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ON-FARM PRODUCTION OF ARBUSCULAR MYCORRHIZAL FUNGUS INOCULUM IN COMPOST AND VERMICULITE MIXTURES: RESULTS OF ON-FARM DEMONSTRATIONS AND IMPACT OF COMPOST MICROBIOLOGICAL QUALITY

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ABSTRACT

The sustainability and profitability of many agricultural systems can be enhanced through the utilization of inoculum of arbuscular mycorrhizal [AM] fungi. Inocula are commercially available, but inoculum can also be produced on-farm in mixtures of compost and vermiculite with a nurse host plant. Demonstration of the on-farm system at a network of cooperating farms produced inocula with an average potency of 297 ± 43 propagules of AM fungi cm^{-3} (mean ± SEM, n= 40 site years). Spread of colonization in host roots was greater for bacterial- vs. fungal-dominated compost, but compost dilution ratio with vermiculite was a more important determinant of AM fungus spore production than compost type.

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INTRODUCTION

Arbuscular mycorrhizal [AM] fungi are naturally occurring soil fungi that form a mutualistic symbiosis with the majority of crop plants. The fungi are considered to be obligate symbionts, dependent upon receipt of photosynthetically fixed carbon by fungal tissues within the roots for further growth and reproduction (Shachar-Hill et al., 1995). Among the benefits to the host plant that are ascribed to the symbiosis are enhanced nutrient uptake, disease resistance, and drought resistance (Smith & Read, 2008). The most commonly reported benefit is the enhancement of uptake of nutrients that are immobile in the soil solution, i.e. P, Zn, and Cu. The extraradical phase of the fungus acts, in effect, as an extension of the plant's root system to explore a greater volume of soil for these nutrients. Positive effects of AM fungus colonization upon plant growth and yield in experimental situations are primarily noted in soils of low nutrient status.

The potential benefits to crop growth and yield make utilization of the AM symbiosis essential for agricultural systems which reduce or eliminate synthetic chemical inputs. Generally speaking, there are two ways to take better advantage of the symbiosis: 1) adopt agricultural practices that enhance the functioning of the indigenous community of AM fungi in the soil, and 2) inoculate with effective isolates of AM fungi (Bagyaraj, 1992). The first strategy fits most effectively with row crop farms and the second with vegetable and horticulture growers. AM fungus inoculum applied to the field at the time of sowing row crops must have a large enough propagule density to compete with the indigenous AM fungus population, which can be cost prohibitive. These farmers can utilize the symbiosis better through the adoption of AM fungus-friendly management practices such as overwintering cover crops (Galvez et al., 1995), reduced tillage (McGonigle & Miller, 1993), and crop rotation (Johnson et al., 1992). Vegetable crop farmers who produce their own seedlings for later outplanting to the field can more economically utilize inoculum of AM fungi by adding inoculum to the potting mix in which seedlings are grown during the greenhouse phase of production. These seedlings then have the benefit of a fully functional symbiosis upon transplant to the field, and do not have the lag time that would otherwise be necessary until they are colonized by the indigenous population. This phenomenon likely is a significant contributor to enhanced yields with AM fungus inoculation even in high P soils (Douds & Reider, 2003; Douds et al., 2012).

Farmers seeking to use AM fungus inoculum have two options: purchase it commercially or produce it on-the-farm. Commercially available inocula are produced in a variety of ways (IJdo et al., 2011) and come in many forms ranging from concentrated mixtures to be added to media or already incorporated into potting media, to preparations with instructions for inoculation via watering or emersion of roots prior to transplanting. There are several published reports comparing the efficacy of commercial inocula to researchers' in-house produced inocula and results vary considerably (Corkidi et al., 2004; Tarbell & Koske, 2007; Wiseman et al., 2009). Another option is for farmers to produce inoculum of AM fungi themselves, on-farm. Pioneering research on the on-farm production of AM fungus inoculum occurred in the tropics (Sieverding, 1991; Gaur, 1997; Gaur et al., 2000; Maiti et al., 2009). These methods can produce indigenous or inoculated isolates of AM fungi using raised beds of soil, typically after fumigation.

