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Miranda M. Hart · Richard J. Reader

Does percent root length colonization and soil hyphal length reflect the extent of colonization for all AMF?

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Abstract Percent root length colonization may not be an appropriate measure of root colonization by arbuscular mycorrhizal fungi (AMF) in all cases. We suggest that AMF will differ in how well percent root length colonization measures the amount of AMF colonization in the root due to differences among AMF in hyphal structure and hyphal aggregation. Although soil hyphal length accounts for hyphal density, we suggest that it does not consider differences in hyphal structure in measurements of external colonization and thus might also misrepresent the true amount of AMF in the soil. To test these suggestions, we measured and compared percent root length colonization and soil hyphal length with root ergosterol and soil ergosterol, respectively, for 21 different species of AMF from three families in a greenhouse experiment. Percent root length colonization predicted intra-radical colonization best for Glomaceae and Acaulosporaceae isolates, while soil hyphal length best represented soil ergosterol for Gigasporaceae isolates. The results show that conventional methods for estimating AMF colonization are not universal for all AMF. Caution is advised when drawing inferences for different groups of AMF.

Keywords Arbuscular mycorrhizal fungi · Root length colonization · Ergosterol · Fungal biomass · Glomaceae

Introduction

Quantifying arbuscular mycorrhizal fungi (AMF) colonization in roots and soil is important for determining both the abundance and functioning of AMF in ecosystems. Percent root length colonization is currently the most widely used measure of AMF colonization within roots. Methods for determining percent root length colonization are mostly based on the original gridline intersect method for determining root length by Newman (1966). Giovannetti and Mosse (1980) modified Newman's method by accounting for AMF structures at grid line intercepts, thereby obtaining a measure of fungal presence in the roots along with total root length. McGonigle et al. (1990) addressed this issue through further modification of Newman's method, whereby root segments were analyzed at the cellular level for fungal structures. While McGonigle's approach offers greater resolution of AMF structures, ability to describe the amount of AMF in a root is limited because it does not account for total root length.

Despite these difficulties, percent root length colonization using the adaptations of Giovannetti and Mosse or McGonigle remains the most widely used measure of AM status. However, there exist further confounding factors which may compromise the ability of this technique to accurately predict AMF colonization.

First, percent root length colonization does not account for structural differences among hyphae. Some AMF have more substantial hyphae than others. For example, members of the family Gigasporaceae (*Gigaspora* and *Scutellospora*) tend to have very thick and robust hyphae compared with AMF belonging to the families Glomaceae (*Glomus*) and Acaulosporaceae (*Acaulospora* and *Entrophospora*). This difference allows researchers to differentiate among AMF genera based on hyphal morphology alone (Abbott 1982, 1985; Lopez-Aguillon and Mosse 1987; Merryweather and Fitter 1998). Thus, a length of hyphae may represent a far greater biomass investment for Gigasporaceae than for Glomaceae and Acaulosporaceae isolates.

Second, percent root length colonization does not differentiate among AMF which have diffuse hyphae within a root and those whose hyphae occur in dense aggregations. For example, Jakobsen et al. (1992) showed that *Scutellospora calospora* external mycelium was densely aggregated near the roots of *Trifolium subterraneum* while other AMF isolates had a more uniform distribution (*Acaulospora laevis*, *Glomus* sp.). Differential aggrega-

M.M. Hart (✉) · R.J. Reader
Department of Botany, University of Guelph, Guelph, Ontario N1G 2W1, Canada
e-mail: mhart@uoguelph.ca
Tel.: +1-519-8244120 ext. 6718, Fax: 1-519-7671991

tion of hyphae has also been noted within roots (Gazey et al. 1992; Rillig et al. 1998, 1999). Because percent root length colonization measures only presence or absence, the extra biomass of densely aggregated hyphae would be missed. Others have attempted to circumvent this problem by qualifying the degree of colonization. Gazey et al. (1992) used the line intercept method (Newman 1966) and simultaneously assessed hyphal density by classifying each root intersecting the line into one of five different colonization intensities. Similar methods were utilized by Rillig et al. (1998, 1999). In all cases, however, such classification is an arduous, lengthy procedure.

