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Developmental aspects of Gigaspora rosea and Glomus etunicatum, alone or in association with Alnus glutinosa

Ned B. Klopfenstein

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Iowa State University

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Developmental aspects of *Gigaspora rosea*
and *Glomus etunicatum*, alone or in
association with *Alnus glutinosa*

by

Ned B. Klopfenstein

A Dissertation Submitted to the
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DEDICATION

To my Father
INTRODUCTION

Vesicular-arbuscular mycorrhizae (VAM) are ubiquitous in nature and occur with species from a majority of higher plant families, including most of our present agricultural crops (Gerdemann, 1968, 1976; Nicolson, 1967; Smith, 1980). The contribution of vesicular-arbuscular mycorrhizal fungi (VAMF) to the nutrient status of the host plant has been well-documented (Gerdemann, 1968; Mosse, 1973; Nicolson, 1967; Smith, 1980). In spite of extensive investigations aimed at elucidating the nature of these symbiotic relationships in situ, many questions remain.

Interactions among VAMF, host plants, physical environments, and other biological components of each surrounding ecosystem are diverse and complex. Nevertheless, beneficial effects upon growth of numerous plant species in various environments frequently are attributed to VAMF (Gerdemann, 1968; Hayman, 1983; Mosse, 1973; Tinker, 1978). Attempts to clarify the diverse functions of VAM in host plant health require specific information about the nature of the mycobionts that are involved in each particular symbiosis.

Study of individual VAMF is currently restricted by the inability to maintain these fungi in independent culture throughout a complete life cycle. Taxonomic status and phylogeny of VAMF therefore are based primarily upon morphological and physical characteristics of fungal structures that are associated with formation of an endomycorrhizal relationship (Abbott, 1982; Abbott and Robson, 1978; Gerdemann and Trappe, 1974; Hall and Fish, 1979; Mosse and Bowen, 1968; Nicolson and Gerdemann, 1968; Nicolson and Schenck, 1979; Old et al., 1973; Schenck
and Smith, 1982; Tinker, 1975; Trappe, 1982; Trappe and Schenck, 1982; Walker, 1983). Structural studies also contribute information on the development and physiology of VAM relationships between specific endophytes and specific host plants.

A refined in vitro VAM culture system could achieve the following:

1. Assure the integrity of the VAMF strain.

2. Supply additional information about the growth and development of the fungus outside the root.

3. Aid in the justification of phenotypic bases for species classification, provided that the morphological characteristics of the spore are maintained in subsequent generations.

4. Contribute information on the ability of different strains of mycobionts to hybridize through hyphal anastomosis.

5. Allow for the development and maintenance of specialized 'lines' of VAMF, derived from single spore types, for use as a uniform inoculum source.

6. Provide a method by which the role of VAMF hyphal connections between the root systems of separate plants can be investigated.

7. Furnish a method to study the interactions of VAM and VAMF with other organisms such as soil microbes.

8. Obtain information on the in vitro conditions necessary for sustained, independent culture of VAMF.

This study is associated with research concerning the adaptation of woody plants toward the production of woody-biomass or quality timber with intensively managed plantations. Further understanding of basic
interactions between specific woody hosts and specific VAMF should prove useful for these purposes because VAM possess the potential to dramatically affect plant growth in these situations. This specific study was designed to contribute observational and technical information that will facilitate development of future experiments on VAM interactions.

The first objective of this study was to obtain structural information about representative stages in the life cycle of the specific VAMF, Gigaspora rosea Nicolson and Schenck (1979) and Glomus etunicatum Becker and Gerdemann (1977). Information on anatomical development was obtained by examining the VAMF in an independent state and in association with roots of a host plant, Alnus glutinosa (L.) Gaertn. In vitro and in vivo cultured specimens were utilized to observe various developmental aspects.

The second objective was directed toward development of techniques for in vitro investigations on endogonaceous fungi and their interactions with their host plants. This included attempts to establish gnotobiotic in vitro cultures of VAMF associated with host tissue. In addition, various antibiotics were evaluated for their effects upon VAMF spore germination and hyphal growth. These antibiotic trials were intended to facilitate the maintenance of gnotobiotic conditions within future VAMF cultures.

In the final analysis, it is hoped that the positive results presented here will assist future attempts to establish in vitro culture systems for VAMF and(or) their symbioses with host plants. It also is
hoped that the information presented on structure and development will contribute to a basic understanding about the fundamental nature of specific mycobionts and their interactions with specific hosts.
LITERATURE REVIEW

Structure and Cytochemistry of VAMF

Fine structure and cytochemical composition of various vesicular-arbuscular mycorrhizae are examined in four current reviews (Brown and King, 1982a; Carling and Brown, 1982; Nemec, 1982; Scannerini and Bonfante-Fasolo, 1983). These reviewers also present interpretations of possible physiological mechanisms of host-mycobiont interactions that are based upon the developmental morphology and molecular localization studies of VAM. In addition, a recent summary presents the procedures and techniques utilized in electron-microscopic examinations of VAM and VAMF (Brown and King, 1982b). As these authors indicate, mycorrhizae are among the most difficult of biological samples to prepare for ultrastructural studies. Thus, the assistance offered by their review is greatly appreciated.