We have developed a method for the on-farm production of AM fungus inoculum in compost and vermiculite mixtures that is suitable for temperate climates (Douds et al., 2006). This method can produce isolates of AM fungi introduced from off-site via colonization of the host plant prior to transplant into the mixture. Communities of AM fungi indigenous to the site/farm can be propagated through the introduction of a small amount of field soil to the compost and vermiculite mixture (Douds et al., 2010). A similar method was recently reported which utilizes lignocellulose agrowastes such as sugar cane bagasse instead of compost, and sand and rice shell as diluents instead of vermiculite (Schlemper & Stürmer, 2014).

The productivity of the on-farm AM fungus inoculum production system depends in part upon the selection of an optimal compost and vermiculite mixture ratio. Predictive equations were developed which utilize compost nutrient analyses to calculate the fraction of compost to be used in the production system (Douds, et al., 2008). No consideration was given in the prior work to the compost microbiological quality, i.e. whether it is fungal- or bacterial-dominated (Ingham & Slaughter, 2004). The objectives of this work are 1) to present the on-farm system as a viable alternative to commercially-available inocula by demonstrating the reliability of the system on working farms, 2) to contrast the performance of the method when conducted with fungal, bacterial, and a 1:1 [v/v] mixture of the two composts over a range of compost:vermiculite dilution ratios, and 3) compare the resulting optimal ratios to optimal ratios calculated by the equations produced earlier using nutrient analyses (Douds et al., 2008).

MATERIALS AND METHODS

ON-FARM DEMONSTRATIONS OF INOCULUM PRODUCTION

Inoculum of AM fungi was produced on-farm from 2003 through 2012 on several farms in southeastern Pennsylvania and one each in Maine and Rhode Island according to the method previously described (Douds et al., 2006). Briefly, a 1:4 [v/v] mixture of yard clippings compost and vermiculite, respectively, was prepared and placed into seven gallon black plastic bags ("Grow Bags," Worm's Way, Bloomington, IN 47404). The compost for the SE Pennsylvania farms was produced in windrows at the Lehigh Valley Composting Facility in Allentown, PA from yard clippings and leaves. Five bahiagrass (*Paspalum notatum* Flugge) seedlings, colonized by one of the following AM fungi: *Rhizophagus intraradices* Schenck & Smith (DAOM 181602); *Funneliformis geosporum* (Nicolson & Gerdemann) Walker, *Claroideoglomus etunicatum* Becker & Gerdemann, *Claroideoglomus claroideum* Schenck & Smith, and *Funneliformis mosseae* (Nicol. & Gerd.) Gerdemann & Trappe, originally isolated from the Rodale Institute Experimental Farm; and *Glomus* sp., isolated from the Stoneleigh Estate, Villanova, PA were transplanted into the bags typically during the last two weeks of May (Figure 1). Bags were weeded and watered as needed throughout the growing season, with no supplemental fertilization necessary. The P. notatum host plants were winter killed, and the AM fungi over wintered outdoors in the growth medium.

Figure 1. On-farm production of inoculum of AM fungi. Seven-gallon bags contain mixtures of compost and vermiculite. Bahiagrass (*Paspalum notatum*) seedlings, pre-colonized by AM fungi, are transplanted into the bags and act as the nurse host plant.

COMPOST MICROBIOLOGICAL QUALITY EXPERIMENT

Composts were produced in piles at The Rodale Institute Experimental Farm, Kutztown, PA. The fungal-dominated compost was initiated with a ratio of manure: green vegetation: woody material of 3:3:4 [v/v/v]. The bacterial compost began with a manure:green vegetation: woody material ratio of 3:4:3 [v/v/v]. Piles were hand turned and stored outdoors until use. Composts were sieved through a 1 cm mesh prior to use. Samples of the composts were withheld for microbiological and chemical analyses. Chemical analyses were conducted by the Penn State Agricultural Analytical Services Laboratory.

A quantitative analysis of soil microbiology was conducted according to the methods of Ingham (2004). Briefly, four replicate 1g samples from the fungal and bacterial composts were taken and diluted 1:5 [wt/vol] with distilled water. A microscope slide was prepared using a drop of the suspension and covered with a cover slip. Bacteria were counted in 20 replicate one quarter fields of view under a microscope at 400X magnification. Mean number of bacteria per field of view was converted to total bacteria per g compost using an appropriate multiplication factor, and biomass of bacteria was calculated assuming an average mass per bacterium of 2 pg (Hall, 1996). Fungi were quantified microscopically at 400 X magnification (Ingham & Klein, 1984). Twenty randomly selected fields of view were observed, and width and length of each fungal hypha encountered were recorded. Biomass of fungal hyphae then was calculated first by converting these values to volume, followed by appropriate multiplication factors to calculate total volume of hyphae per g of compost, and finally volume was converted to biomass using the average density of fungi of 0.33 g cm^{-3}.