The measurement of AMF mycelia in the soil avoids some of these problems. The external mycelium is typically measured using the modification by Miller et al. (1995) of the Newman (1966) method for root length determination. Because this approach considers hyphal density as well as total hyphal length, it should account for the hyphae from densely aggregating AMF. Structural differences among hyphae, however, may go undetected by this method, similar to percent root length colonization. A further obstacle associated with this approach is that there is no way of distinguishing between AMF and non-AM hyphae. Thus, values for soil hyphal length may be elevated where non-AMF densities greatly exceed AMF densities in the soil. There is some evidence for large differences in the amount of external mycelium produced for different groups of AMF. Hart and Reader (2002) showed that the external mycelium of Gigasporaceae isolates greatly exceeds that of Glomaceae and Acaulosporaceae isolates. It is not yet known how the presence of AMF affects the density of non-AM fungi. If non-AMF hyphae are more dense where AMF external mycelia are limited, then soil hyphal length may overestimate soil hyphal length for AMF with limited external mycelia.

Biomass measurements of AMF might be a better indicator of AMF colonization than percent root length colonization and soil hyphal length because biomass represents the total amount of fungus, not merely its presence or absence. For fungi, biomass is most commonly estimated by measuring the content of ergosterol, a fungal sterol found in cell membranes (Martin et al. 1990) which, thus, provides a measure of metabolically active fungal biomass (Ekbald et al. 1988; Nylund and Wallander 1992; Frey et al. 1994). Ergosterol has several advantages over percent root length colonization for quantifying AMF. First, it gives an estimate of quantity (versus occurrence) thereby accounting for particularly robust or dense hyphae. Second, because ergosterol estimates total amount of biomass, it accounts for differences in hyphal aggregations. Finally, this compound can be measured for both intra-radical and external hyphae, whereas percent root length colonization reflects only intra-radical colonization.

To date, there has been no attempt to establish the relationship between AMF colonization and soil hyphal length obtained through percent root length colonization versus biomass (ergosterol). Therefore, we compared percent root length colonization and soil hyphal length with ergosterol for 21 different species of AMF from three families.

Materials and methods

AMF isolates

We acquired 21 isolates from a collection at the University of Guelph and Premier Tech (Riviere du Loup, Quebec, Canada). These isolates were part of a larger study investigating life history strategies in AMF (Hart and Reader 2002). The isolates were chosen to represent each of the three AMF families Acaulosporaceae, Glomaceae and Gigasporaceae, and to provide replication at the genus level:

1. Family Acaulosporaceae
   – *Acaulospora morrowiae* Spain and Schenck
   – *Acaulospora spinosa* 1 Walker and Trappe
   – *Acaulospora spinosa* 2
   – *Entrophospora columbiana* Spain and Schenck

2. Family Gigasporaceae
   – *Gigaspora gigantea* (Nicol. & Gerd.) Gerdemann & Trappe
   – *Gigaspora margarita* (Becker & Hall)
   – *Scutellospora calospora* (Nicol. & Gerd.) Walker & Sanders
   – *Scutellospora heterogama* (Nicol. & Gerd.) Walker & Sanders
   – *Scutellospora pellucida* (Nicol. & Schenck) Walker & Sanders

3. Family Glomaceae
   – *Glomus aggregatum* Schenk & Smith emend. Koske
   – *Glomus claroideum* Schenck & Smith
   – *Glomus constrictum* Trappe
   – *Glomus etunicatum* Becker & Gerdemann
   – *Glomus geosporum* (Nicol. & Gerd.) Walker
   – *Glomus intraradices* Quebec1 (Q1) Schenck & Smith
   – *Glomus intraradices* Quebec 2 (Q2)
   – *Glomus intraradices* Kansas (K)
   – *Glomus intraradices* Israel (I)
   – *Glomus intraradices* France (F)
   – *Glomus intraradices* (J)
   – *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe

Inoculum

Isolates were equalized in terms of ergosterol. Samples of whole inoculum (spores and root fragments) from each isolate were homogenized and the ergosterol extracted with hexane by HPLC following the method of Grant and West (1986). The standard used was commercial ergosterol (5,7,22-ergostatrien-3b-ol). Based on the concentration of ergosterol in whole inoculum, each isolate was diluted down to a common ergosterol density.

