root colonization is not necessarily the means by which disturbance reduces P absorption. Data supporting this hypothesis were obtained in a subsequent greenhouse study (Miller and McGonigle 1992) in which increased P absorption by plants in undisturbed soil partially re-

sulted from the ability of the existing external mycelium to take up and translocate nutrients to the plant.

Reduction in VAM colonization following soil disturbance was observed in soil cores taken from a zero-till maize field in early spring after being frozen (Evans and Miller 1988). If, as discussed above, the external mycelium is an important component of the inoculum, this result implies that the external network does remain viable and infective over winter. Research by Jasper et al. (1989a, b, 1991) has shown that external hyphae of VAM species can survive drying conditions and retain their infectivity, except when the soil has been disturbed. However, the ability of VAM hyphae to survive winter freezing has not been directly demonstrated. Warcup (1967) stated that the hyphae of VAM endophytes are relatively long-lived in the soil. Both saprophytic and parasitic fungi can survive as hyphae in soil over winter (Park 1968).

The goal of this experiment was to investigate (1) whether the external mycelium of VAM fungi survives winter freezing in the absence of plant roots and acts as a source of inoculum, and (2) whether soil disturbance alters the infectivity of the mycelium following winter freezing.

Materials and methods

The experiment consisted of three stages: (1) establishment of external VAM mycelia in mesh pouches under controlled growth conditions; (2) transplantation of pouches to field soil; (3) establishment of bioassays to assess infectivity of external mycelia.

Establishment of external mycelium

Silt loam soil was collected from a zero-till maize field at the Elora Research Station near Guelph, Ontario, Canada (43°31′N, 80°14′W). The soil was partially air-dried, pulverised mechanically, passed through a 5-mm sieve and pasteurized. The soil was then packed at a bulk density of approximately 1 g cm^{-3} into 20-cm diameter pots to a depth of 4 cm. A pair of sealed pouches of 43-μm nylon mesh (250 cm^3 volume) containing 270 g of pasteurized soil were placed on top of this soil layer (Fig. 1). The nylon mesh was impervious to roots but not to hyphae. A layer of VAM inoculum consisting of colonized root pieces, spores, and hyphae in Turface (Applied Industrial Materials Corp., Deerfield, Ill.) was placed around the pouches. The inoculum was obtained from leek (*Allium porrum* L.) pot cultures of Elora 500, an undescribed *Glomus* isolate. Pasteurized soil was placed on top of the inoculum and pouches. Unpasteurized field soil was mixed with sterile 0.1% Bacto-Peptone (Difco Laboratories, Detroit, Mich.; 1 g soil to 10 ml BP) and the suspension filtered through 45-μm mesh (Ames et al. 1987). Aliquots of this filtrate (50 ml) were added to all pots to reintroduce soil bacteria eliminated by pasteurization. The pots were watered to a gravimetric water content of 22% every other day throughout the experiment. A solution of aqueous NH_4NO_3 was added to all pots (at a rate of 50 mg N kg^{-1}) at the start of the experiment.

Six germinated maize seeds (*Zea mays* L. cv Pioneer 3949) were planted in each pot and thinned to three seedlings per pot after 1 week. The shoots were harvested after 3 weeks, and a second cycle of maize was established in the same pots without disturbance of the soil. The growth chamber regime for the first 6 weeks consisted of a 17-h photoperiod at 70 Wm^{-2} (day and night temperatures of 22° C and 13° C, respectively) with a relative humidity of 70%. After 6 weeks, the conditions in the growth chamber were altered to a 12-h photoperiod with day and night temperatures of 13° C and 8° C, respectively, to allow for acclimatization prior to transplantation of pouches. Maize plants were maintained in the pots during the acclimatization period.