The experiment was initiated on June 7, 2013 and the experimental design was similar to that previously reported for an experiment to predict compost:vermiculite dilution ratios based upon compost chemistry (Douds et al., 2008). The experiment was conducted as a complete factorial with three

factors: compost (fungal, bacterial, and fungal + bacterial mix), compost:vermiculite dilution ratio (1:0, 1:2, 1:9, 1:49 v/v), and AM fungus (*Gigaspora margarita* Becker & Hall [INVAM PA201] and *Glomus* sp., both originally isolated from The Rodale Institute). There were three replicate seven gallon "Grow Bags" per compost X dilution ratio X AM fungus treatment combination. Five *P. notatum* seedlings transplanted into each bag, one AM fungus per bag. The seedlings had been grown for two months in a greenhouse in the presence of one of the two AM fungi to establish colonization prior to outplanting to the bags. Preliminary sampling of the roots of the *P. notatum* indicated average percentage root length colonized of 19.1 ± 0.7 and 1.6 ± 1.6 (mean ± SEM, n=3) for the plants inoculated with *Glomus* sp. and *G. margarita*, respectively. Bags were weeded and watered as needed with no supplemental fertilization. Data was collected after the *P. notatum* shoots were winter killed.

DATA COLLECTION AND ANALYSIS

Samples were collected from the bags on the various farms in the late autumn, after *P. notatum* was frost killed yet before the compost and vermiculite mixture froze for the winter to quantify on-farm AM fungus inoculum production. One pooled sample was taken from each bag, each the result of sampling the media from two to three positions per bag, between the planting positions of the original *P. notatum* seedlings to avoid the original rhizospheres. Samples were stored in zip lock bags at 4 ºC until analysis. AM fungus propagule density was determined via Most Probable Number [MPN] bioassay using a pooled sample from all bags of a given farm (Alexander, 1965). The pooled sample was used because the farmers would be mixing the inoculum prior to use, hence producing a mixed species inoculum. Dilutions for the MPN assays ranged from 10^{-1} to 10^{-5}, with three to five replicates per dilution and *P. notatum* as the bioassay host. Plants were grown in a controlled environment chamber for four weeks, after which time entire roots systems were recovered, stained with trypan blue (Phillips & Hayman, 1970), and scored under a dissecting microscope for presence or absence of AM fungus colonization.

Data collection for the compost microbiological quality experiment began November 22, 2013. Shoots were removed and total fresh weight was recorded. Subsamples of each shoot were rinsed and dried in a forced draft oven at 80 ºC for 3 days. Total shoot dry weights were calculated from the mean dry:fresh wt ratios of an additional six larger subsamples per AM fungus treatment. The media plus roots of each bag was transferred to a bin, mixed thoroughly, and root and media samples were removed (one each per bag) and stored in zip lock bags at 4 ºC until analysis. Roots were stained with trypan blue (as above) and percentage root length colonized by AM fungi was quantified via the gridline intersect method (Giovannetti & Mosse, 1980). Spores of AM fungi were isolated from the compost and vermiculite mixture via wet sieving and centrifugation (Jenkins, 1964; Gerdemann & Nicholson, 1963) and quantified under a dissecting microscope at 20X magnification. Shoot tissue was ground to pass a 20 mesh sieve, and N and P were determined after H_2SO_4 and H_2O_2 digestion via the methods of Wall and Gehrke (1975) and Murphy and Riley (1962), respectively.

Data were analyzed using analysis of variance. Percentage root length colonized data were first arc sin transformed. Measurements for which significant treatment effects were seen were characterized further using Tukey's Method of Multiple Comparisons (α=0.05).

RESULTS

ON-FARM INOCULUM PRODUCTION

Inoculum of AM fungi was successfully produced every attempt at cooperating farms (Table 1). Mean inoculum density over the study period was 297 ± 43 propagules cm^{-3} (mean ± SEM, n=40 site years). Inoculum density ranged from a high of 1200 cm^{-3} to lows of 12 and 16 cm^{-3}. The low values were likely due to location of the inoculum production bags in a shady site in one instance and intermittent grazing due to the bags being located too close to a horse pasture in another.

