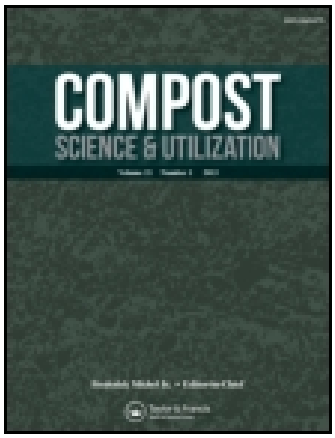

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ABSTRACT. On farm production of arbuscular mycorrhizal (AM) fungi is suitable for vegetable and horticultural crop production because the inocula may be efficiently mixed into horticultural potting media for plant production in the greenhouse. These inocula are not amenable for use in row crop production because they are not in a form suitable for mechanical application. Experiments were conducted in which light expanded clay aggregates (LECA) and pelletized biochar were used in the media for the on-farm production of AM fungus inoculum utilizing compost and vermiculite with *Paspalum notatum* Flugge as the nurse host plant. Subsequent colonization assays using *P. notatum* failed to detect any infectivity of LECA granules, indicating that the AM fungi did not infest the granules. However, as little as 0.1 g fresh wt of biochar was sufficient to produce colonization of test plants. Biochar pellets recovered from the on-farm system used to propagate *Rhizophagus intraradices* exhibited 24 propagules g^{-1} fresh wt. These results indicate the promise of pelletized biochar as a carrier for AM fungi in inoculum production systems.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are naturally-occurring soil fungi that form a mutualistic symbiosis with the majority of crop plants. Among the benefits to the host plant attributed to the symbiosis are enhanced uptake of immobile soil nutrients, notably P; and increased drought and disease resistance (Smith and Read 2008). Furthermore, AM fungi are thought to contribute to the stability of soil aggregates through the physical action of extra-radical hyphae and via the action of glomalin released to the soil (Miller and Jastrow 2000; Wright and Upadhyaya 1998). These benefits make the utilization of the symbiosis essential for the sustainability of agriculture, especially in organic systems prohibited from applying synthetic chemical fertilizers or pesticides.

Farmers can take better advantage of the AM symbiosis in two ways: through management of

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the community of AM fungi indigenous to their soils or through inoculation with effective isolates of AM fungi (Bagyaraj 1992). These two methods largely divide themselves between row crop farmers and vegetable crop farmers, respectively. Row crop farmers can adopt a variety of management practices to improve the health and functioning of the indigenous AM fungus community (Douds and Seidel 2013). Vegetable farmers who grow their own seedlings for later outplanting to the field can efficiently mix AM fungus inoculum into potting media for the production of seedlings ready to take advantage of the symbiosis (Koltai et al. 2010; Ortas 2012).

Inocula of AM fungi are available commercially in a variety of forms and are produced through a variety of methods (Ijdo et al. 2011). An alternative to purchasing inocula is for the farmer to produce it him/herself on-farm. These methods were first developed for the tropics (Gaur 1997; Gaur and Adholeya 2000; Maiti et al. 2009; Sieverding 1991), but a method also was developed for temperate climates that can be used to produce inoculated or indigenous isolates of AM fungi (Douds et al. 2006, 2010). The latter method utilizes compost and vermiculite as the growth medium and requires no utilization of synthetic chemicals, making it attractive to organic farmers. Inocula produced via these methods are readily mixed into horticultural potting media for the production of seedlings in the greenhouse or applied to the field by hand in labor-intensive farms. However, these inocula are not amenable to mechanical application to the field for use by row crop farmers. Inoculation of row crops has been shown to increase yield (Baltruschat 1987; Khan 1975), so the modification of the growth medium or development of a formulation for the final product to make it amenable to mechanical application during sowing of seeds could expand the utilization of inocula of AM fungi produced on-farm.