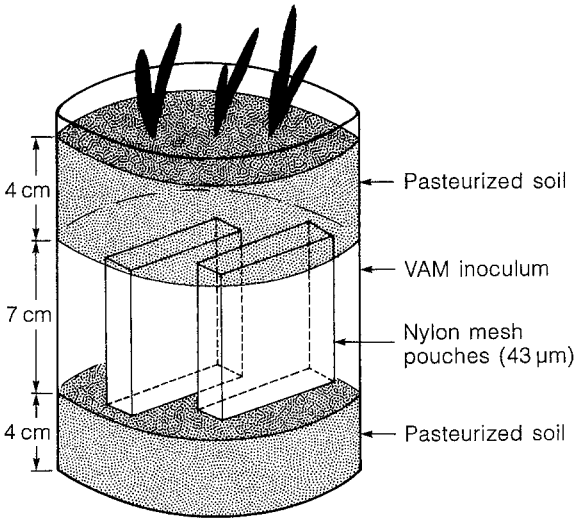

Fig. 1 A schematic diagram showing the arrangement of nylon mesh pouches, inoculum, pasteurized soil, and maize seedlings within a pot

Transplantation and sampling procedures

After 1 week of acclimatization, 12 pairs of pouches were buried at a depth of 15 cm in the same field at the Elora Research Station from which the soil had been collected. One pouch of each pair was sealed in a 1-l plastic bag prior to burial to isolate the hyphae from plant roots and to prevent entry of indigenous soil fungi. In the growth chamber, an additional four pairs of pouches were transferred to pots containing pasteurized soil only, to simulate the physical handling associated with transplanting. The intent of including these control pouches was to assess the status of the mycelium at the time when the other pouches were transplanted to the field. Since there were no plant roots or viable VAM fungal hyphae in the surrounding pasteurized soil, none of these pouches were placed in bags. These pouches were removed from the pots after 2 weeks, which we thought would be sufficient time for any deleterious effects of transplanting to become obvious, yet brief enough to prevent excessive degradation of the mycelium in the absence of plant roots.

Two pairs of pouches were buried at each of six locations in the field in early November 1991, when the soil temperature at 10-cm depth under a nearby sod plot was 6.5° C. The soil froze in mid-December of 1991 and reached minimum temperatures under sod of −3.3° C (5-cm depth) and −1.7° C (10-cm depth) on 21 March 1992. After 7 April 1992, the soil temperature remained above 0° C. Pouches from each of three locations were removed at each of two sampling times: once in March 1992, while the soil was still frozen, and once in April 1992, 16 days after the soil had thawed (soil temperature of 6.3° C at 10-cm depth).

At all three sampling times, the pouches were kept at 4° C overnight following removal from the soil or pots. Soil from each pouch of one pair (one bagged and one unbagged) was removed from the pouch, mixed, and passed through a 2-mm sieve. Subsamples (5 g each) of this soil were taken for measurement of soil water content, hyphal length density, hyphal viability, and VAM spore density. The remaining soil was replaced in the pouch. The soil from each pouch of the second pair from each location was left undisturbed.

Bioassays

Four seeds of sudan grass (*Sorghum sudanense* Staph.) were planted directly into both disturbed and undisturbed pouches. The pouches were placed in 4-inch pots, and the space around the pouch filled in with Turface. Given the small size of the pouches, sudan grass was used for the bioassays rather than faster-growing maize. Following emergence, seedlings were thinned to two per pouch. After 4 weeks, the roots were washed free of soil, fixed, cleared, bleached in alkaline H_2O_2 for 15–30 min (Kormanik and McGraw 1984), and stained with chlorazol black E (Brundrett et al. 1984). The percentage root length colonized by VAM was determined (McGonigle et al. 1990a). The bioassays were done following each sampling time, under the same conditions, and in the same growth chamber.

