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Sheared-Root Inocula of Vesicular-Arbuscular Mycorrhizal Fungi†

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For efficient handling, vesicular-arbuscular mycorrhizal fungi should be processed into small and uniform inocula; however, processing can reduce the inoculum density. In this article we describe the preparation and use of sheared-root inocula of *Glomus* spp. in which inoculum densities were increased during processing. Our objectives were to determine inoculum viability and density after shearing and to ascertain if the sheared inocula could be pelletized or used with a gel carrier. Root samples were harvested from aeroponic cultures, blotted dry, cut into 1-cm lengths, and sheared in a food processor for up to 80 s. After shearing, the inoculum was washed over sieves, and the propagule density in each fraction was determined. Sheared inocula were also encapsulated in carrageenan or used in a gel carrier. Shearing aeroponically produced root inocula reduced particle size. Propagule density increased with decreasing size fraction down to a size of 63 μm, after which propagule density decreased. The weighted-average propagule density of the inoculum was 135,380 propagules g (dry weight) of sheared root material^{-1}. Sheared roots were encapsulated successfully in carrageenan, and the gel served as an effective carrier. Aeroponic root inoculum was stored dry at 4°C for 23 months without significant reduction in propagule density; however, this material was not appropriate for shearing. Moist roots, useful for shearing, began to lose propagule density after 1 month of storage. Shearing proved to be an excellent method to prepare viable root inocula of small and uniform size, allowing for more efficient and effective use of limited inoculum supplies.

Selected vesicular-arbuscular mycorrhizal (VAM) fungi have been shown to enhance the growth of numerous plants of economic importance (18), including vegetables (11, 20, 26), field crops (1, 12, 19), and native plants used for revegetation (5, 28). Nonetheless, VAM fungi are not used widely in crop production, partially because inoculum sources are limited and application technologies are not well developed. The VAM fungi are difficult to culture on a commercial scale because they are obligate symbionts. These fungi can be grown with host plants in pot cultures containing soil (9), sand (31), or expanded clay (1, 6). They have also been grown by using hydroponics (7, 23), aeroponic culture (15), and root organ culture (22, 24). Various strategies have been proposed to apply inocula of VAM fungi (17). Inocula containing soil are considered impractical because of their bulk and the risk of contamination; however, chopped roots in peat blocks (32) and spores within a clay matrix (6) have been proposed for field application.

Since the cost of inoculum production and application is high, better methods must be found to process inocula of VAM fungi. For efficient handling, inocula should be processed into small and uniform pieces. Such inocula could then be pelletized (4) or used in fluid-drill systems (3, 25). Roots colonized by *Glomus* spp. can serve as inocula because of the presence of intraradical vesicles (2) and, for a few species, spores (e.g., *Glomus intraradix* [10]). Roots colonized by *Glomus* spp. have been processed by grinding (4), maceration (2), and milling (16). However, these processes usually reduced inoculum density. For example, a drop of nearly 50% in propagule number has been reported when air-dried peat inoculum was milled to a size of 850 μm (32).

In this article we describe the preparation and use of sheared-root inocula of *Glomus* spp. Our objectives were (i) to determine if root inocula of VAM fungi produced in aeroponic culture would remain viable when sheared, (ii) to quantify the inoculum density of the resulting size fractions, (iii) to ascertain if the sheared inocula could be pelletized or used in a gel carrier, and (iv) to evaluate storage methodology for aeroponic root inocula.

MATERIALS AND METHODS

Shearing method and particle size distribution. Root samples were removed from eight plants freshly harvested from a 12-week-old aeroponic culture of an undescribed *Glomus* sp. (isolate S328, INVAM 925) started on nodal stem cuttings of industrial sweet potato (*Ipomoea batatas* (L.) Lam., cv. White Star). This isolate is similar to *Glomus macrocarpum* Tul. and Tul. but lacks an expanding outer wall. The aeroponic culture system was described previously (15, 29). The root material was blotted on paper towels and cut into 1-cm lengths with scissors. Separate root samples (5 g [fresh weight]) were then placed in 50 ml of water in the work bowl of a Little Pro food processor (Cuisinart, Norwich, Conn.) and sheared for periods of 0, 5, 10, 20, 40, and 80 s. Processing was interrupted halfway through each run or every 10 s, and roots adhering to the side of the bowl were scraped back into the water. After processing, the contents of the bowl were washed over a stack of four sieves that had openings ranging from 63 to 425 μm. The smallest fraction was collected on a 5-μm-pore-size nylon membrane (Micron Sep, Honeoye Falls, N.Y.). Each fraction was dried at 65°C and weighed. A U.S. patent application has been filed for this method (30).