Because the taxonomy of vesicular-arbuscular mycorrhizal fungi (VAMF) is based primarily upon the structure and formation of their soil-borne resting spores, information on the anatomy of these spores is found regularly with species descriptions (Gerdemann and Trappe, 1974; Hall and Fish, 1979; Mosse and Bowen, 1968; Nicolson and Gerdemann, 1968; Nicolson and Schenck, 1979; Schenck and Smith, 1982; Sward et al., 1978; Trappe, 1982; Trappe and Schenck, 1982). There are relatively few studies, however, that examine the ultrastructure of VAMF resting spores. Mosse (1970 a,b,c) performed elaborate investigations on the development of "honey-colored, sessile spores" (Acaulospora laevis Gerdemann and Trappe). Light and transmission-electron microscopy
provided detailed anatomical observations on the formation, maturation, dormancy, and germination of this azygospore. Ultrastructural transformations in Gigaspora margarita Becker and Hall during spore dormancy, spore germination, and germ-tube growth have been thoroughly examined (Sward, 1981 a,b,c). Histochemical tests also were completed by Sward to determine chemical composition of components within the dormant azygospore. Transmission-electron microscopy (TEM) also was utilized by Macdonald and coworkers to investigate the ultrastructure of VAMF spores (Macdonald and Chandler, 1981; Macdonald et al., 1982). They demonstrated the presence of bacterium-like organelles (BLOs) within Glomus caledonicum (Nicol. and Gerd.) Trappe and Gerd., Glomus mosseae (Nicol. and Gerd.) Gerd. and Trappe, Gigaspora margarita Becker and Hall, Gigaspora heterogama (Nicol. and Gerd.) Gerd. and Trappe, and "white reticulate" spores. Scanning electron microscopy (SEM) occasionally has been employed to observe surface morphology of selected VAMF resting spores (Ames and Linderman, 1976; Bhattacharjee et al., 1982; Brown and King, 1982a; Brown and King, 1982b; Janos and Trappe, 1982; Nemec et al., 1981; Old et al., 1973; Rose et al., 1979; Tewari et al., 1982; Tzean and Huang, 1980; Varma et al., 1981; Walker and Trappe, 1981).

Information on the ultrastructure of various vesicular-arbuscular mycorrhizal associations has increased considerably over the last 15 years. The vast majority of VAM ultrastructural examinations involved mycobionts from the genus Glomus. Host plants associated with Glomus spp. in these studies include the following: 1) Star of Bethlehem,
Ornithogalum umbellatum L., (Bonfante-Fasolo, 1982; Bonfante-Fasole et al., 1981; Bonfante-Fasolo and Scannerini, 1977 cited by Scannerini and Bonfante-Fasolo, 1983; Scannerini, 1972 cited by Scannerini and Bonfante-Fasolo, 1983; Scannerini and Bellando, 1968; Scannerini and Bonfante-Fasolo, 1975; Scannerini and Bonfante-Fasolo, 1977; Scannerini et al., 1975); 2) onion, Allium cepa L., (Bonfante-Fasolo et al., 1981; Cox and Sanders, 1974; Cox et al., 1975; Dexheimer et al., 1979; Gianinazzi et al., 1979; Schoknecht and Hattingh, 1976); 3) garden pea, Pisum sativum L., (Protsenko, 1973 cited by Scannerini and Bonfante-Fasolo, 1983; Protsenko and Shemakanova, 1971 cited by Scannerini and Bonfante-Fasolo, 1983); 4) bean, Phaseolus vulgaris L., (Holley and Peterson, 1979); 5) soybean, Glycine max (L.) Merr., (Carling et al., 1977; King and Brown, 1981; White and Brown, 1979); 6) white clover, Trifolium repens L., (Walker and Powell, 1979; Lim et al., 1983; Lim et al., 1984); 7) Trifolium parviflorum (Macdonald and Chandler, 1981); 8) tobacco, Nicotiana tabacum L., (Kaspari, 1973; Kaspari, 1975); 9) Ammophila arenaria (L.) Link., (Old and Nicolson, 1975); 10) corn, Zea mays L., (Toth, 1981); 11) Zea diploperennis Iltis, Doebley and Guzmán (Kariya and Toth, 1981); 12) raspberry, Rubus idaeus L., (Gianinazzi-Pearson et al., 1981); 13) grapevine, Vitis vinifera L., (Baertschi and Garrec, 1980; Bonfante-Fasolo, 1978 cited by Scannerini and Bonfante-Fasolo, 1983; Bonfante-Fasolo et al., 1978 cited by Scannerini and Bonfante-Fasolo, 1983; Bonfante-Fasolo and Grippiolo, 1982; Grippiolo, 1981 cited by Scannerini and Bonfante-Fasolo, 1983); 14) yellow poplar, Liriodendron tulipifera L., (Kinden and Brown, 1975 a,b,c, 1976); and
15) English yew, *Taxus baccata* L., (Strullu, 1978; Strullu et al., 1981). Ultrastructural investigations of VAM that examine mycobionts other than *Glomus* spp. are rare. TEM was used to observe colonization of *Leptospermum juniperinum* Sm. by *Gigaspora margaritae* (Sward, 1978). Reports on the fine structure of VA mycorrhizal associations with *Acaulospora* spp. and *Sclerocystis* spp. are apparently unavailable.