Hyphal length and viability

Fungal hyphae were extracted from the soil using a modified membrane filter technique (R. M. Miller, unpublished work). The 5-g subsample of soil from each disturbed pouch was soaked overnight in a sodium hexametaphosphate solution [0.028% (w/v)] to disperse the soil. This mixture was vigorously stirred at the end of the soaking time to free the hyphae from soil particles. The resulting suspension was diluted so that approximately 50 mg of soil was collected on a 20-μm nylon mesh filter. The filter was incubated for 1.5 h in 0.05% trypan blue stain (in lactophenol; R. M. Miller, unpublished work). The hyphae were then collected on a cellulose nitrate filter (1.2 μm pore size diameter). Hyphal length was determined by a gridline intersect method (Tennant 1975) using a microscope ocular grid.

In the vital staining procedure, the soil subsample was soaked for only 30 min to reduce any effect on hyphal viability. The hyphae collected on the nylon mesh filter were stained for 5 min in a fluorescein diacetate (FDA) solution of 50 μg ml^{-1} (Hamel et al. 1990). The hyphae were then filtered onto a cellulose nitrate filter (as above) and immediately observed using fluorescence microscopy. Hyphal segments intersecting the filter grid were classified as live if fluorescent or dead if nonfluorescent. The ratio of live to total intersections was multiplied by the total hyphal length density determined from trypan blue-stained hyphae to obtain the viable hyphal length density. Total hyphal length density could not be obtained using FDA-stained hyphae as the fluorescence microscope was not equipped with an ocular grid.

Isolation of spores

Spores were isolated by wet sieving (Daniels and Skipper 1984) and counted. Spore viability was not assessed.

Data analysis

Data were processed using analysis of variance. Percentage data were arc-sine transformed prior to analysis of variance. Means were separated using protected LSDs. Unless otherwise indicated, statistical significance was accepted at $P<0.05$.

Results

General

The level of arbuscular colonization of maize roots at the end of the 6-week establishment phase was approximately 60%.

Placing the pouches in polyethylene bags did not result in significant differences in arbuscular colonization of bioassay plants ($P=0.59$), total hyphal length density ($P=0.20$), hyphal viability ($P=0.93$), or spore density ($P=0.96$). Data from both bagged and unbagged pouches were, therefore, pooled for all analyses.

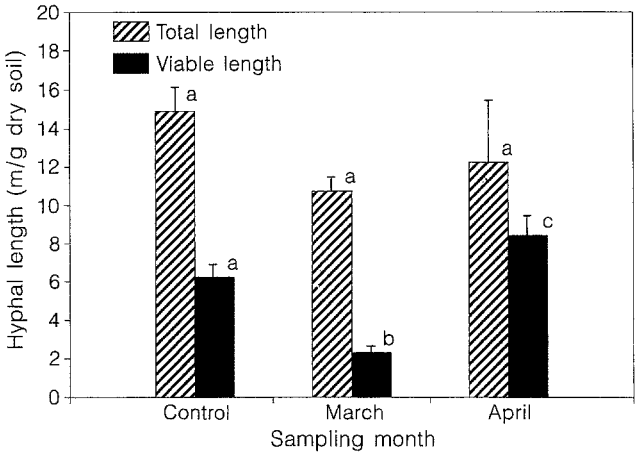

Fig. 2 Total hyphal length density and viable hyphal length density in pouch soil at three sampling times. *Bars* for each parameter labelled with different letters are significantly different ($P<0.05$). *Lines above bars* indicate standard error of the mean

Hyphal length and viability

After the filtration procedure, hyphae were retrieved primarily as short fragments. Total hyphal length density did not change significantly from one sampling period to another. Hyphal length density was slightly lower in the March samples than in the control samples, but this decrease was statistically significant only at $P=0.08$ (Fig. 2). There was no significant increase in hyphal length density between the March and April samples (Fig. 2). The length of viable hyphae decreased significantly from the control samples to the March samples (Fig. 2), and increased significantly between the March and April samples (Fig. 2). The viable hyphal length in the April samples was significantly greater than in the controls. When stained with FDA, viable segments of hyphae were observed to have a fluorescent yellow-green colour. The fluorescence was discontinuous in the hyphae from all samples, i.e. there were segments of the hyphae which did not become fluorescent. This discontinuous arrangement of viable cytoplasm gave many of the hyphae a banded appearance. In samples removed from frozen soil in March, the length of the viable segments within most hyphae was markedly less than in the control samples. The short viable segments of cytoplasm were separated within a hypha by longer nonviable segments. This type of hypha was also observed in samples taken in April. In general, however, the viable segments observed in April were longer than those sampled in March. Fine branching hyphae originating from some viable segments were also observed in April.