Propagule density in size fractions. Aeroponically grown roots of sweet potato, colonized with *Glomus* sp. (isolate S328), were harvested from an 18-week-old aeroponic culture. Roots were blotted on paper towels and cut with scissors into 1-cm lengths, and fresh and dry weights were determined for a portion of the material. Six 5-g (fresh

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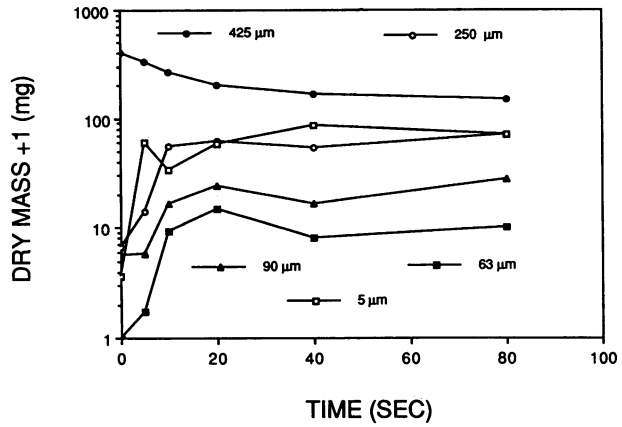

FIG. 1. Effect of shearing time on the dry mass of fractions of various sizes of root inoculum of *Glomus* sp. (isolate S328).

weight) subsamples were processed as described above for 40 s with 50 ml of distilled water. The scissor-cut material was also mixed with 50 ml of water.

Size fractionation was accomplished by washing the contents of the work bowl over a stack of four sieves that had openings of from 63 to 425 µm. The smallest fraction was then collected on 33-µm-pore-size polyester mesh (Spectra/Mesh Polyester; Spectrum Medical Industries, Inc., Los Angeles, Calif.). The root material from each fraction was collected, and known amounts (dry weight basis) were added to the first dilution of a most-probable-number (MPN) assay (27) as follows. For the scissor-cut fractions of >425 µm and 250 to 425 µm in size, the root material was collected by vacuum filtration to remove excess water and weighed directly for addition to the growth medium. A subsample for dry-weight determination was also removed from these fractions. For the fractions of 90 to 250, 63 to 90, and 33 to 63 µm in size, the root material was collected by vacuum filtration and resuspended in 50 ml of water. Subsamples for addition to the MPN assay and for dry-weight determination were removed from the stirred suspension. Three 10-fold dilutions of processed roots were made in Metro-Mix 200 (W. R. Grace & Co., Fogelsville, Pa.) beginning at a dilution of 1:3,529 (wt/vol). Fifty milliliters of each dilution was

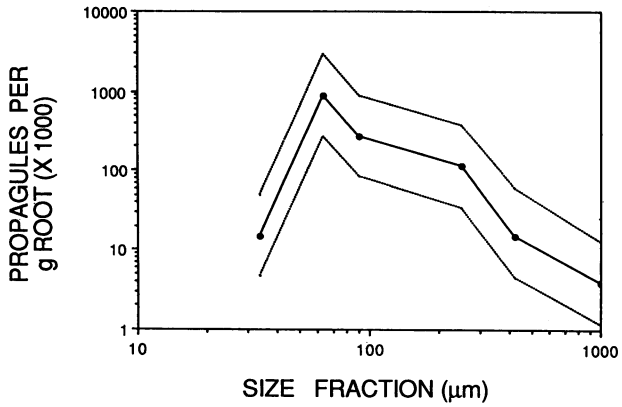

FIG. 2. Propagule densities of *Glomus* sp. (isolate S328) root inoculum (estimated by an MPN assay) of fractions of various sizes after shearing for 40 s. Dotted lines represent 95% confidence limits.

added to each of five "Pinecell" Conetainers (63-ml capacity; Stuewe and Sons, Inc., Corvallis, Oreg.), five surface-disinfested (15 min in 25% Clorox bleach) seeds of sea oat (*Uniola paniculata* L.) were added to each Pinecell, and then the seeds were covered with fine horticultural vermiculite. Plants were grown in a high-intensity-discharge (HID) growth chamber and watered with 0.25×-strength Hoagland's solution (13) for 42 days, at which time complete root systems were harvested and assessed for the presence or absence of colonization by VAM fungi.