Farm	**Inoculum (propagules cm^{-3})**									
	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Cedar Meadow Farm Holtwood, PA	16	350	717							
Meadow View Farm Kutztown, PA		250	400							
Eagle Point Farm Kutztown, PA		155	155	1200	400	400	155	250	155	400
Shenk's Berry Farm Lititz, PA	250	155	400		155	250	40	12	25	40
Covered Bridge Farm Oley, PA	72	250	1073							
Somerton Tanks Farm Philadelphia, PA		72	250							
The Rodale Institute Kutztown, PA				400	717	250	717	400	183	155
Bacon Bros Farm Prudence Island, RI							500		400	
Highmoor Farm Monmouth, ME								25	16	72

Table 1. Inoculum of AM fungi produced on-farm in bags containing mixtures of compost and vermiculite using *Paspalum notatum* as the nurse host plant. Results of Most Probable Number bioassays on a pooled sample from 6-8 bags per farm.

COMPOST MICROBIOLOGICAL QUALITY EXPERIMENT

CHARACTERISTICS OF BACTERIAL VS. FUNGAL COMPOSTS

The composts utilized were equivalent in N status, but since the fungal compost tended to be higher in P it had a lower N:P ratio compared to the bacterial compost (Table 2). The fungal compost had significantly higher organic matter and carbon contents than the bacterial compost, reflecting the differences in feedstock percentages. Bacterial biomass did not differ significantly between the two composts, however no fungi were observed in the bacterial compost (Table 2).

Compost Chemistry			
Analyte	**Fungal Compost**	**Bacterial Compost**	**ANOVA (Pr>F)[1]**
pH	7.2	7.7	0.0020
Organic matter (%)	51.0 ± 1.1	39.3 ± 0.4	0.0006
Carbon (%)	29.9 ± 1.1	25.2 ± 0.1	0.0119
Total N (%)	2.5 ± 0.1	2.4 ± 0.1	0.8203
P_2O_5 (%)	2.67 ± 0.17	1.79 ± 0.28	
P (elemental)	1.17 ± 0.07	0.78 ± 0.12	0.0553
K_2O (%)	0.87 ± 0.02	0.72 ± 0.10	
K (elemental)	0.73 ± 0.02	0.60 ± 0.08	0.2109
C:N (ratio)	12.2 ± 0.9	10.4 ± 0.3	0.1380
N:P (ratio)	2.12 ± 0.11	3.28 ± 0.52	0.0976
Compost Biology			
Bacteria (µg g^{-1} compost)	1569 ± 262	1134 ± 93	0.1686
Fungi (µg g^{-1} compost)	311 ± 88	0 ± 0	0.0120

[1]Pr>F less than or equal to 0.05 indicates statistically significant difference in values across the columns.

Table 2. Compost chemistry (data from the Penn State Agricultural Analytical Services laboratory, n=3 +/- SEM, dry wt basis) and microbiological status (n=4 +/- SEM) at time of initiation of the experiment.

IMPACT UPON GROWTH OF *P. NOTATUM*

All factors studied had significant impacts upon the growth of *P. notatum* (Table 3). Pre-colonization of plants by *Glomus* sp. resulted in greater eventual biomass than did colonization by *G. margarita* (Pr>F <0.0001). Plants growing in the bacterial dominated compost produced less biomass than those growing in the other composts (Pr >F=0.0001) while, as may have been expected, shoot biomass declined significantly with decreasing amount of compost in the growth media. P concentration in the shoots after senescence was affected by AM fungus, dilution ratio, and the interaction of compost and dilution ratio. Plants inoculated with *G. margarita* had a higher P concentration than those inoculated with *Glomus* sp. (Pr>F=0.0004) and P concentration tended to increase with decreasing compost concentration (Pr>F=0.0005). These observations indicate the impact of a dilution effect: higher concentrations were found in treatments with lower biomass, rather than AM fungus-mediated differences in P uptake per se.

IMPACT UPON AM FUNGI

AM fungus species, compost type, and compost:vermiculite mixture ratio all had significant effects upon percentage of P. notatum root length colonized by AM fungi (Pr>F <0.0001, Table 3). Plants initially inoculated with *G. margarita* were more colonized than those inoculated with *Glomus* sp. at the end of the experiment, even though the situation was reversed at the time the inoculum production bags were initiated. Development of colonization progressed in the order bacterial > fungal + bacterial > fungal-dominated compost, while the 1:9 compost vermiculite dilution produced the greatest colonization and the 1:0 (100% compost) treatment the least.