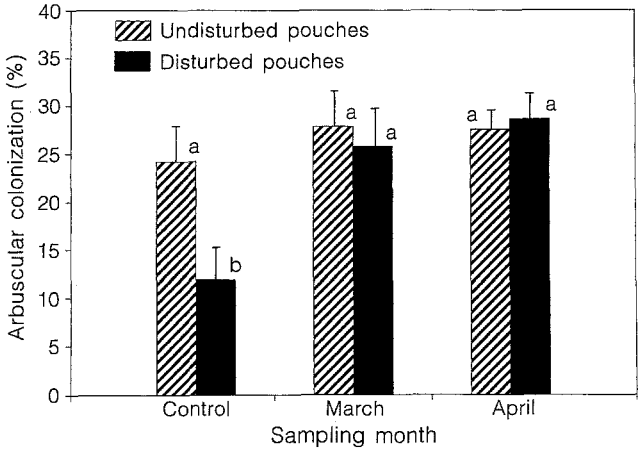

Fig. 3 Arbuscular colonization of bioassay plant roots (*Sorghum sudanense*) after 4 weeks. *Bars* labelled with different letters are significantly different ($P<0.05$). *Lines above bars* indicate standard error of the mean

Spore density

The spore density of inoculum added to the pots in which the external mycelia were established was approximately 298 spores g^{-1} dry soil. At all three harvests, total spore density in the pouches was very low, ranging from 1 to 5 spores g^{-1} soil.

Bioassay colonization

Sudan grass roots were colonized by the Elora 500 isolate in all three bioassays (Fig. 3). Disturbance of control pouch soil prior to the bioassay significantly reduced arbuscular colonization in the roots of sudan grass (Fig. 3). The percentage arbuscular colonization of sudan grass roots in undisturbed pouches reached the same levels in both the March and April bioassays as in the control bioassay (Fig. 3). Soil disturbance had no significant effect, even at $P=0.98$, on arbuscular colonization in sudan grass roots established in the pouches harvested in March or April (Fig. 3).

Discussion

The results of this experiment suggest that external hyphae of the *Glomus* isolate Elora 500 survive winter freezing, as viable hyphae were observed in pouches removed from both frozen soil in March and from thawed soil in April. Moreover, the hyphae can survive in the absence of plant roots, as hyphae in the pouches that were placed inside plastic bags were isolated from both live and dead plant roots for 6 months.

Colonization by VAM fungi was observed in roots of all bioassay plants. Given the very low observed spore densities and probable low spore viability (based on previous studies of the Elora 500 isolate by T. McGonigle, personal communication), it is unlikely that spores played a significant role in the colonization of bioassay roots. Therefore, the colonization data suggest that the external mycelium not only survives freezing, but also retains its colonization potential and proliferates after thawing, independent of plant roots.

We observed a decrease in the amount of viable hyphae between the unfrozen control samples and samples taken from frozen soil. This decrease could be a result of freezing temperatures, or alternatively, it could be related to the length of time that the external hyphae were isolated from a photosynthetic carbon supply. Bethlenfalvay et al. (1982) found a decrease in external VAM mycelial length with time which appeared to be related to changing source-sink relations within plants at the onset of seed production. Schubert et al. (1987) observed a similar decline in viable external mycelium with time after inoculation. However, the decline appeared to be unrelated to the formation of reproductive structures or plant senescence. In the present experiment, the amount of viable hyphae was greater in the pouches harvested after spring thaw than in pouches harvested from frozen soil. This increase occurred without a source of photosynthetic carbon for the hyphae. It appears that the amount of viable external mycelium may be independent of the host plant to some extent, as was also observed by Schubert et al. (1987).

