

USE OF VESICULAR-ARBUSCULAR MYCORRHIZAL ROOTS, INTRARADICAL VESICLES AND EXTRARADICAL VESICLES AS INOCULUM*

BY BRENDA BIERMANN†

Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331, USA

AND

R. G. LINDERMAN

US Department of Agriculture, Agricultural Research Service, Horticultural Crops Research Laboratory, Corvallis, Oregon 97330, USA

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SUMMARY

The presence of intraradical vesicles increases the inoculum potential of excised mycorrhizas. High infectivity was obtained with root pieces colonized by species of vesicular-arbuscular (VA) mycorrhizal fungi that formed intraradical vesicles (i.e. *Glomus fasciculatum*, *Glomus mosseae* and *Acaulospora spinosa*). Root pieces colonized by mycorrhizal fungi which generally do not form intraradical vesicles (i.e. *Gigaspora margarita* and *Gigaspora gigantea*) were not infective. Extraradical vesicles of *Gigaspora margarita* were also not infective propagules. Intraradical vesicles separated from the colonized roots remained highly infective but hyphae and cell debris from the same roots were not. Mycorrhizal fungi in root pieces treated for short times in sodium hypochlorite were apparently killed.

INTRODUCTION

Regrowth of hyphae from root pieces colonized by VA mycorrhizal fungi has been observed by several workers (Magrou, 1946; Stahl, 1949; Tolle, 1958) and is stimulated by root exudates (Tolle, 1958). Peuss (1958) and Winter and Meloh (1958) first successfully used colonized roots as inoculum. Recent research indicates that VA mycorrhizas may be the most important source of new infections in many ecosystems (Read, Koucheki and Hodgson, 1976; Hayman and Stovold, 1979; Abbott and Robson, 1981; Kianmehr, 1981). Plants are colonized more rapidly when inoculated with excised VA mycorrhizas than when inoculated with spores (Hall, 1976; Powell, 1976; Abbott and Robson, 1981). One potential disadvantage of using mycorrhizas instead of spores as inoculum is the increased chance of introducing pathogens (Ames and Linderman, 1978), but this need not be a problem if proper sanitation is used in pot culturing.

Not all root pieces colonized by VA mycorrhizal fungi are equally infective. We observed that subterranean clover root segments colonized by *Gigaspora margarita*

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† Present address: Native Plants, 360 Wakara Way, Salt Lake City, Utah 84108, USA.

failed to colonize geranium plants under conditions in which root inoculum of *Glomus mosseae*, *Glomus deserticola*, *Glomus fasciculatum* and *Acaulospora spinosa* resulted in colonization. We hypothesized that this difference was due to the absence of vesicles in roots colonized by species of *Gigaspora* (Gerdemann and Trappe, 1974). Vesicles were present in those roots colonized by the other four species. Intraradical vesicles are considered to function mainly as storage organs (Gerdemann, 1968; Mosse, 1973). Their ability to act alone as propagules has not been demonstrated. However, Gerdemann and Trappe (1974) noted for *Glomus radiatus* that intraradical vesicles were identical to chlamydo-spores. They also noted for *G. fasciculatum* that 'vesicles in senescent roots often become thick-walled and converted to chlamydo-spores'.

The purpose of this research was to test the hypothesis that the presence of intraradical vesicles increases the inoculum potential of excised mycorrhizal roots, and to determine whether individual vesicles (intraradical or extraradical) could act as propagules. Another objective was to evaluate the viability of root inoculum treated with sodium hypochlorite to remove potentially pathogenic surface microorganisms.