Structural examinations of VAM are frequently coupled with tests that involve elemental analysis, histochemistry, or enzyme cytochemistry. X-ray microanalysis is frequently employed in conjunction with electron microscopy to determine the elemental composition of cellular constituents. This process has been used to detect various chemical elements in VA mycorrhizae of bean (Holley and Peterson, 1979), clover (Strullu et al., 1981; Walker and Powell, 1979), grape (Baertshi and Garrec, 1980; Gay and Baertschi, 1981), onion (Cox et al., 1980; Schoknecht and Hattingh, 1976), soybean (White and Brown, 1979), and yew (Strullu et al., 1981). In these studies, X-ray analysis was used most frequently to detect phosphorus. Aluminum, calcium, chlorine, copper, iron, magnesium, potassium, silicon, and sulfur occasionally were examined. Ferric chloride has been utilized during the specimen fixation procedures so that iron could be used as an indirect indicator for phenolic compounds (Holley and Peterson, 1979). In other ultrastructural studies, the mineral composition of clover VAM was determined by laser probe mass spectrography (Strullu et al., 1983).

Additional information about the composition of VA mycorrhizae can be obtained from histochemical staining procedures. A variety of
carbohydrate, lipid, protein, and phenolic histochemical tests have been used to help determine the composition of VAM citrus tissue (Nemec, 1981). In this light microscopic (LM) study with citrus, additional attempts were made to localize specific enzymatic activities that were unique in the VAM interaction. Additional LM analyses of VAM histochemical compositions also have been performed (Cooper and Lösel, 1978; Cox et al., 1975; Holley and Peterson, 1979; Ling-Lee et al., 1975).

In ultrahistochemical examinations of VAM, specific staining procedures are applied during preparation of specimens for electron microscopy. These processes assist in determining the chemical nature of cellular structures within VA mycorrhizae. Considerable information about the physiology of VA symbioses also is attained by assessing the cytochemical modifications that result from host–mycobiont interactions. Ultrahistochemical tests have been used to identify and localize various polysaccharides, proteins, polyphosphate granules, and plasma membranes within VAM (Bonfante-Fasolo et al., 1981; Bonfante-Fasolo, 1982; Cox et al., 1975; Cox et al., 1980; Dexheimer et al., 1979; Gianinazzi-Pearson et al., 1981; Kinden and Brown, 1976; Scannerini and Bonfante-Fasolo, 1979).

Structural studies of VAM have generated additional physiological information through the use of enzyme cytochemistry. These techniques detect the specific position of many enzyme activities. Ultrastructural sites of various phosphatase activities in VAM have been demonstrated with enzyme cytochemistry (Gianinazzi et al., 1979; Gianinazzi-Pearson
et al., 1978; Marx et al., 1982; Protsenko, 1973 cited by Scannerini and
Bonfante-Fasolo, 1983; Scannerini, 1975 cited by Scannerini and
Bonfante-Fasolo, 1983). Peroxidase activity has also been examined in
endomycorrhizal roots at the EM level (Bonfante-Fasolo and Scannerini,
1980 cited by Scannerini and Bonfante-Fasolo, 1983). In additional LM
studies of VAM, several phosphatases and dehydrogenases were localized
(Macdonald and Lewis, 1978). Results of this investigation led to
proposed metabolic systems that are possessed by the fungal endophyte.