We also observed a change in the distribution of viable cytoplasm within a hypha from the unfrozen control samples compared to the samples taken from frozen soil. In the samples from frozen soil, the viable hyphal segments were smaller and were separated by much larger nonviable segments than in the unfrozen control samples. Schubert et al. (1987) also observed discontinuous distribution of viable cytoplasm within a hypha. They noted that the fluorescent portions of hyphae stained with FDA were usually separated by nonfluorescent tracts. Very few hyphae were completely fluorescent, and such hyphae were observed only early in the experiment, while plants were becoming established. In their later harvests, no hyphae were completely fluorescent. We observed that in the samples taken following spring thawing of the soil, the live cytoplasmic segments were larger than in the samples from frozen soil, and branching from some live segments of hyphae was observed.

Based on the observations and results of this experiment, we hypothesize that this observed change in the distribution of viable cytoplasm represents an adaptation of the fungus to the freezing stress, i.e. the external hyphae survive freezing conditions by forming such modified or "resting" hyphae. In the spring, following soil thawing, the viable hyphal segments proliferate to re-form an external mycelium. A few studies have shown that intraradical VAM hyphae regrow within the lumina of old hyphae. Schaffer (1993) observed intrahyphal hyphae in moribund soybean roots sampled from field soil in early spring. No intrahyphal hyphae were observed in root samples taken from the same

field in fall or winter, which suggests the intrahyphal growth may be part of "re-invasion" of the plant early in the growing season. Lim et al. (1983) suggested such re-invasion as one possible interpretation of the intrahyphal hyphae they observed in white clover roots. They suggest that intrahyphal hyphae may develop following damage or degeneration of portions of the hyphal system and that regrowth from within the parent hypha would be more efficient than penetration of intact root material. Such re-invasion was also observed by Tommerup and Abbott (1981). They found that hyphae survived inside roots of subterranean clover after drying to −50 MPa for 6 months. When the plants were watered after this extensive drought period, growth of new intraradical hyphae within old hyphae was observed.

Schaffer's study (1993) suggests that intrahyphal hyphae may develop following winter freezing. If such regrowth occurs in extra- as well as intraradical hyphae following freezing, it may result in rapid re-establishment of an intact external network, allowing the VAM fungus to become an effective nutrient uptake system and root colonizer early in the spring. New root systems could link up with this established network in the soil, thereby eliminating the need to establish their own external mycelia (McGonigle et al. 1990b). The persistence of external hyphae over winter as resting hyphae could thus be a very important factor in the early P nutrition of crop plants.

In bioassays conducted on pouches that had been exposed to freezing, soil disturbance did not cause a reduction in arbuscular colonization. This result is similar to the seasonal nature of the disturbance effect observed by Miller et al. (unpublished work). A corollary of the above hypothesis is that soil disturbance during times of the year when the external mycelium consists of resting hyphae may not reduce subsequent root colonization, as it would merely disperse the viable fragments, rather than destroy an intact hyphal network. At some point in the spring, when a certain degree of interconnectedness of the new hyphae has been achieved by regrowth and branching, soil disturbance would again reduce root colonization.

This preliminary study indicates the importance of monitoring changes in the state of the external mycelium, particularly the spatial distribution of viable segments within hyphae, to fully understand the seasonal pattern of VAM colonization.