Biochar is the result of thermal decomposition of organic matter under limited oxygen supply (pyrolysis). Physical and chemical properties of the resultant biochar are a function of both the identity of the initial feedstock material and the pyrolysis conditions (Han et al. 2013; Mukome et al. 2013). The aromatic structure of biochars contribute to their longevity in soils (Baldock and Smernik 2002), which makes them attractive tools for carbon sequestration, and to their ability to retain plant nutrients (Mao et al. 2012), making them attractive as agricultural soil amendments (Novak et al. 2009; Spokas et al. 2011). In addition to their potential physical or chemical impacts upon the productivity of the soil, there is evidence for beneficial impacts upon soil biology, such as selection for plant growth promoting bacteria or fungi (Graber et al. 2010). Further, though generalizations are difficult to make across the wide variety of biochars used and experimental conditions studied, biochar largely has been shown to have a positive impact upon colonization of roots by AM fungi (reviewed by Warnock et al. 2007).

One potential mechanism whereby biochar may have a positive impact upon the functioning of AM fungi is that it serves as a refuge for fungal hyphae due to its porous nature (Warnock et al. 2007). Another porous substance that could provide refuge for AM fungi, and hence become a unit of inoculum to be applied to the field, is light expanded clay aggregate (LECA) commonly used in hydroponics. We sought to take advantage of the interfacial porous microstructure of biochar and LECA to study the potential of these materials to be carriers for AM fungi in the on-farm production of inoculum in compost and vermiculite mixtures. This work would serve as a test of the concept to work toward potential mechanical application of inoculum for row crop production.

MATERIALS AND METHODS

Two experiments were conducted to determine the suitability of light expanded clay aggregates (LECA) and pelleted biochar derived from switchgrass (*Panicum virgatum* L.) as constituents in the on-farm system for the production of AM fungus inoculum and their use as units of inoculum for potential mechanical delivery to the field. These porous materials were incubated in the rhizospheres of

host plants colonized by one of three AM fungi for one growing season. The biochar or LECA particles then were recovered and their infectivity determined in bioassays.

Experiment 1. Light expanded clay aggregates

Inoculum of AM fungi was produced on-farm at the Rodale Institute, Kutztown, PA as described earlier (Douds et al. 2006) with a significant modification. Typically, seven-gallon (26 L) plastic bags (Black & White Poly Grow Bags, Sunleaves Garden Products, Bloomington, IN, USA) are approximately three-fourths filled with a 1:4 [v/v] mixture of yard clippings compost and vermiculite, respectively. The compost was produced in windrows by the Lehigh County Compost Facility, Allentown, PA (table 1). The vermiculite functions as a relatively inert, from a plant nutrient standpoint, diluent of the compost. In this experiment, however, LECA granules (Hydro-Korells, Worm's Way, Bloomington, IN, USA) functioned as the diluent. Three bags were utilized, each containing a 1:3 [v/v] mixture of yard clippings compost and LECA, respectively. These bags then received six three-month-old *Paspalum notatum* Flugge seedlings colonized by either *Funneliformis mosseae* (Nicol. & Gerd.) Walker & Schuessler or *Claroideoglomus claroideum* (Schenck & Smith) Walker & Schuessler, both originally isolated from the Rodale Institute Experimental Farm, or *Rhizophagus intraradices* (Schenck & Smith) Walker & Schuessler (DAOM 181602). A sample of these seedlings (mean ± SEM, $n = 5$) prior to transplant indicated levels of AM fungus colonization of 25.2 ± 1.5, 23.8 ± 4.9, and 59.5 ± 4.6% root length, respectively. The experiment was initiated June 24, 2004 at the Rodale Institute, Kutztown, PA USA, and the bags were weeded and watered as needed through the growing season. The *P. notatum* host plants winter killed and the bags overwintered outdoors.

TABLE 1. Summary of chemical characteristics of the composts used in the experiments*

Characteristic	2004 (LECA)	2012 (Biochar)
pH	7.7	7.6
Total N	2.0%	2.5%
P	0.27%	0.44%
K	1.00%	0.86%
C:N	13.1:1	12.4:1
N:P	7.4:1	5.7:1

*Analyses were conducted by the Penn State Agricultural Analytical Services Laboratory. Dry weight basis; elemental N, P, and K.