In Vitro Studies on VAMF

Axenic cultures of sporulating VAMF have not been maintained and
subcultured in exclusion of host roots (Cooper and Lösel, 1978;
Gerdemann, 1968; Lewis, 1975; Macdonald and Lewis, 1978; Mosse and
Bowen, 1968; Tinker, 1975; Tommerup and Kidby, 1979; Watrud et al.,
1978a). Various surface-sterilization techniques have been developed
for utilization with spores of various species of endogonaceous fungi
(Allen et al., 1979; Allen et al., 1980; Daniels and Graham, 1976;
Gerdemann, 1955a; Godfrey, 1957; Graham, 1982; Green et al., 1976;
Hepper, 1979; Hepper and Smith, 1976; Koske, 1981a; Macdonald, 1981;
Macdonald and Lewis, 1978; Mertz et al., 1979; Mosse, 1959, 1961; Mosse
and Hepper, 1975; Mosse and Phillips, 1971; Schenck et al., 1975;
Siqueira et al., 1982; St. John et al., 1981; Sylvia and Schenck, 1983;
Tommerup and Kidby, 1980; Watrud, 1982; Watrud et al., 1978a). Effects
of the following parameters upon spore germination and(or) hyphal growth
processes of VAMF also have been investigated: 1) pH (Daniels and
Trappe, 1980; Green et al., 1976; Hepper, 1984; Mosse, 1959; Siqueira et
al., 1982; You and Chien, 1981); 2) temperature (Clarke, 1978; Daniels and Menge, 1980a; Daniels and Trappe, 1980; Graham, 1982; Green et al., 1976; Godfrey, 1955; Koske, 1981a; Schenck et al., 1975; Tommerup and Kidby, 1980; Tommerup, 1983b; You and Chien, 1981); 3) moisture (Daniels and Trappe, 1980; Godfrey, 1955; Koske, 1981a; Sylvia and Schenck, 1983); 4) light (Hayman, 1978, cited by Siqueira et al., 1982; Schenck et al., 1975; Tommerup and Kidby, 1980; Watrud et al., 1978a); 5) oxygen and carbon dioxide concentration (Tacon et al., 1983); 6) mineral concentration (Daniels and Trappe, 1980; Hepper, 1979, 1983a; Hepper and Smith, 1976; Hirrel, 1981; Koske, 1981a; Mosse, 1959; Mosse and Hepper, 1975; Siqueira et al., 1982); 7) various nutritive amendments and(or) cofactors (Daniels and Duff, 1978; Daniels and Graham, 1976; Gerdemann, 1955a; Hepper, 1979, 1983b; Hepper and Smith, 1976; Koske, 1981a; Mosse, 1959; Mosse and Hepper, 1975; Siqueira et al., 1982); 8) soil and(or) root extracts (Daniels and Graham, 1976; Hepper, 1983b; Mosse, 1959); 9) root exudates (Graham, 1982; Hepper and Mosse, 1975; Mosse and Hepper, 1975); 10) presence of host plant (Daniels and Duff, 1978; Daniels and Trappe, 1980; Powell, 1976; Tommerup, 1983b, 1984); 11) soil type (Daniels and Duff, 1978; Daniels and Trappe, 1980; Hepper, 1979; Hepper, 1984; Hepper and Mosse, 1975; Mosse, 1959; Tommerup and Kidby, 1980; Tommerup, 1983b); 12) various contaminating microbes (Koske, 1981a,b; Mayo and Davis, 1983; Mertz et al., 1979; Paulitz and Menge, 1983; Schenck et al., 1975; Sylvia and Schenck, 1983); 13) soil sterilization (Daniels and Graham, 1976; Daniels and Trappe, 1980; Koske, 1981a; Mosse, 1959; Sylvia and Schenck, 1983; Tommerup, 1984); 14) spore age
and (or) storage conditions (Daniels and Duff, 1978; Daniels and Graham, 1976; Daniels and Menge, 1980a; Daniels and Menge, 1981; Godfrey, 1957; Hepper, 1979; Hepper and Smith, 1976; Koske, 1981a; Mertz et al., 1979; Mosse, 1959; Tommerup, 1983a; Tommerup and Kidby, 1979); 15) previous spore germination (Koske, 1981b; Mosse, 1959); 16) spore population density (Daniels and Trappe, 1980; Koske, 1981a); 17) possible self inhibitors (Watrud et al., 1978b); 18) antibiotics, growth inhibitors, and (or) agricultural chemicals (Beilby, 1983; Beilby and Kidby, 1982; Daniels and Menge, 1980b; Hepper, 1979; Mertz et al., 1979; Mosse, 1959; Tommerup and Briggs, 1981; Tommerup and Kidby, 1980); and 19) physical stresses (Godfrey, 1955; Tommerup and Kidby, 1979, 1980). After separating G. caledonicum (Nicol. & Gerd.) Trappe & Gerd. hyphae from their parent spores, limited hyphal growth that was dependent upon nutrients in the medium has been obtained (Hepper, 1983b). Gnotobiotic in vitro endomycorrhizal culture systems also have been developed with root organs (Hepper and Mosse, 1975; Miller-Wideman and Watrud, 1984; Mosse and Hepper, 1975) and whole plants (Allen et al., 1979; Allen et al., 1980, 1981; Allen and St. John, 1982; Hepper, 1981; Macdonald, 1981; Mosse, 1962; Pearson and Tinker, 1975; St. John et al., 1981) serving as host tissue. However, the establishment of the two-membered endomycorrhizal associations on specialized agar media is difficult to reproduce (St. John et al., 1981). Nevertheless, establishment of two-membered culture systems on a chemically-defined medium remains as an essential step toward differentiating between the interacting influences of other organisms, particularly those present in the flora of the
rhizosphere. Establishment of VAM on a chemically-defined medium under gnotobiotic conditions is a means by which several previously unmonitored variables can be controlled. Such a system would provide an indirect method to study the physiological requirements of the VAMF. By observing the physiological response of these associations in vitro, inferences can be drawn as to the nutritional and environmental requirements of the fungus (Allen et al., 1979; Beilby, 1983; Beilby and Kidby, 1980, 1982; Daniels and Graham, 1976; Daniels and Trappe, 1980; Graham, 1982; Green et al., 1976; Hepper, 1979, 1983a, 1983b; Hepper and Smith, 1976; Koske, 1981a; Lewis, 1975; Lösel and Copper, 1979; Macdonald and Lewis, 1978; Mosse, 1959, 1962; Mosse and Hepper, 1975; Schenck et al., 1975; Siqueira et al., 1982; Sylvia and Schenck, 1983; Tacon et al., 1983; Watrud et al., 1978b). Therefore, development of techniques to facilitate axenic culture of specific endogonaceous fungi is a prerequisite toward determination of conditions which will contribute to the independent growth of endomycorrhizal endophytes.