MATERIALS AND METHODS

Preparation of inoculum

Experiment 1. Geranium (*Pelargonium × hortorum* L. H. V. Bailey 'Sprinter Scarlet') and subterranean clover (*Trifolium subterraneum* L. 'Mt. Barker') were inoculated with spores, pieces of mycorrhizal root or extraradical vesicles of various species of VA mycorrhizal fungi. Fresh spores and root pieces were obtained from 3- to 4-month-old pot cultures of subterranean clover. Approximately 50 spores of *Gigaspora margarita* were used per plant as inoculum, 30 of *Gigaspora gigantea* and 100 of *Glomus fasciculatum*. Mycorrhizae were washed free of spores, cut into 0.1 to 0.5 cm pieces, and applied at the rate of 0.25 g per plant, then mixed into the soil. Root pieces used were from pot cultures of *Gigaspora gigantea*, *Gigaspora margarita*, *Glomus fasciculatum*, *Glomus mosseae* or *Acaulospora spinosa* which had colonized approximately 25, 40, 70, 65 and 40% of the root length respectively (Biermann and Linderman, 1981). Examination of stained roots from pot cultures used as inoculum showed that intraradical vesicles were abundant ($\geq 5 \text{ cm}^{-1}$) on root pieces colonized by the latter three species, but absent on root pieces colonized by either species of *Gigaspora*. Intraradical chlamydo-spores resembling spores formed in soil were not found in stained samples of any of the inocula used. Adhering spores were manually removed from root pieces under a dissecting microscope. Extraradical vesicles and attached hyphae of *Gigaspora margarita* were sieved from a pot culture of subterranean clover, and approximately 100 clusters of vesicles were isolated and used to inoculate each plant. Control plants were inoculated with the combined leachates of the various inocula passed through a 37 μm sieve.

Experiment 2. Plants of subterranean clover were inoculated with intraradical vesicles isolated from mycorrhizal roots, or with hyphae and cell debris from the same roots. Roots were from freshly harvested 3-month-old pot cultures (subterranean clover) of *Glomus fasciculatum* or *Glomus mosseae*, or from a 3-month-old pot culture (strawberry) of *Glomus fasciculatum* which had been stored for 2 years at 4 °C. Roots were washed free of spores and gently macerated with a small amount

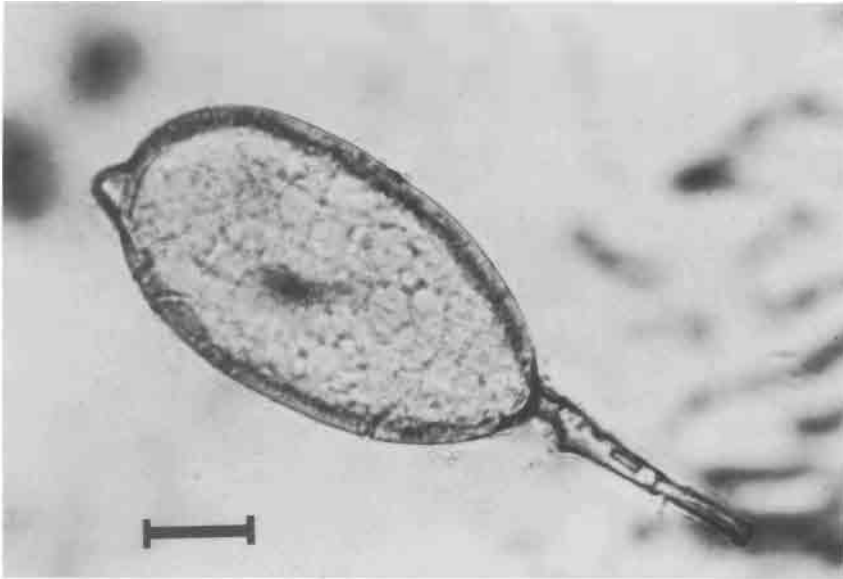


Fig. 1. Intraradical vesicle isolated from strawberry roots colonized by *Glomus fasciculatum* and used to infect subterranean clover roots. The papilla-like structure is an artifact due to slide preparation. Scale = 10 μ m.

of water in a ground-glass maceration tube. The macerate was then washed through a series of sieves. Most of the isolated intraradical vesicles were decanted from the 63 and 90 μ m sieves. Approximately 50 intraradical vesicles, freed of host material and hyphae (Fig. 1), were used to inoculate each plant. Intraradical vesicles of *Glomus fasciculatum* ranged from thin- to thick-walled; most had occluded hyphal attachments but some had an open hyphal attachment through which cytoplasm extruded. Intraradical vesicles of *G. mosseae* were thin-walled with open hyphal attachments or septa. A control inoculum consisted of the hyphae and host cell debris which remained after the vesicles had been removed from the sieve layer; this was applied at about 1000 63- to 90- μ m fragments per plant. Numerous hyphae were present in the roots from which intraradical vesicles were extracted; hyphae were also abundant in the sieve layer from which the vesicles were extracted.