Bags were removed from the field and taken to the lab for analysis on March 21, 2005. The bags were laid on their sides in a large bin, carefully cut open, and six root samples were collected from each bag: two each from the top, middle, and lower third of the compost and LECA mixture avoiding the original rhizospheres of the *P. notatum* seedlings. These roots then were cleared and stained with trypan blue (Phillips and Hayman 1970) and observed under a dissecting microscope (20×) to determine percentage root length colonized by AM fungi via the gridline intersect method (Giovannetti and Mosse 1980). An average of 143 ± 11 (mean ± SEM) intersections (286 cm) (Newman 1966) were observed per sample. In addition, five LECA granules from each sampling zone of each fungus were added to conical plastic pots (165 cm^3, SC-10 "super cell," Stuewe and Sons, Corvallis, OR, USA) in the center third of the column of soil mix in the pot. The soil mix used was an autoclaved mixture of sand, field soil, vermiculite, and turface (Applied Industrial Materials Corp., Deerfield, IL, USA) (1:0.75:1:0.75 v/v/v/v). There were six replicate conical pots per AM fungus species, two pots per sampling zone. Each pot received two *P. notatum* seedlings. Plants were grown in a controlled environment chamber (685 μmol m^{-2} s^{-1} PAR; 60% RH; day/night 16/8 h, 25/18°C) for nine weeks during which they received an inorganic nutrient solution lacking P (Hoagland and Arnon 1938) twice each week. Entire root systems were recovered at the end of the growth period, and AM fungus colonization was quantified as above.

Experiment 2. Pelletized biochar

Production of Biochars

Biochar was created from switchgrass pellets in a Biochar Experimenter's Kit (BEK) (All Power Labs, LLC, Berkeley, CA, USA) as described in Boateng and Mullen (2013). Pelleted switchgrass biomass was pyrolyzed under two conditions: (1) 2 h at 500°C and (2) 1 h at 600°C. A random sample of 10 pellets of each showed the cylindrical pellets to be 3.6 mm in diameter and 14 mm long. Further, the two biochar samples exhibited similar density, 0.64 versus 0.69 g dry wt. cm^{-3} for the biochars produced at 2 h at 500°C and 1 h at 600°C pyrolysis conditions, respectively.

Production of AM Fungus Inoculum

Inoculum of AM fungi was produced on-farm at the Rodale Institute, Kutztown, PA as described earlier (Douds et al. 2006). Briefly, seven-gallon (26 L) plastic bags (Black & White Poly Grow Bags, Sunleaves Garden Products, Bloomington, IN, USA) were approximately three-fourths filled with a 1:4 [v/v] mixture of compost and vermiculite, respectively. The compost was produced on-site at the Rodale Institute from yard clippings and leaves (table 1). Pouches (11 cm × 13.5 cm) made from 100% polyamide nylon fiber (100 μm mesh; NITEX, Tetko, Inc., Briarcliff Manor, NY, USA), filled with 111 g of pelleted biochar, then were inserted into the center of the bag oriented vertically. *Paspalum notatum* Flugge seedlings colonized by *F. mosseae*, originally isolated from the Rodale Institute Experimental Farm; *R. intraradices* (DAOM 181602); or *Glomus* sp., originally isolated from the Stoneleigh Estate, Villanova, PA, were transplanted into the bags on June 4, 2012 after the threat of frost had passed. Five seedlings were transplanted in a ring around the inserted bag of biochar. There were three replicate on-farm inoculum bags, each containing a mesh bag of biochar, per AM fungus × pyrolysis condition combination, for a total of 18 bags (3 AM fungi × 2 pyrolysis conditions × 3 replicates).

The bags were weeded and watered as needed through the growing season. After the *P. notatum* was freeze killed in autumn, the compost and vermiculite mixture in each bag was sampled and the mesh pouches were carefully removed on November 15, 2012. All samples were stored at 4°C until analysis. Three samples of each type of char were removed for moisture content estimation. Pooled samples of pellets were allowed to air dry in the laboratory for 4 d. Moisture content of the recovered biochar pellets was approximately 100% ((fresh-dry)/dry × 100%). Dry:fresh wt. ratios were 0.51 ± 0.01 (mean ± SEM, $n = 3$) and 0.50 ± 0.02 for the 2 h at 500°C and 1 h at 600°C biochars, respectively.