A. Full model ANOVA				
Source		**Pr>F**		
	Biomass (g)	**%P**	**%N**	**Colon. (%)**
AMF	<.0001	0.0004	<.0001	<.0001
Compost	0.0001	0.1234	0.5604	<.0001
AMF X Compost	0.2660	0.3080	0.1616	0.0280
Dilution Ratio	<.0001	0.0005	<.0001	<.0001
AMF X Ratio	<.0001	0.6067	0.8503	0.0699
Compost X Ratio	0.0312	0.0047	0.4544	<0.0001
AMF X Comp X R	0.0563	0.3552	0.0017	0.6582
B. Main effects separation of means				
1. AM fungus				
G. margarita	93.6 b	0.239 a	0.883 b	36.3 a
Glomus sp	117.1 a	0.202 b	1.165 a	24.7 b
MSD	9.0	0.194	0.0745	4.6
2. Compost type				
Fungal	118 a	0.206 a	1.017 a	12.7 c
Fungal + Bacterial	106 a	0.227 a	1.003 a	27.9 b
Bacterial	92 b	0.229 a	1.051 a	50.9 a
MSD	13.3	0.0286	0.1097	6.7
3. Compost:vermiculite dilution ratio [v/v]				
1:0	162 a	0.194 c	1.246 a	17.1 c
1:2	125 b	0.201 bc	0.849 c	30.0 b
1:9	82 c	0.251 a	0.993 c	52.5 a
1:49	52 d	0.231 ab	1.077 b	22.3 bc
MSD	16.9	0.0364	0.1394	8.6

[1]Numbers in the same column, within a factor, followed by the same letter are not significantly different (α=0.05, Tukey's Method of Multiple Comparisons), means of 36 observations for AM fungus treatment, 24 for compost type, and 18 for dilution ratio, MSD= minimum significant difference.

Table 3. Results of ANOVA on the impact of AM fungus (*G. margarita* or *Glomus sp.), c*ompost microbiological quality (fungal, bacterial, or a mixture of both), and compost and vermiculite mixture ratio (1:0, 1:2, 1:9, and 1:49 v/v) upon aboveground biomass, %P, %N of the host plant (*Paspalum notatum*), and AM fungus colonization of roots.[1]

Though plants initially inoculated with *G. margarita* were well colonized at the end of the experiment, eg. 81 ± 1.8% of root length (mean ± SEM, n=3) for 1:9 bacterial compost:vermiculite, very few *G. margarita* spores were produced and they were outnumbered by the incidental AM fungi introduced to the compost via incorporation of small amounts of soil when the piles were turned and/or inclusion of colonized root systems as part of the vegetative biomass in the compost feedstock (data not shown) (Douds et al., 2006). Further analysis, therefore, will be presented only for the *Glomus* sp. Colonization of roots in bags inoculated with *Glomus* sp. was significantly affected by both compost type and dilution ratio (Pr>F <0.0001) (Figure 2A). Percentage root length colonized averaged 41.3, 22.0, and 10.9 percent for the bacterial, bacterial+fungal, and fungal-dominated composts, respectively. The 1:9 compost:vermiculite dilution ratio produced the greatest colonization, 41.9% of root length, while there was no significant difference among the other three ratios. Compost type and dilution ratio had no significant effect upon spore production by *Glomus* sp., Pr>F =0.7143 and 0.0600, respectively, but the interaction was significant (Pr>F= 0.0197). Sporulation was unaffected by dilution ratio in the bacterial+fungal compost, while the fungal compost tended to produce more spores at the 1:2 dilution (33% compost) and the bacterial compost at the 1:0 (100% compost) treatment (Figure 2B).

Using the compost nutrient analyses (Table 2) and the earlier published equations for the AM fungus *Glomus mosseae* (Douds et al., 2008) to predict optimal fraction of compost in the mixture for sporulation of *Glomus* sp. used here, yielded optimal fractions of compost of 0.37 and 0.39 for the fungal- and bacterial-dominated composts (37 and 39 percent), respectively. The predictive equation did well for the fungal but not for the bacterial-dominated compost (Figure 2B).