In all cases, isolates were cultured prior to the experiment by growing the inoculum with leek (*Allium porrum* L.) as host. Leek, which is commonly used in trap cultures and is known to host a wide variety of AMF, was grown in Conetainers (4 cm × 20.5 cm) (Stuewe and Sons Inc. Corvallis, Ore.) which were two-thirds filled with a sterile, low-P potting soil and silica sand mixture in a 1:1 ratio. Throughout the experiment, soil was sterilized by heating to 120°C for 30 min, left to cool overnight, heated again to 120°C for 30 min, then left for 1 week prior to planting. A layer of inoculum was added to each container (this differed depending on the ergosterol content of each isolate) and each pot was filled with additional soil-sand mixture. Three *A. porrum* seedlings were then added and grown for 30 days, at which point they were harvested by removal of the entire shoot by hand. The inoculum soil was then used in the following experiment.

Experimental treatments

Two factors were tested in a completely randomized factorial design with five replicates for each experimental treatment. The two factors were AMF isolate and host plant. We used 21 different AMF isolates as described above and four host plants: English plantain (*Plantago lanceolata* L.), common plantain (*Plantago major* L.), Kentucky-blue grass (*Poa pratensis* L.) and annual blue grass (*Poa annua* L.). These species were chosen as highly mycotrophic plants common to old fields and meadows (which was the origin of most of the AMF isolates). Three seedlings of each spe-

cies (approximately 1 cm radicle) were added to each Conetainer, at which point 50 ml of soil filtrate (mesh size 3 μm) from each isolate was added to each Conetainer to control for differences in other soil organisms among Conetainers. Plants were randomized on 14 greenhouse benches (1.5 m × 8 m) at Premier Tech, Riviere-du-Loup, Quebec, Canada for 12 weeks between July and October 2000. Each Conetainer was subjected to 14 h of supplemented light (12.2 W/m^2 over a 24-h period). Conetainers were watered and fertilized as needed with a low-P fertilizer.

As dependent variables, we measured intra-radical colonization and external hyphal colonization. Extent of intra-radical colonization was determined at week 12 by measuring percent root length colonization and root ergosterol. For both methods, the root system of the host plant was cleaned by first shaking off excess soil and then washing the roots with water. Next, roots were sonicated for 15 s to remove any residual matter, including external mycelium. The clean root system was then cut into 2-cm fragments and eight fragments were randomly selected for percent root length colonization measurement. These were stained with Chlorazol Black (Brundrett 1991) and mounted on glass slides. Fungal material (AMF hyphae or spores or vesicles) was recorded using a gridline intersect method (McGonigle et al. 1990) and the percentage of 100 intersections 'colonized' was calculated. Root ergosterol was assessed by the method cited above. Eight 2-cm fragments of clean roots were randomly selected for root fungal biomass analysis and homogenized before extraction.