Data Collection, Microscopy, and Analysis

Modified most probable number/colonization bioassays were conducted with both the biochar recovered from the mesh bags and the compost and vermiculite mixtures. Ten-fold dilution series experiments were initiated in which 10 g, 1.0 g, and 0.1 g fresh weight of either the biochar pellets or compost and vermiculite mixtures were added to each conical plastic pot (65 cm^3, RLC 4 "pine cell," Stuewe and Sons, Corvallis, OR, USA) in the center third of the column of soil mix in the pot. The soil mix used was an autoclaved mixture of sand, field soil, vermiculite, and turface (Applied Industrial Materials Corp., Deerfield, IL, USA). There were three replicate pots for each dilution, and dilution series were set up for each inoculum media type (biochar pellet or compost and vermiculite mixture) × AM fungus × biochar combination. Each pot received a *P. notatum* seedling, and plants were grown in a controlled environment chamber for seven weeks (685 μmol m^{-2} s^{-1} PAR; 60% RH; day/night 16/8 h, 25/18°C). Plants received an inorganic nutrient solution lacking P (Hoagland and Arnon 1938) twice each week.

At harvest, root systems were washed clean of soil mix, the upper and lower 1.5 cm sections of the root system were discarded, and the remainder was cleared and stained with trypan blue (Phillips and Hayman 1970). The roots were observed under a dissecting microscope (20×) and scored for percentage root length colonized by AM fungi via the gridline

intersect method (Giovannetti and Mosse 1980). Samples in which colonization was not detected via the gridline method then were thoroughly scanned to score presence/absence of colonization. Presence/absence data were used to determine the most probable number of propagules g^{-1} fresh wt. of inocula (Alexander 1965). Colonization data were plotted with the independent variable (weight of inoculum) on a Log_{10} scale and analyzed via ANOVA after arcsin transformation. Significant treatment and interaction effects were characterized further using Tukey's method of multiple comparisons ($\alpha = 0.05$).

A subsample of biochar pellets from bags used to propagate *R. intraradices* was observed with scanning electron microscopy to detect AM fungal spores and putative AM fungus hyphae. Samples were glued to aluminum mounts (Electron Microscopy Sciences, Hatfield, PA, USA) with Duco cement (ITW Consumer, Riviera Beach, FL, USA) and allowed to dry. Samples were then sputter coated for 2×60 sec using an Edwards Scancoat 6 (West Sussex, UK). They were then observed at $1000\times$ with a FEI Quanta 200F scanning electron microscope (Hillsboro, OR, USA).

RESULTS

Experiment 1. Light expanded clay aggregates

P. notatum roots recovered from bags containing LECA were well colonized by AM fungi. Roots from bags in which *R. intraradices*, *C. claroideum*, and *F. mosseae* were propagated exhibited 59.7 ± 9.2, 50.1 ± 5.8, and 45.3 ± 7.5 (mean ± SEM, $n = 6$) percentage root length colonized, respectively. However, hyphae and spores of these fungi did not appear to colonize the LECA granules. No colonization was found in any of the *P. notatum* assay plants grown for nine weeks with five of these granules (data not shown).

Experiment 2. Pelletized biochar

AM fungi proliferated in the compost and vermiculite mixture outside the original rooting volume of the *P. notatum* seedlings as evidenced by the colonization produced on roots of test plants in the colonization bioassay (figure 1). Further, extraradical hyphae of all AM fungi tested grew into the mesh bags containing biochar pellets, and fungi on or within these pellets were infective in the colonization bioassay as well (figure 2). Scanning electron microscopy showed colonization of biochar pellets by hyphae and spores of putative AM fungi (figure 3).