DISCUSSION

The AM fungus productivity of the on-farm inoculum production system appeared to be affected more by the compost's nutrient levels rather than its fungal- vs. bacterial-dominated microbiological quality. The development of the AM symbiosis can be considered from the perspectives of the plant or fungus. From the plant's perspective, the AM symbiosis is governed by the availability of P: the lower the P availability the more the plant will allow colonization by the fungus in order to take advantage of the symbiosis for nutrient uptake. This occurs through the root exudation of signal molecules that stimulate AM fungus hyphal growth and branching in the rhizosphere. These are produced in greater quantities under P limiting conditions (Nagahashi & Douds, 2000). Conversely, the plant has several strategies to limit colonization under high P availability. First, root exudates have low hyphal-branching activity under high P availability (Nagahashi & Douds, 2000). In addition, the plant can limit the supply of sugar available to the fungus within the root, thereby limiting the spread of colonization (Treseder & Allen, 2002). From the perspective of the AM fungi, the benefit of the symbiosis is photosynthetically-produced sugar (Shachar-Hill et al., 1995). AM fungi are considered to be obligate symbionts, dependent upon receipt of photosynthetically fixed carbon by fungal tissues within the roots for further growth and reproduction.

Therefore, one can envision the optimal situation in which the available P is low enough for abundant colonization by AM fungi, yet N and other nutrients are in sufficient quantities to allow for healthy shoot growth and rates of photosynthesis to make sugar available for the fungi. On this basis, the nutrient analyses of the fungal- and bacterial-dominated composts (Table 2) would lead to the prediction that the bacterial compost, being lower in P and higher in N:P ratio than the fungal-dominated compost, would not require as much dilution with vermiculite as would the fungal compost. This prediction was correct with respect to AM fungus colonization of roots: the bacterial compost exhibited significant colonization at 100% compost while there was little colonization in the fungal compost (Figure 2A). Spore production and colonization are often not directly proportional, however (Hetrick & Bloom, 1986) as was seen here (Figure 2).

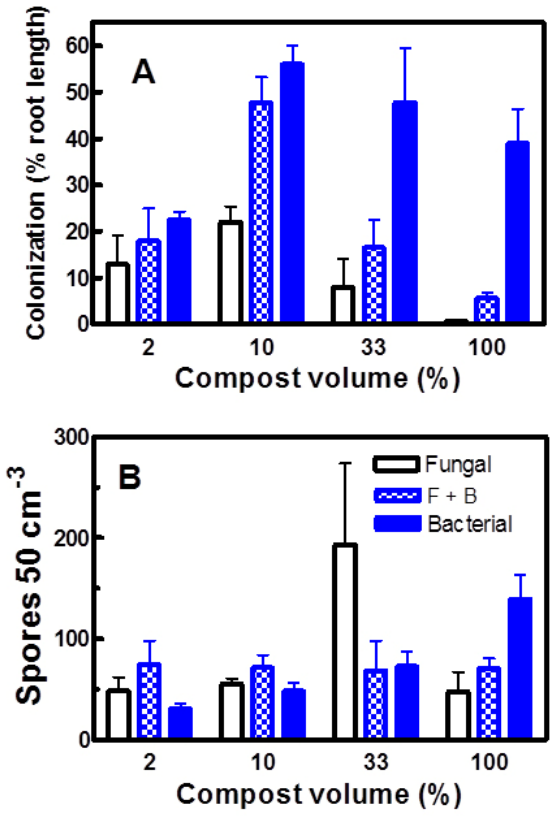

Figure 2. AM fungus colonization of roots of *Paspalum notatum* (A), and spore population of *Glomus* sp. (B) in the on-farm AM fungus inoculum production bags containing compost:vermiculite dilution ratios of 1:0, 1:2, 1:9, and 1:49 [v/v] (100%, 33%, 10% and 2% compost, respectively), n=3 ± SEM.

On-farm production of AM fungus inoculum is a reliable and viable alternative to commercially-available inocula. The average propagule density produced over 40 site years of 297 propagules cm^{-3} is significantly greater than the 80-100 propagules cm^{-3} necessary to qualify as mass production (Feldmann & Grotkass, 2002). Producing or purchasing inoculum of AM fungi is only the first step to utilizing the mycorrhizal symbiosis to increase the sustainability of an agricultural system. Growers may need to modify their greenhouse culture regimes to allow for colonization of roots by AM fungi to fully realize the benefits of the inoculum (Meikle & Amaranthus, 2008). Indeed, this challenge, particularly as faced by organic farmers, is a focus on ongoing research.

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