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References

Ames RN, Mihara KL, Bethlenfalvay GJ (1987) The establishment of microorganisms in vesicular-arbuscular mycorrhizal and control treatments. Biol Fertil Soils 3:217–223

Bethlenfalvay GJ, Brown MS, Pacovsky RS (1982) Relationships between host and endophyte development in mycorrhizal soybeans. New Phytol 90:537–543

Brundrett MC, Piche Y, Peterson RL (1984) A new method for observing the morphology of vesicular-arbuscular mycorrhizae. Can J Bot 62:2128–2134

Daniels BA, Skipper HD (1984) Methods for the recovery and quantitative estimation of propagules from soil. In: Schenck NC (ed) Methods and principles of mycorrhizal research. American Phytopathological Society, St Paul, Minn, pp 29–37

Evans DG, Miller MH (1988) Vesicular-arbuscular mycorrhizas and the soil-disturbance-induced reduction of nutrient absorption in maize. I. Causal relations. New Phytol 110:67–74

Evans DG, Miller MH (1990) The role of the external mycelial network in the effect of soil disturbance upon vesicular-arbuscular mycorrhizal colonization of maize. New Phytol 114:65–71

Fairchild GL, Miller MH (1988) Vesicular-arbuscular mycorrhizas and the soil-disturbance-induced reduction of nutrient absorption in maize. II. Development of the effect. New Phytol 110:75–84

Hamel C, Fyles H, Smith DL (1990) Measurement of development of endomycorrhizal mycelium using three different vital stains. New Phytol 115:297–302

Jasper DA, Abbott LK, Robson AD (1989a) Soil disturbance reduces the infectivity of external hyphae of vesicular-arbuscular mycorrhizal fungi. New Phytol 112:93–99

Jasper DA, Abbott LK, Robson AD (1989b) Hyphae of a vesicular-arbuscular fungus maintain infectivity in dry soil, except when the soil is disturbed. New Phytol 112:101–107

Jasper DA, Abbott LK, Robson AD (1991) The effect of soil disturbance on vesicular-arbuscular mycorrhizal fungi in soils from different vegetation types. New Phytol 118:471–476

Kormanik PP, McGraw AC (1984) Quantification of vesicular-arbuscular mycorrhizae in plant roots. In: Schenck NC (ed) Methods and principles of mycorrhizal research. American Phytopathological Society, St Paul, Minn, pp 37–47

Lim LL, Fineran BA, Cole ALJ (1983) Ultrastructure of intrahyphal hyphae of *Glomus fasciculatum* (Thaxter) Gerdemann & Trappe in roots of white clover (*Trifolium repens* L.). New Phytol 95:231–239

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990a) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol 115:495–501

McGonigle TP, Evans DG, Miller MH (1990b) Effect of degree of soil disturbance on mycorrhizal colonization and phosphorous absorption by maize in growth chamber and field experiments. New Phytol 116:629–636

Miller MH, McGonigle TP (1992) Soil disturbance and the effectiveness of arbuscular mycorrhizas in an agricultural ecosystem. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. CAB, Wallingford, UK, pp 156–163

O'Halloran IP, Miller MH, Arnold G (1986) Absorption of P by corn (*Zea mays* L.) as influenced by soil disturbance. Can J Soil Sci 66:287–302

Park D (1968) The ecology of terrestrial fungi. In: Ainsworth GC, Sussman AS (eds) The fungi, vol 4. Academic Press. New York, pp 5–39

Schaffer GF (1993) Assessment of the over-winter survival of intra-radical VAM fungi in field-grown *Glycine max* (soybean) root pieces. MSc thesis, University of Guelph

Schubert A, Marzachi C, Mazzitelli M, Cravero MC, Bonfante-Fasolo P (1987) Development of total and viable extraradical mycelium in the vesicular-arbuscular mycorrhizal fungus *Glomus clarum* Nicol. & Schenck. New Phytol 107:183–190

Tennant D (1975) A test of a modified line intersect method of estimating root length. J Ecol 63:995–1001

Tommerup IC, Abbott LK (1981) Prolonged survival and viability of VA mycorrhizal hyphae after root death. Soil Biol Biochem 13:431–433

Warcup JH (1967) Fungi in soil. In: Burges A, Raw F (eds) Soil biology. Academic Press, London, pp 51–110