Extent of external hyphae was determined at week 12 by measuring soil ergosterol and soil hyphal length. The ergosterol of the external mycelium was determined by the extraction methods given for root ergosterol, but using 20 ml of root-free soil. Soil hyphal length was determined by a modified gridline intercept method from Miller et al. (1995). A 10-g portion of soil from each Conetainer was suspended in 250 ml of water and sodium hexametaphosphate (3.6% w/v) was added for 16–18 h to break up soil aggregates. The soil suspension was then agitated in a blender at high speed for 2 min and stirred with an electronic stir bar. One 6-ml aliquot per sample was removed from halfway between the beaker edge and the vortex and added to 250 ml of distilled water plus 30 ml of sodium hexametaphosphate solution. This mixture was stirred to resuspend hyphae and 10-ml aliquots were transferred to 50-ml centrifuge tubes and centrifuged five times at 1,000 *g*. Pellets were resuspended in glycerol and centrifuged again at 75 *g* for 30 s. The supernatant was filtered onto a 20-μm polyester filter which was then stained with Chlorazol Black and decanted over 1.2-μm nitrocellulose filter paper. These filters were mounted on glass slides, dried and made transparent by mounting in immersion oil. Hyphae were recorded at 140 intersections per sample and hyphal length per gram dry soil was then calculated as described by Miller et al. (1995).

Background fungi

To control for non-AM fungi, we set up identical treatments but without AMF inoculum.

Statistical analysis of predictions

We used SPSS for Windows. Release 7, 1995 in all analyses. Linear regression analysis was used to measure the relationship between percent root length colonization and root ergosterol or soil ergosterol. To test whether the relationship between percent root length colonization and fungal biomass depended on the species of host plant, a two-way analysis of variance (ANOVA) was carried out with AMF family and host species as the two factors.

Results

Background fungi

Our control pots did not contain AMF; no intra-radicle colonization by AMF structures (arbuscules, vesicles, coils) was observed. Root ergosterol, however, was 0.07–0.41 μg/g dry root, indicating the presence of non-AM fungi. Similarly, soil ergosterol was found at 0.15–0.37 μg/g dry soil.

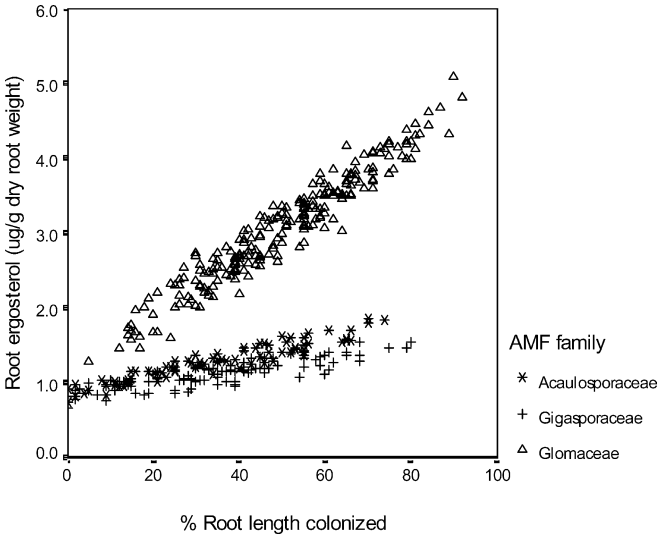

Fig. 1 Relationship between percent root length colonization and root ergosterol for three AMF families

Root ergosterol

There was a significant interaction between AMF family and host plant species in ANOVA ($F_{6,419}$=2.24, P=0.04). However, since the trends were similar for all four hosts, data were pooled for further analysis.

Overall, percent root length colonization and root ergosterol had a positive, linear relationship (R^2=0.51, P<0.01) (Fig. 1, Table 1). However, this relationship was stronger when we compared different groups of AMF. Most variation in root ergosterol for Glomaceae and Acaulosporaceae isolates was accounted for by percent root length colonization (R^2=0.93 and 0.89, respectively, P<0.01 for both). For Gigasporaceae isolates, there was also a positive, linear relationship between percent root length colonization and root ergosterol, with most variation explained by percent root length colonization (R^2=0.75, P<0.01).

There was a large difference between the AMF families in the slope of the relationship between percent root length colonization and root ergosterol (Fig. 1, Table 1). While all families had similar *y* intercepts, Glomaceae isolates had a much greater slope (1.1) than Acaulosporaceae (0.87) and Gigasporaceae (0.85) isolates.