Exclusion of extraneous organisms from VAMF cultures also could be useful in taxonomic studies. Classification of endogonaceous fungi is presently based primarily on phenotypic characteristics of the spores and mycelium (Abbott, 1982; Abbott and Robson, 1978; Gerdemann and Trappe, 1974; Hall and Fish, 1979; Mosse and Bowen, 1968; Nicolson and Gerdemann, 1968; Nicolson and Schenck, 1979; Old et al., 1973; Schenck and Smith, 1982; Tinker, 1975; Trappe, 1982; Trappe and Schenck, 1982; Walker, 1983). Species of VAMF are currently maintained using open-pot cultures that are open to contamination by a multitude of undesirable
organisms including other endomycorrhizal fungi.

In previous studies, various antibiotics have frequently been utilized as effective supplements to surface-sterilization procedures for spores of VAMF fungi (Beilby and Kidby, 1980; Cooper and Lösel, 1978; Hepper, 1979; Hepper and Smith, 1976; Mertz et al., 1979; Mosse, 1959, 1961, 1962; Mosse and Hepper, 1975; Pearson and Tinker, 1975; St. John et al., 1981; Sylvia and Schenck, 1983; Watrud, 1982; Watrud et al., 1978a,b). Antibiotics also have been incorporated occasionally into culture media to control bacterial contamination in studies with VAMF (Godfrey, 1957; Mertz et al., 1979; Tommerup and Kidby, 1980). Although antibiotics provide a valuable means to control undesired microorganisms, their effects upon the growth and development of the VAMF have been generally neglected. The few studies which are available seem to indicate that some antibiotics have the potential to influence adversely the germination and hyphal elongation processes of certain mycobionts (Tommerup and Kidby, 1980). The successful use of bacterial inhibitors would seem to depend on the interaction of the particular antibiotic, its respective concentration, the type of VAMF, and the conditions under which the antibiotic is used.
MATERIALS AND METHODS

Structural Studies of VAMF and VAM

**Derivation, maintenance, and general handling of biological material**

Azygospores of *Gigaspora rosea* were originally isolated from a sandy terrace near the Des Moines River at the 4H Camping Center, Boone County, Iowa. The origin of the *Glamus etunicatum* was used in these studies is unknown. Chlamydospores of *Gl. etunicatum* were first observed as contaminants in several open-pot cultures which were designed to perpetuate other species of VAMF. Dr. Christopher Walker¹ identified in this investigation. Seeds of *Alnus glutinosa* originated in central Europe, and were obtained from germplasm collected in 1976 (Robison et al., 1979).

VAMF were sustained and increased in open-pot culture (Gilmore, 1968). Cultures were initiated in plastic pots (20 cm in diameter) containing an autoclaved mixture of 3 parts sand and 1 part vermiculite. Surface-disinfested spores served as the endomycorrhizal inoculum. Decontamination of spores was accomplished by a 20-min treatment with 5.0% (wt/vol) chloramine-T, followed by several rinses in sterile deionized water (Tommerup and Kidby, 1980). Between 5 and 50 spores were pipetted into the pot culture substrate at 3-5 cm below the surface. When only a few spores were to be applied, a filter paper

¹Forestry Commission, Northern Research Station, Roslin, Midlothian, Scotland.
funnel was incorporated into the pot culture. The paper funnel helps to restrict early root growth to the vicinity of the spores, but it decomposes later to allow for root system expansion. Seeds of *A. glutinosa* and/or *Sorghum vulgare* Pers. were surface-disinfested and planted above the VAMF spores. Alder seeds were immersed in deionized water for one hour, then treated with 30% hydrogen peroxide for 10 min to decontaminate the seed surface (Neal et al., 1967). A 10-min soak in 10% Clorox® was used to disinfect the sorghum seeds. Several rinses with sterile deionized water followed all seed disinfestation procedures. All pot cultures were maintained in a greenhouse. Efforts to keep individual VAMF isolates separated from other isolates were attempted by either suspending each pot culture from above, or placing the open-pot culture on inverted pot that was segregated from other cultures. Pot cultures were watered as needed and fertilized weekly with a solution of Peter's® 15-0-15 water soluble fertilizer.