The overall colonization produced by the compost and vermiculite mixture was greater than that produced by biochar pellets, however, 27.2 ± 2.6 versus 18.2 ± 2.8% of root length (mean ± SEM; $Pr > F < 0.0001$, table 2), respectively. The two biochars did not differ significantly in the colonization produced in the bioassay (21.6 ± 2.7 versus 23.9 ± 2.9 for the 2 h at 500°C and 1 h at 600°C conditioned biochars, respectively ($Pr > F = 0.2224$, table 2)). Colonization produced by potting mixture and biochar pellets from bags with plants originally colonized by *R. intraradices* produced more colonization in the bioassay than did mixture and pellets from bags with *Glomus* sp., 25.9 ± 3.4 versus 18.7 ± 2.9, respectively. The significant interaction of AM fungus and biochar type was due to there being no difference in colonization produced among the different fungi when grown in the presence of biochar pellets produced at 2 h at 500°C while *R. intraradices* and *F. mosseae* produced more colonization than *Glomus* sp. grown in the presence of biochar pellets produced by pyrolysis for 1 h at 600°C ($Pr > F = 0.0002$). Most probable number bioasssays showed that the compost and vermiculite mixture tended to have more propagules g^{-1} fresh wt. than did the biochar pellets, 15.8 ± 3.7 versus 10.5 ± 4.5, respectively. Further, *R. intraradices* inocula tended to be more infective across inocula types than those of *F. mosseae* and *Glomus* sp. (24.0 ± 0, 5.0 ± 2.5, and 10.5 ± 4.7 propagules g^{-1} fresh wt., respectively).

DISCUSSION

Vegetable farmers who outplant seedlings to the field have an efficient option for the use of

FIGURE 1. Colonization of *Paspalum notatum* roots grown in the presence of variable amounts of compost and vermiculite mixture used in the on-farm production of AM fungus inoculum. Means of three observations ± SEM. *A*, mixture removed from outside a mesh bag containing biochar pyrolyzed for 2 h at 500°C; *B*, mixture removed from outside a mesh bag containing biochar pyrolyzed for 1 h at 600°C. LINR = *Rhizophagus intraradices*; LMSS = *Funneliformis mosseae*.

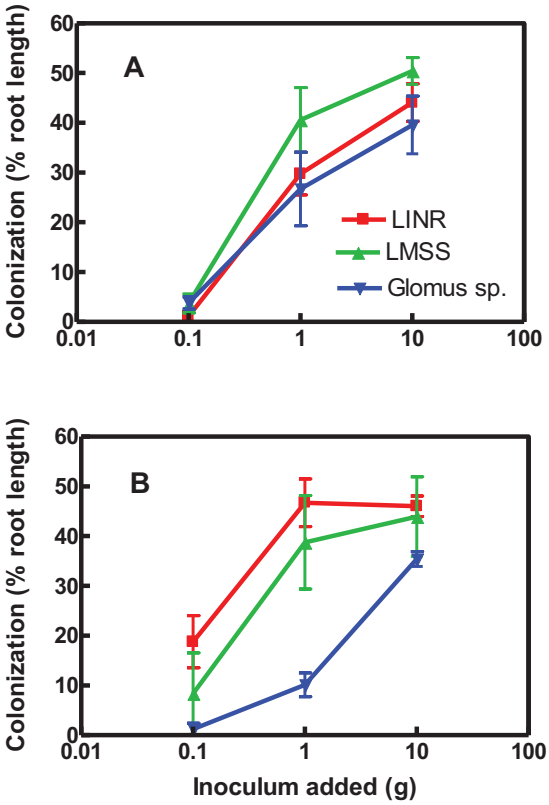
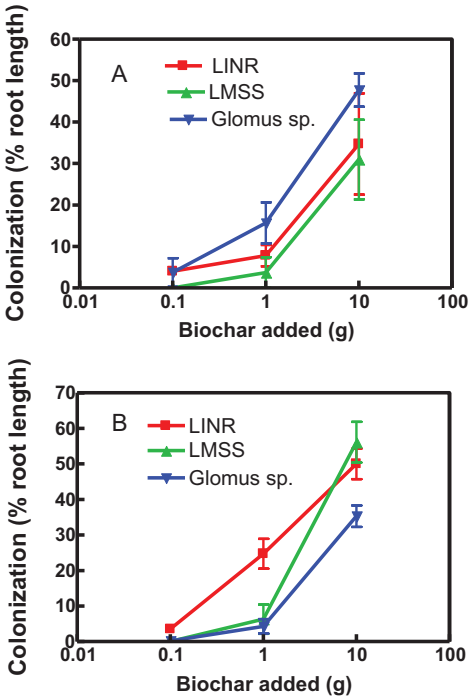