Soil ergosterol

There was a significant interaction between AMF family and host plant species in ANOVA ($F_{6,419}$=8.38, P<0.001). Again, trends were similar for all four host plants and data were pooled for further analysis.

Table 1 Results of linear regression analysis of the relationship between percent root length colonization (independent variable) and fungal biomass (dependent variable). All values of F are significant at $P<0.001$. For all measures of fungal biomass, degrees of freedom are equal to 1,239 (Glomaceae), 1,79 (Acaulosporaceae), 1,99 (Gigasporaceae) and 1,419 (All AMF families) (R^2 coefficient of determination, F F-value testing the statistical significance of the regression)

Analysis	AMF family	R^2	F	Slope	y Intercept
Root ergosterol versus percent root length colonization	Glomaceae	0.93	2970	0.04	1.1
	Acaulosporaceae	0.89	653	0.01	0.87
	Gigasporaceae	0.75	288	0.008	0.85
	All AMF families	0.51	434	0.04	0.60
Soil ergosterol versus soil hyphal length	Glomaceae	0.43	177	0.26	0.11
	Acaulosporaceae	0.47	70	0.26	0.12
	Gigasporaceae	0.92	1112	0.37	0.22
	All AMF families	0.99	35179	0.42	–0.18

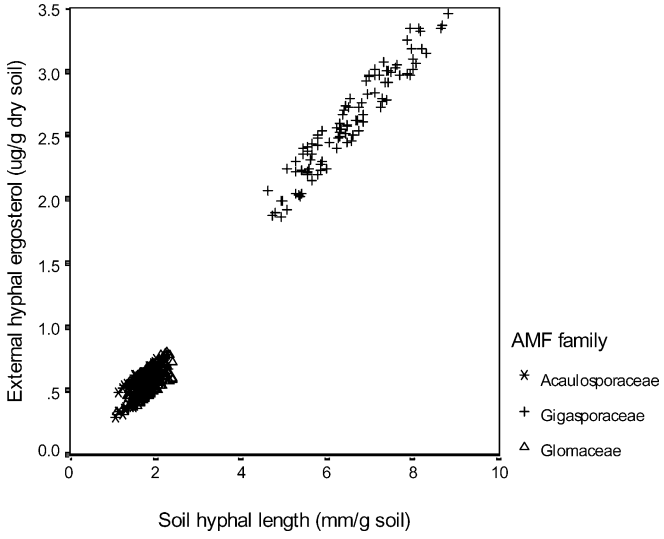

Fig. 2 Relationship between soil hyphal length and soil ergosterol for three AMF families

Overall, there was a very strong relationship between soil hyphal length and soil ergosterol for the external mycelium with almost all variation in soil ergosterol accounted for by soil hyphal length (R^2=0.99, P<0.01) (Fig. 2, Table 1). However, the relationship was not as strong for different groups of AMF. Soil ergosterol for Glomaceae and Acaulosporaceae isolates, though related to soil hyphal length (R^2=0.43 and 0.47, respectively, P<0.01 for both), was less reliably predicted from soil hyphal length than Gigasporaceae isolates (R^2=0.92, P<0.01).

There was little difference between the AMF families in the slope of the relationship between soil hyphal length and soil ergosterol, with slopes for all families ranging between 0.26 and 0.37 (Fig. 2, Table 1). In this case, the y intercept for Gigasporaceae isolates (0.22) was different to those of Glomaceae and Acaulosporaceae isolates (0.11 and 0.12, respectively)

Discussion

Percent root length colonization is a good predictor of root ergosterol for all groups of AMF. For all families there was a significant positive, linear relationship and much of the variation in root ergosterol was accounted for by percent root length colonization. However, there was some variation. While root ergosterol for Glomaceae and Acaulosporaceae isolates was highly correlated with and predicted by percent root length colonization, the relationship between these variables was less consistent for Gigasporaceae isolates. This may be due to differences among the families in their mycelia. Gigasporaceae isolates have very thick and robust hyphae compared with the other AMF families. This reflects a sizable investment on the part of Gigasporaceae isolates, but may not be detected by the presence/absence approach of percent root length colonization. In the same way, densely aggregated hyphae, as reported for a Gigasporaceae isolate by Jakobsen et al. (1992), would also be poorly represented by percent root length colonization. Such discrepancies could account for the high variation in the relationship between root ergosterol and percent root length colonization for Gigasporaceae isolates.