Inoculum encapsulation. Sweet potato roots harvested from a 13-week-old aeroponic culture were stored at 4°C for 51 days and then processed as described above for 40 s. The inoculum was fractionated over standard sieves. The fraction sized between 90 and 250 µm was washed over 0.8-µm-pore-size GA4-S Metricel membranes (Gelman Sciences Inc., Ann Arbor, Mich.) with sterile water or a disinfecting solution (2% chloramine T plus 0.02% streptomycin sulfate) (21). This material was then suspended in 2.5% Kappa-carrageenan (C-1263; Sigma, St. Louis, Mo.) (8). The carrageenan was prepared by being dissolved in warm water and then being autoclaved at 121°C for 20 min. The solution was cooled to 28°C in a water bath, and pellets were produced by extruding the carrageenan suspension through a 16-gauge needle from a 30-ml syringe into 0.3 M KCl at room temperature. Pellets were separated from the KCl by being sieved over a surface-disinfested, 425-µm-pore-size sieve and then were blotted on sterile paper towels. A bioassay for infectivity was conducted in the HID growth chamber. Thirty milliliters of Metro-Mix 200 was placed in each Pinecell. Pellets (5, 10, or 15) were placed on the growth medium, and an additional 20 ml of growth medium was added to five replicates per treatment. Five surface-disinfested seeds of sea oats were placed in each Pinecell and covered with vermiculite. Plants were watered with 0.25×-strength Hoagland's solution for the first 28 days of the assay and with deionized water during the final 14 days. The assay was harvested after 42 days of growth in the HID growth chamber, and the MPN of propagules per pellet was determined.

Encapsulation was tested with a second VAM fungus. Sweet potato roots colonized with *Glomus etunicatum* Becker and Gerdemann (isolate S329, INVAM 906) were grown in an aeroponic culture for 18 weeks, colonized roots were processed as described above for 40 s, and the fraction sized between 63 and 90 µm was collected on sieves and washed with water onto a 0.8-µm-pore-size Metricel membrane. This material was then suspended in 2.5% carrageenan. The carrageenan pellets were prepared as described above except that the solution was cooled to 34°C in a water bath. The infectivity assay was conducted in Pinecells by using *Zea mays* cv. Early Sunglow as the host and pasteurized Arredondo loamy sand (loamy, siliceous, hyperthermic, Grossarenic Paleudult) as the growth medium. Seven replicates of 0, 1, 5, 10, or 20 pellets were placed on 30 ml of soil and covered with an additional 20 ml of soil. Two seeds were placed on the soil surface and covered with vermiculite. The plants were watered with 0.25×-strength Hoagland's solution and harvested after 42 days of growth in the HID growth chamber, at which time the MPN of propagules per pellet was determined.

Inoculum in gel carrier. Sweet potato roots colonized by *Glomus* sp. (isolate S328) were harvested from an 8.5-week-old aeroponic culture and processed as described above for 40 s. The fraction sized between 90 and 425 µm was used to make dilutions in a 2.5% hydroxyethylcellulose carrier (Na-

trosol; Aqualon, Wilmington, Del.). The dilutions resulted in concentrations of 0, 0.1, 0.5, 1, 10, and 100 spores ml^{-1}. A no-gel control was also established.

A 42-day bioassay was conducted in the HID growth chamber. Thirty milliliters of growth medium was placed in a Pinecell, 1 ml of an inoculum suspension was added, and this mixture was covered with an additional 20 ml of growth medium. There were seven replicates per treatment. Disinfested seeds of sea oats were placed on the surface of the medium and then covered with vermiculite. Plants were watered with 0.25×-strength Hoagland's solution for the first 28 days of the assay and with deionized water during the final 14 days, at which time the MPN of propagules in the initial inoculum was determined.

Storage of inocula. Sweet potato roots colonized by *Glomus* sp. (isolate S328) were harvested from a 13-week-old aeroponic culture, blotted dry with a paper towel, and stored moist in a sealed container at 4°C for 21 days before the experiment was initiated. Roots were then cut into 1-cm-long sections and either air dried for 72 h at 24°C or left moist (moisture content, 92%). Fifty grams (fresh weight basis) of roots was added to 500 ml of oven-dried vermiculite (dry storage) or 500 ml of vermiculite moistened with 100 ml of distilled water (moist storage) and placed in 1-liter screw-cap Nalgene bottles (Nalge Company, Rochester, N.Y.). The bottles were stored in the dark at 4°C. After 0, 1, 3, 6, and 23 months of storage, MPN assays were established to assess propagule density. The MPN assays consisted of three 10-fold dilutions of the initial root-vermiculite mixture.

RESULTS AND DISCUSSION

Shearing aeroponically produced root inoculum for up to 20 s reduced the particle size; increasing amounts of inoculum were found on the fine sieves (Fig. 1). By 20 s, the mean length of the root pieces was reduced from 10 to 1 mm. Processing from 20 to 80 s had little effect on the size distribution of the inoculum.

Propagule density increased dramatically with decreasing size fraction down to a size of 63 μm, after which propagule density decreased sharply (Fig. 2). The distribution of total dry weight in each fraction was 17, 63, 11, 3, and 5%, respectively, for the >425-, 250- to 425-, 90- to 250-, 63- to 90-, and 33- to 63-μm-size fractions. The weighted-average propagule density (dry mass of each fraction × propagule density) of the inoculum was 135,380 propagules g (dry mass) of $root^{-1}$. Our results differ from those of previous studies that show that other types of processing (including grinding, maceration, and milling) reduce inoculum density. Graham and Fardelmann (10) found that the number of root fragments was directly related to propagule density, as determined by an MPN assay. The shearing process cuts roots so cleanly that there is little loss of inoculum viability during processing. By reducing the size of particles, the number of particles per gram of root is greatly increased. Shearing proved to be an excellent method to obtain viable inocula of small and uniform size.