FIGURE 2. Colonization of *Paspalum notatum* roots grown in the presence of variable amounts of pelleted biochar. Means of three observations ± SEM. *A*, pyrolyzed for 2 h at 500°C; *B*, pyrolyzed for 1 h at 600°C. LINR = *Rhizophagus intraradices*; LMSS = *Funneliformis mosseae*.



AM fungus inoculum: mixing the inoculum into horticultural potting media to produce plants with pre-established mycorrhizas. This allows the plants to immediately take advantage of the symbiosis in the field, without a delay necessary for the colonization of roots by the indigenous community of AM fungi in the field soil (Koide et al. 1999). This option is not available for row crop farmers. There are situations in which utilization of inoculum of AM fungi with row crops may be beneficial. These include situations in which the indigenous AM fungus population has been negatively impacted by agricultural practices or other phenomena. For example, long fallows in dry climates can be associated with reduced yield of the subsequent mycotrophic crop due to reduced AM fungus populations (Thompson 1987). Similar to that situation, crops grown in soils that have been flooded for extended periods have exhibited reduced yields due to P deficiency exacerbated by reduced AM fungi activity in those soils (Ellis 1998). The impact of AM fungus inoculation has been shown to be inversely related to the health of the indigenous, background population of the field soil (Hamel et al. 1997; Sieverding 1991).

Manual delivery of inoculum to the field was recognized early on as impractical (as reviewed

FIGURE 3. Scanning electron micrographs of surface of biochar pellets (2 h at 500°C) incubated in a mesh pouch for one growing season. Mesh bags were surrounded by a 1:4 [v/v] mixture of compost and vermiculite in which grew *Paspalum notatum* plants colonized by the AM fungus *Rhizophagus intraradices*. *A*, Surface of pellet covered with fungal hyphae. *B*, Spore produced within a pore of the biochar.

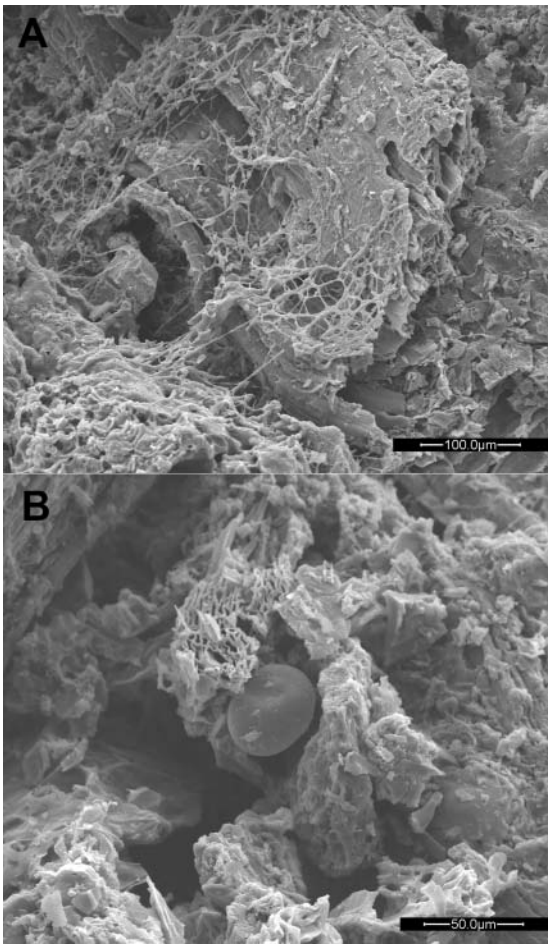


TABLE 2. Summary of the AM fungus colonization bioassay using biochar pellets or compost and vermiculite mixture as inoculum*

Source†	df	F	Pr > F
Media	1	22.5	<0.0001
Biochar	1	1.51	0.2224
Media X Biochar	1	0.46	0.4992
AM fungus	2	5.17	0.0075
AMF X Media	2	4.24	0.0175
AMF X Biochar	2	9.48	0.0002
Dilution	2	143.04	<0.0001
Dilution X Media	2	11.88	<0.0001
Dilution X AMF	4	1.07	0.3764
Dilution X Biochar	2	0.11	0.8955

*Results of Analysis of Variance, three way, and higher interactions were assumed to be zero.