Initial extractions of VAMF spores from soil or pot culture material were performed by a slightly modified centrifugal-sugar flotation method from Jenkins (1964) (Walker et al., 1982; Warnke, 1982). Spore samples were placed in a petri dish with Ringer's solution at 4°C (Furlan and Fortin, 1975). A dissecting microscope was used for primary observation, selection, and manipulation of individual spores. Single spores were handled with stork-billed microdissecting forceps or by a finely-drawn Pasteur pipette. Specialized containers were designed to facilitate the processing of spore samples through the various treatments. These containers were assembled by modifying Beem® capsules
into a small, open-ended cylinder with removable top and bottom caps. Holes were punched into the center of each cap. Each opening in the caps was then covered on the inside with a fitted disc of porous styrene. An additional fitted disc of 400-mesh sieve was placed over the styrene, so that it was on the inside when the cap was in place. These receptacles allow fluids to be exchanged easily during spore treatments, and also prevent rapid drying by retaining moisture.

For anatomical studies, cultures of *A. glutinosa* were initiated and maintained in a manner similar to the pot cultures. After surface-disinfestation procedures, spores of *G. rosea*, or *G. etunicatum* were introduced with the alder seeds to establish VAM. No spores were added to alder cultures that served as nonmycorrhizal controls. Root samples for microscope observation were taken after 5 to 7 months growth. All cultures were monitored periodically for spore production before, during and after root sampling to assure the integrity of each culture.

**Light microscopy**

Various methods were applied to clear and stain VA mycorrhizal root samples. For rapid evaluations, a procedure similar to that of Kormanik et al. (1980) was utilized. The following steps were employed in this procedure: 1) Whole, intact *A. glutinosa* seedlings were collected. The fresh root systems were gently rinsed in water or 0.1M Na-Na₂ phosphate buffer at 4°C to remove adhering debris. After rinsing, the root system was severed from the plant and submersed in a petri dish. Roots were cut into 0.5 to 2.0 cm segments and placed into basket-like containers. 2) Root samples were immersed in 1N KOH within covered dishes, and
steamed in an autoclave for 30 min at atmospheric pressure. This was followed by a 5-min treatment with fresh 1N KOH at room temperature. 3) To remove the KOH, specimens were rinsed with at least 5 changes of tap water. 4) Further bleaching of the roots was performed in an alkaline H₂O₂ solution for 15 min. The alkaline H₂O₂ bleaching solution contains 60 ml of 30% H₂O₂, 18 ml of 58% NH₄OH, and 3522 ml H₂O. 5) Samples were rinsed again with 5 changes of tap water to remove the H₂O₂. 6) Roots were acidified in 1% HCl for 10 min. 7) A lactic acid-glycerin mixture containing 0.1% (wt/vol) stain was applied to the root samples. To prepare this solution, 875 ml of 85% lactic acid, 63 ml of glycerin, 62 ml of water, and 0.1 gm stain were thoroughly mixed. The stain consisted of either trypan blue, aniline blue, acid fuchsin, or combinations of the aforementioned stains. Staining was accelerated with steam in an autoclave at atmospheric pressure for 30 min. 8) Roots were destained with a lactic acid-glycerin mixture that did not contain any stain. A milder version of this clearing and staining protocol also was utilized when time allowed. With this procedures, the KOH clearing (step #2) and the staining (step #7) were performed at room temperature for at least 24 h. Before the forementioned methods were developed, a procedure similar to Walker's (1979a) modification of a method derived by Bevage (1968) was utilized. With this procedure, roots were bleached with 10% Clorox® instead of alkaline H₂O₂, and lactophenol was used in place of the lactic acid-glycerin mixture. Saturated chloral hydrate also was implemented as an alternative bleaching agent in an additional clearing technique.
Permanent microscope slides of stained specimens were prepared by two different modes. Samples that were previously immersed in the lactic acid-glycerin mixture or in lactophenol were occasionally mounted directly in polyvinyl alcohol lactophenol (PVL) (Walker, 1979b). Permount® also was used frequently as a slide mounting medium; however, this required additional specimen preparation. Before mounting in Permount®, stained samples were first dehydrated gradually in a series of solutions containing sequentially higher proportions of ethanol. Additional counter staining of the biological material was incorporated into the dehydration schedule in some instances. At the end of the dehydration series, subjects were placed in three successive changes of 100% ethanol to ensure total dehydration. These samples were then placed in a mixture containing equal proportions of ethanol and xylene. This was followed with three changes of pure xylene. After this process, the specimens were amenable to mounting in Permount®.