Sheared-root inocula from aeroponic culture may provide an economical source of VAM fungi for research and agriculture. With the propagule densities achieved by this process, we have estimated a cost of 2 to 3 cents per 1,000 propagules. To achieve adequate colonization of container-grown plants, we have found that an inoculation rate of approximately 20 propagules per plant is necessary (unpublished data), resulting in an inoculum cost of 5 cents per 100 plants.

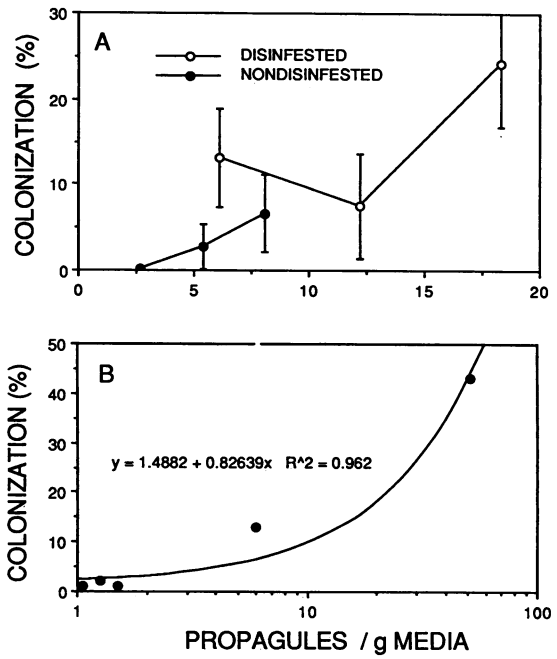

FIG. 3. Effect of propagule density (estimated by an MPN assay) on the infectivity of inoculum encapsulated in carrageenan (A) or in a gel carrier (B). Symbols are the means of five and seven replicates for panels A and B, respectively, and bars represent the standard error of the mean at $P \leq 0.05$.

Sheared roots were encapsulated successfully in carrageenan. For *Glomus* sp. (isolate S328), the disinfested inoculum contained 1.2 propagules per pellet, while the nondisinfested inoculum contained 0.5 propagules per pellet. For *G. etunicatum*, the inoculum contained 0.3 propagules per pellet, although each pellet had an average of 4.0 ± 0.85 spores. Increasing the density of propagules encapsulated in carrageenan resulted in improved colonization, demonstrating the viability of this inoculum (Fig. 3A). Natrosol served as an effective carrier of a processed inoculum. The inoculum contained 0.5 propagules ml^{-1}, and increasing inoculum density also resulted in increased colonization (Fig. 3B).

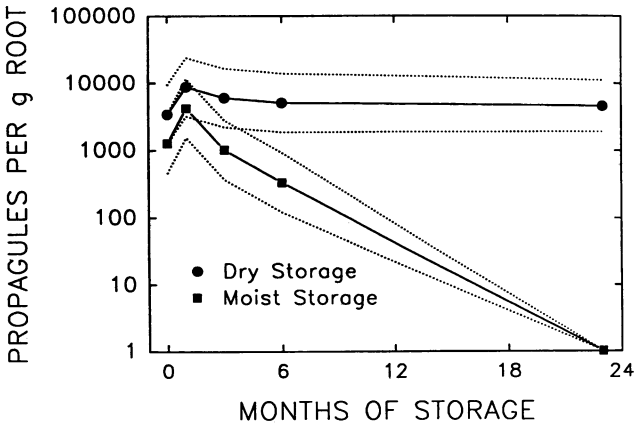

FIG. 4. Propagule densities of *Glomus* sp. (isolate S328) root inoculum (estimated by an MPN assay) stored dry or moist at 4°C in vermiculite. Dotted lines represent 95% confidence limits.

This confirms the observation of Hung et al. (14) that Natrosol is a good carrier of VAM fungi.

Dry storage was clearly superior to moist storage for aeroponic root inoculum (Fig. 4). Propagule densities of dry roots were nearly constant over the 23-month evaluation period. Hung and Sylvia (15) reported that aeroponic root inocula stored moist at 4°C retained infectivity for at least 9 months; however, our present results indicate that propagule densities of moist roots began to decline after 1 month. Unfortunately, air-dried roots cannot be effectively sheared. Therefore, for maximum inoculum densities, sheared-root inocula should be prepared from moist roots stored at 4°C for less than 3 months.

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