Experiment 3. Geranium plants were inoculated with root pieces which had been treated with a surface-sterilant. Roots were from a 6-month-old pot culture (subterranean clover) of *Glomus fasciculatum* or from non-mycorrhizal subterranean clover grown under the same conditions. The roots were left untreated or were agitated for 30 or 90 s in 0.5% sodium hypochlorite. Roots were then rinsed several times in sterile, distilled water, cut into 0.1 to 0.5 cm pieces, and applied at the rate of 0.20 g per plant and mixed into the potting medium. Ten 1.0 to 1.5 cm root pieces from each treatment were plated on each of three plates of potato dextrose agar. The number of pieces from which bacteria or fungi were isolated was recorded after 2 days.

Plant culture. In all experiments, seeds were germinated on water agar and

Table 1. *Colonization and growth response of geranium and subterranean clover 6 weeks after inoculation with various types of inoculum from different species of VA mycorrhizal fungi*

Species	Inoculum type	Percentage root length mycorrhizal		Shoot dry weight (as proportion of control)	
		Subclover	Geranium	Subclover	Geranium
Control		0	0	1.00	1.00
<i>Gigaspora margarita</i>	Roots	0	0	1.25	1.01
<i>Gigaspora gigantea</i>	Roots	0	0	1.12	0.82
<i>Glomus fasciculatum</i>	Roots	70.5	70.8	1.45**	1.75**
<i>Glomus mosseae</i>	Roots	—	65.2	—	1.69**
<i>Acaulospora spinosa</i>	Roots	41.2	—	1.32*	—
<i>Gigaspora margarita</i>	Spores	12.8	27.7	1.41*	1.05
<i>Gigaspora gigantea</i>	Spores	35.2	33.6	1.17	0.99
<i>Gigaspora margarita</i>	Extra-radical vesicles	0	—	1.06	—

*, ** Significantly different from controls at $P = 0.05$ and $P = 0.01$, respectively (t test).

planted in 50 ml plastic tubes (Ray Leach Nursery, Canby, OR) containing the various inocula. Inocula in Experiments 1 and 3 were mixed into the plant growth medium, but in Experiment 2 the inocula were pipetted on to the medium in a layer and covered with 3 cm of medium. Plants were grown in river sand in Experiment 1, and in silt loam soil in Experiments 2 and 3. Plant growth media were steam-pasteurized (with aeration) at 60 °C for 30 min. In all experiments, plants were fertilized weekly with Long Ashton nutrient solution (Hewitt, 1966) with NaH_2PO_4 reduced from 43 to 11 mg phosphorus l^{-1} . Supplemental greenhouse lighting of 150 $\mu\text{E m}^{-2} \text{s}^{-1}$ was provided 16 h daily with high-pressure sodium vapour lamps. The night temperature was maintained at 16 °C, day temperature at 21 °C.

Collection of data. There were ten single-plant replications of each treatment in Experiments 1 and 3, and three to six in Experiment 2 (Table 2). Data were collected 6 weeks after planting. Roots were washed, cleared and stained (Phillips and Hayman, 1970), and colonization by VA mycorrhizas measured as percentage of root length that was colonized (Biermann and Linderman, 1981). Shoot weight was measured in Experiments 1 and 3 after drying at 60 °C for 72 h.

RESULTS

Glomus fasciculatum, *Glomus mosseae* and *Acaulospora spinosa* all colonized host plants when mycorrhizal root pieces were used as inoculum (Table 1). Subterranean clover and geranium were colonized after inoculation with spores, but not by pieces of mycorrhizal root from pot cultures of *Gigaspora margarita* and *Gigaspora gigantea*. Extraradical vesicles of *Gigaspora margarita* did not colonize subterranean clover. Host growth response was generally related to amount of root colonization, and no significant change in dry weight of shoots of the host, compared to controls,

Table 2. Colonization of plants of subterranean clover inoculated with vesicles or hyphae and cell debris from roots colonized by *Glomus mosseae* or *Glomus fasciculatum*

Treatment	Percentage root length mycorrhizal	No. plants colonized/ no. inoculated
Non-inoculated	0.0	0/6
Inoculum from cold-stored strawberry roots:		
<i>Glomus fasciculatum</i> vesicles	27.3	5/6
<i>Glomus fasciculatum</i> hyphae and cell debris	0.0	0/6
Inoculum from freshly harvested subterranean clover:		
<i>Glomus fasciculatum</i> vesicles	4.5	3/4
<i>Glomus fasciculatum</i> hyphae and cell debris	0.0	0/6
<i>Glomus mosseae</i> vesicles	8.1	3/3
<i>Glomus mosseae</i> hyphae and cell debris	0.0	0/6