†Media = biochar pellets vs. compost and vermiculite mixture; Biochar = switchgrass biomass pyrolyzed for 2 h at 500°C or 1 h at 600°C; AM fungus = *Funneliformis mosseae*, *Rhizophagus intraradices*, or *Glomus sp.*; Dilution = 0.1, 1.0, or 10.0 g fresh wt. of media inoculated into the rhizosphere of *Paspalum notatum*.

by Baltruschat 1987). Two strategies may be employed to overcome this problem. One strategy is the encapsulation of AM fungus tissue in beads of polysaccharide molecules such as alginate, hydrogel, or carrageenan (Declerck et al. 1996; Hung et al. 1991; Strullu et al. 1991; Sylvia and Jarstfer 1992). These methods are amenable more for commercial operations because they require preliminary isolation and purification of the fungal tissue from the media and root tissues with which they were produced. The second strategy is more applicable to on-farm inoculum production methods. This approach entails modifying the media in which the host plants are grown to provide colonized/infested particles that can be delivered to the field via either fertilizer applying or seeding machinery. Clay-brick granules and light expanded clay aggregates have been used successfully in inoculum production systems (Aboul-Nasr 1997; Baltruschat 1987; Dehne and Backhaus 1986; Gaur et al. 2000). LECA granules readily became infested with AM fungi when used as part of hydroponic inoculum production systems (Baltruschat 1987; Dehne and Backhaus 1986). Application of 2–4 mm expanded clay particles containing AM fungi either within the row via a rotating seeder or as a sidedress fertilizer increased both the AM fungus colonization and yield of maize (Baltruschat 1987). Our attempt to utilize LECA granules in the on-farm system to produce units of AM fungus inoculum amenable to mechanical application was unsuccessful because the granules failed to produce colonization of test plants. Even *R. intraradices*, used successfully by Aboul-Nasr (1997) and Gaur and Adholeya (2000), was unsuccessful in our

system. We have not pursued further the use of this type of material.

Previous work indicated the potential for biochar pellets or particles to become colonized by AM fungi and used as units of inoculum. Saito (1989) applied charcoal to soil used to grow *Glycine max*, and upon recovery of the charcoal after harvest of the plants noted many spores of AM fungi and their hyphae attached. This was attributed to the porous charcoal providing micro-habitat for AM fungi and a refuge from competition from other fungi (Ezawa et al. 2002; Ogawa 1989) though no details about the charcoal or micrographs were given.

We present here support for the amendment of media used for the on-farm production of AM fungus inoculum with pelleted biochar produced from *P. virgatum* for the production of units of inoculum suitable for mechanical application to the field. Incubation of pouches containing biochar in the compost and vermiculite mixture can produce inocula with a potency up to 24 propagules g^{-1} fresh wt. (32 propagules cm^{-3}) of pellets. Though this result compares favorably with the nine propagules cm^{-3} of clay-brick granules (Gaur and Adholeya 2000) and the utilization of AM fungus colonized light expanded clay aggregates at the rate of 0.1% to 1% of greenhouse pot volume for the colonization of plants in greenhouse experiments (Aboul-Nasr 1997; Dehne and Backhaus 1986) it is lower than the 80–100 propagules cm^{-3} target inoculum density for mass production of inoculum (Feldmann and Grotkass 2002). Further experimentation with different pelletized biomass feedstocks or other AM fungi may be necessary if the propagule density achieved here is not great enough to compete with or significantly supplement indigenous populations (Sieverding 1991).

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