**Electron microscopy**

**Spores** Spores were extracted from freshly collected pot culture material, placed in Ringer's solution, and examined under the dissecting microscope. Approximately 500 mature spores that appeared to be healthy and newly formed were selected from the spore sample. The selected spores were then placed within the specialized container that was designed to facilitate further treatments. A temperature of approximately 4°C was maintained throughout the extraction, selection, and brief storage of the spores.
Before fixation procedures, spores were first subjected to the 20-min surface-disinfestation treatment with 5% (wt/vol) Chloramine T at room temperature. After rinsing, each spore sample was divided into two subsamples. The first subsample was immediately immersed in the primary fixative solution for subsequent structural studies of the quiescent spores. Spores in the second subsample were involved in preliminary tests for germination, and were handled aseptically. Some spores of *Gi. rosea* and *Gl. etunicatum* from the second subsample were placed into 1% water agar (Difco Bacto) in petri dishes. Other *Gi. rosea* spores were placed upon sterile dialysis tubing that overlayed 1% water agar inside the petri dishes. At least six petri dishes were used for each germination test, each containing an average of 15 spores. The petri dishes were sealed with Parafilm® strips, and incubated at room temperature in the dark. Periodic examinations were performed to assess spore germination within the succeeding 230 days. During the germination test, representative samples of germinating spores were fixed and prepared for electron microscopic observation.

Spores were fixed initially in a mixture of 4% (vol/vol) glutaraldehyde and 4% (wt/vol) paraformaldehyde in 0.1M Na-Na₂ phosphate buffer at pH 6.8. Primary fixations was performed for 2 h at room temperature, followed by 24 h at 4°C. This was followed by five 0.1M phosphate buffer rinses at 4°C, with each rinse lasting at least 30 min. Spores were post-fixed for 60 h at 4°C with three changes of 1% (wt/vol) osmium tetroxide in the Na-Na₂ phosphate buffer. This was followed by four, 30-min phosphate buffer rinses. Samples were dehydrated in a
sequentially graded ethanol series (12, 25, 37, 50, 60, 70, 82, 95, 100%) for 5-10 min at each stage. To insure complete dehydration, spores were subjected to three additional 30-min changes of absolute (100%) ethanol.

Some of the spores in absolute ethanol were freeze-fractured to expose sectional views for SEM observation. Individual spores were frozen in liquid nitrogen and placed in a trough in a metal block that was equilibrated thermally with the liquid nitrogen. With the aid of a dissecting microscope, each spore was fractured by the impact of a sharp scalpel. Spore fragments were returned to absolute ethanol immediately following each fracture.

Spores were dried in preparation for SEM in two different manners. With one sample, spores were air-dried slowly from ethanol over a period of 3-4 days. This was accomplished while the spores remained within the specialized processing capsule. The capsule was placed within an unsealed petri dish containing filter paper that was premoistened with absolute ethanol. The petri dish was placed inside a sealed desiccator.

Spores in the other sample were prepared for critical-point drying. This sample was processed for 1 h in each solution of a graded ethanol-freon 113 series (3:1, 1:1, 1:3, 100% freon). This was followed by three more changes of pure freon over a 16-h period. Spores within the specialized container were critical-point dried with liquid CO₂ under pressure. A desiccator was used to store SEM specimens that had been critical point dried.

In preparation for mounting SEM specimens, pieces of metal tape
were fastened onto brass discs with electrically conductive silver paste. The metal tape was positioned with the adhesive side facing upward on the brass disc. Specimens were then oriented under a dissecting microscope, and firmly applied to the adhesive of the metal tape. A sputter coater (Polaron Instruments Inc. SEM Coating Unit E5100) was used to apply a gold-palladium coating (ca. 540 Å) over the specimens. Coated specimens were examined with a JEOL JSM 35 scanning electron microscope operating at 15 KV.

Spores that were to be embedded with resin for sectioning were selected after secondary fixation with osmium tetroxide and the subsequent buffer rinses. Intact rupture-free spores that exhibited no other visible damage from earlier processing were selected. To facilitate further processing, some spore samples were incorporated into 1% buffered water agar containing 0.1M Na-Na₂ phosphate at pH 6.8. Between five and ten spores were positioned in a row within narrow troughs which were formed previously in the solidified buffered agar. Molten buffered agar at 40°C was applied over the spores to seal them within the agar. Before additional processing, the agar which contained the spores was cut into small blocks that were approximately 8 mm³. Supplementary spore samples of *G*. *rosea* and *G*. *etunicatum* also were processed within the modified Beem® capsules, and were not incorporated into agar blocks. With one sample that was processed in this manner, the dormant *G*. *rosea* azygospores were first punctured with a finely-sharpened needle before resin infiltration. Intact, germinated spores of *G*. *rosea* also were processed within the specialized containers. All
spore samples were dehydrated through a sequentially graded ethanol series.