Table 3. Colonization and growth response of geranium inoculated with mycorrhizal (*Glomus fasciculatum*) root pieces treated with 0.5% sodium hypochlorite

Treatment time (s)	Percentage root length mycorrhizal	Host growth response (as proportion of shoot dry weight of non-mycorrhizal controls)
0 (Untreated)	60.1 c	221/142 = 1.56*
30	13.9 b	160/175 = 0.91
90	0.0 a	154/156 = 0.97

a to c Means within a column not followed by the same letter significantly different (Duncan's multiple range test, 5% level).

* Significantly different at $P = 0.05$ (t test).

was caused by any inoculum which failed to colonize roots. In a few cases, the inoculum colonized roots but did not promote growth of the host. This occurred on geranium inoculated with spores of both *Gigaspora* species, and on subterranean clover inoculated with *Gigaspora gigantea*.

Roots of subterranean clover were colonized by intraradical vesicles of *Glomus mosseae* isolated from roots of subterranean clover, and by intraradical vesicles of *Glomus fasciculatum* isolated from fresh roots of subterranean clover or cold-stored roots of strawberry (Table 2). Non-inoculated plants, or plants receiving hyphae and cell debris from the same roots from which intraradical vesicles were extracted, were not colonized.

Treatment of *Glomus fasciculatum* mycorrhizas with 0.5% sodium hypochlorite

Table 4. *Isolation of bacteria and fungi from mycorrhizal (Glomus fasciculatum) pieces of root treated with 0.5% sodium hypochlorite*

Treatment time (s)	Bacteria isolated (% of pieces)	Fungi isolated (% of pieces)
0	100 c*	30 a
30	63 b	33 a
90	20 a	40 a

a to c Means within a column not followed by the same letter are significantly different (Duncan's multiple range test, 5% level).

* Significantly different at $P = 0.05$ (t test).

for 30 s reduced the extent to which the inoculum colonized geraniums, and treatment for 90 s completely prevented colonization (Table 3). With either treatment, no host growth response to inoculation occurred, but the untreated inoculum enhanced growth significantly. Bacteria were isolated from fewer segments when they were treated in sodium hypochlorite, but isolation of fungi was unaffected (Table 4).

DISCUSSION

Several lines of evidence indicate that intraradical vesicles formed in mycorrhizas act as propagules and contribute significantly to the infectivity of these roots. Segments of root colonized by species which form only extraradical vesicles (*Gigaspora* spp.) were not infective, whereas those colonized by species which form intraradical vesicles were infective both in our experiments with freshly collected roots and in experiments by Crush and Pattison (1975) with lyophilized roots. The fact that spores of *Gigaspora* were infective suggests that this phenomenon was related to inoculum infectivity rather than to host or environment. Also, roots of lily, which had mostly arbuscules and few intraradical vesicles early in the growing season, were not infective at that time (Ames and Linderman, unpublished data). Moreover, the grinding of lyophilized roots reduced their infectivity, possibly because intraradical vesicles were destroyed (Crush and Pattison, 1975). The observation that infectivity of VA mycorrhizas is probably related to the amount of nutrient reserves in fungal structures in roots (Powell, 1976; Abbott and Robson, 1981) supports the idea that intraradical vesicles contribute to inoculum potential. Intraradical vesicles are filled with large quantities of lipid and are thought to function as organs for storage of food (Gerdemann, 1968; Mosse, 1973). Direct evidence that intraradical vesicles function as propagules was provided here in that intraradical vesicles, separated from roots and hyphae, colonized host roots.

Tommerup and Abbott (1981) did not observe direct growth from intraradical vesicles. This is not inconsistent with our hypothesis, however, because nutrients stored in intraradical vesicles could increase infectivity when translocated through the hyphal network to a growing point. In this way, more than one intraradical vesicle could nourish one hypha, allowing it to traverse a greater distance to reach a new site for colonization of a root. Individual hyphae have been observed to regrow (Tommerup and Abbott, 1981), but probably do not have as great a potential for colonization when not linked to the nutrient reserves provided by

intraradical vesicles. Such hyphae plus cell debris from macerated roots were not infective in our study, but they may not have been viable.