Soil hyphal length was a less reliable predictor of soil ergosterol when considering AMF families separately. While all families showed a positive, linear relationship between soil ergosterol and soil hyphal length, there was considerable variation among the AMF families in how well soil hyphal length accounted for variation in soil ergosterol. For Gigasporaceae isolates, soil hyphal length and soil ergosterol had a near perfect relationship. For Glomaceae and Acaulosporaceae isolates, however, soil hyphal length accounted for little of the variation in soil ergosterol. The reasons for this discrepancy are unclear. It suggests that soil hyphal length determination is less precise for Glomaceae/Acaulosporaceae isolates. It may be that the particularly limited and fine external mycelia of these AMF compared with Gigasporaceae isolates makes them difficult to observe and therefore quantify. It is unlikely that the non-specific nature of soil hyphal length determination was responsible for the discrepancy because soil ergosterol measurements are also non-specific. Therefore, both measurements should give a value for the total amount of fungal hyphae present in the soil.

It is important to note the differences in slope among AMF families for root colonization (Fig. 1). This discrepancy suggests one of two things. First, it may be that

AMF families are uniform in the concentration of ergosterol they contain per unit tissue. If this were true, then the differences observed would be due to differences in the amount of fungal tissue (i.e., biomass) per unit percent root length colonization, as we predicted. However, it may also be that AMF families differ in ergosterol concentration per unit tissue. In this case, fungal biomass per unit percent root length colonization may be similar and the families differ only in the concentration of ergosterol in their tissues. However, soil hyphal length measurements (which account for differences in hyphal aggregation and therefore total hyphal quantity) were strongly correlated with measurements of soil ergosterol. Further, there was no difference among AMF families in the slope of the relationship between soil hyphal length and soil ergosterol measurements (Fig. 2). These facts suggest that ergosterol is a good indicator of biomass for all AMF fungi and that differences among families observed in Fig. 1 were due to differences in AMF biomass and not to difference in tissue ergosterol concentrations. In order to fully exploit ergosterol as a tool for measuring AMF colonization, it would be extremely useful to establish the relationship between ergosterol and AMF biomass for different groups of AMF.

Because this study used ergosterol measurements as an estimate of fungal biomass and ergosterol is non-specific among fungi, it is possible that our measurements were influenced by background, non-AM fungi. However, the results from control treatments suggest that background ergosterol for non-AMF treatments were substantially lower than the minimal ergosterol values for all AMF treatments. It is possible that AMF differentially affect the growth of non-AM fungi. However, while the nature of the interaction between AMF and non-AM fungi in the AMF treatments is unknown, it is unlikely that non-AM fungi contributed significantly to our results due to such low levels of non-AMF fungi in the absence of AMF.

In general, percent root length colonization is a good indicator of AMF colonization within the roots for all groups of AMF, but is less precise for Gigasporaceae isolates. However, due to large differences between AMF in the ratio of internal:external colonization, it should not be used as a surrogate measurement of total AMF colonization. Soil hyphal length is an excellent estimator of the external mycelium for AMF for Gigasporaceae isolates, but is less reliable for AMF with limited external mycelia (Acaulosporaceae and Glomaceae). These differences are likely due to family differences among AMF in the structure and distribution of hyphae.

The results of this study show that an estimation of colonization which is both qualitative and quantitative (such as ergosterol content) might provide a more accurate representation of total AMF colonization (roots and soil). The results suggest that ergosterol estimation of AMF biomass, despite being non-specific to AMF, is a reliable method for estimating the size of AMF colonization in the roots and soil.

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