The thin-walled extraradical vesicles formed by *Gigaspora margarita* did not function as propagules in our experiments, supporting Gerdemann's (1975) statement that the extraradical vesicles of *Gigaspora* sp. are not analogous to those in roots. They may function as temporary storage organs preceding spore formation, similar to the larger vesicles of species of *Acaulospora*. It has also been observed (J. Kough, pers. comm.) that extraradical vesicle formation always preceded sporulation in pot cultures of *Gigaspora margarita* and *Gigaspora gigantea*.

Effectiveness of VA mycorrhizal fungi in increasing uptake of phosphorus and growth of the host has been related to inoculum potential (Daft and Nicholson, 1969; Sanders *et al.*, 1977; Abbott and Robson, 1981). When used as inoculum, fragments of root containing intraradical vesicles have induced more rapid colonization and a greater response in growth of the host than have spores of the same species (Hall, 1976; Powell, 1976; Warner and Mosse, 1980). Besides this increased infectivity, mycorrhizas containing intraradical vesicles offer other advantages over soil and spores for large-scale inoculation of plants. We have found intraradical vesicles to be viable after 2 years of cold storage of pot cultures, and others have found stored, lyophilized pieces of root to be infective (Jackson, Franklin and Miller, 1972, Crush and Pattison, 1975). Pieces of root are also lighter, and therefore more economical to handle, than blended pot culture material. A disadvantage of root inoculum is the potential for introducing pathogens, but it could be treated with a surface-sterilant to eliminate some of these organisms. Mycorrhizas of yellow poplar were infective after being dipped in 0.5% sodium hypochlorite by Clark (1963), but a 30-s treatment in our experiments reduced infectivity, and a 90-s treatment apparently killed the fungus. It has also been observed that a 60-s treatment eliminated infectivity of roots colonized by *Glomus* sp. (J. Parke, pers. comm.). A treatment time less than 30 s may be possible without reducing inoculum infectivity but would not eradicate fungal pathogens.

The apparent killing of mycorrhizal fungi in pieces of root by brief treatment in sodium hypochlorite may be due to its uptake, to effects on exposed hyphae on the root surface or to the elimination of root surface microorganisms necessary for hyphal regrowth. Germination of spores of VA mycorrhizal fungi was not reduced by 2 min (Koske, 1981) or 3 min (Schenck, Graham and Green, 1975) exposure to 0.5% sodium hypochlorite. Intraradical vesicles separated from roots treated with surface-sterilant could be tested for viability to determine whether the hypochlorite treatment affected only the surface of the pieces of root.

We have demonstrated that intraradical vesicles separated from VA mycorrhizas retain their infectivity. Development of techniques such as sonication and gradient flotation to remove more efficiently intraradical vesicles from mycorrhizas could make intraradical vesicles a practical source of inoculum for fungi which form them abundantly. An enzymic method for separating internal hyphae, vesicles and arbuscules from roots has recently been described (Capaccio and Callow, 1982). Contamination by pathogens could be substantially reduced compared to that occurring when pieces of root are used.

Our findings indicate that infectivity of root inoculum can be increased by increasing the abundance of intraradical vesicles. Fertilization with phosphorus at levels higher than that optimal for plant growth eliminated formation of intraradical vesicles in roots of pasture grasses (Abbott and Robson, 1979). The number of

intraradical vesicles has been observed to increase at the time of flowering (Mmbaga, in Gunze and Hennessy, 1980) and under supraoptimal supply of nitrogen (Bevege, Bowen and Skinner, 1975), and to decrease with shading or defoliation (Gunze and Hennessy, 1980).

Further research is necessary to determine the viability of intraradical vesicles stored in decomposing roots and in soil for various times. Spores sieved from six field soils under peppermint were extremely abundant in the 63- to 90- μm layer, but no sporocarp was found, and few spores larger than 90 μm (Biermann and Linderman, unpublished results). Because intraradical vesicles are the same size as small chlamydospores and can be thick-walled like chlamydospores (Gerdemann and Trappe, 1974; Gerdemann, 1975), it is possible that these 'spores' were actually intraradical vesicles released from decaying roots. However, we do not know whether intraradical vesicles remain viable after decomposition of host roots. If they do, they would represent a considerable source of inoculum for those species which form them abundantly.

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