After complete dehydration, specimen samples were processed for infiltration with either LR White acrylic resin or Spurr's epoxy resin (Spurr, 1969). Infiltration with LR White resin was initiated with a graded ethanol-resin series (3:1, 1:1, 1:3) over a three-day period. This was followed by six daily changes of pure resin. All infiltration steps were carried out under constant rotation. Polymerization was performed within sealed gelatin capsules at 60°C for two days.

Before infiltration with Spurr's resin, specimens were first subjected to three changes of propylene oxide. This was followed by a graded propylene oxide-resin series (3:1, 1:1, 1:3) over the course of three days. Each day during the subsequent six days specimens were immersed in fresh changes of pure resin. All phases of infiltration with Spurr's resin were conducted under desiccation with constant rotation. The Spurr's resin was cured under desiccation at 60°C for three days.

Resin-embedded specimens were sectioned with glass knives on a Reichert-Jung Ultracut E or an LKB Utratome III ultramicrotome. Sections for light microscopy were 0.3 to 1μ in thickness.

**Preparation of A. glutinosa roots for microscopy**

VAM and noninfected control root samples were collected from pot cultures 150 days after the seeds were planted. Entire root systems were briefly rinsed in five changes of 0.1M Na-Na₂ phosphate buffer (pH 6.8) at 4°C. Root samples were immediately placed into a primary
fixative solution containing 4% formaldehyde and 4% glutaraldehyde in the phosphate buffer. After immersion in the primary fixative the roots were cut into 1-5 mm segments. A portion of the root segments was removed from the fixative, and subjected to clearing procedures for light microscopic observation. The remaining root segments were fixed for electron microscopy by the same basic procedures as those used for fixing spores. A check for VAMF species purity within each pot culture was performed by extracting the spores from root washings and from samples of the pot culture substrate.

To allow scanning electron microscopic observation of root surfaces, roots were prepared similarly to the procedure used for spores. After post-osmification, roots were washed in buffer, dehydrated in ethanol, transferred to freon 113, critical-point dried, mounted on foil tape over brass discs, and coated with gold-palladium. Root specimens were examined with a JEOL JSM-35 scanning electron microscope operating at 15 KV.

To permit SEM observation of internal structures within the root, some root samples were freeze-fractured after their complete dehydration with absolute ethanol. In addition, two different techniques were utilized to remove host cytoplasm from root cells, so that fungal structures could be examined more thoroughly.

One procedure used to remove host cytoplasm from fixed root specimens was derived from techniques of Kinden and Brown (1975d). Freeze-fractured root fragments were rehydrated initially by reversing the graded ethanol series, followed by placing them in the phosphate
buffer. The clearing procedure began with a three-min treatment in 1.0% aqueous periodic acid at room temperature. Root specimens were rinsed briefly several times in distilled water and treated with 4.0% aqueous KOH for 20 min at 55°C. After immersion in 1.0% acetic acid for one minute, the samples were thoroughly rinsed with several changes of distilled water over a one-h period. These root samples were subjected to two changes of 1.0% (wt/vol) OsO₄ in phosphate buffer over the subsequent 48 h. Following a thorough rinse with four changes of the 0.1M phosphate buffer, the root fragments were dehydrated to absolute ethanol via the graded ethanol series.

The other root clearing technique utilized was based upon a method devised by King et al. (1981). This simpler method began with freeze-fractured root fragments in absolute ethanol. Root samples were processed for 30 min at each stage of a graded ethanol-toluene sequence (3:1, 1:1, 3:1). This was followed by three 15-min changes of pure toluene. Host cytoplasm was then removed with a solution containing equal parts of toluene and soluene®, a tissue solubilizer from Packard Instruments Co. Inc. The root clearing process was conducted for 36 h at room temperature, and for an additional 36 h at 4°C. Five 30-min changes of pure toluene were used to stop the clearing reaction. Specimens were returned to absolute ethanol by reversing the sequence of the graded ethanol-toluene series.

After reaching absolute ethanol, all cleared root fragments were prepared for SEM in a manner similar to that used for spores and intact root segments. The structure within exposed root cells that were devoid
of cytoplasm was observed with a JEOL JSM-35 scanning electron microscope which was functioning at an accelerating voltage of 15 KV.

Attempts Toward In Vitro Culture of VAM

Several attempts were made to attain gnotobiotic, in vitro cultures of VAMF in association with host plant tissue. *Alnus glutinosa* served as the host tissue in the form of whole plants, root organs, or undifferentiated callus tissue. Spores of *Gigaspora rosea* or *Glomus etunicatum* were utilized as VAMF inoculum.

**VAM associations with intact alder seedlings**

Attempts to establish in vitro VAM associations with roots of intact plants were initiated within glass culture tubes (50 ml). Each tube contained approximately 20 ml of a solidified nutrient medium that was a modification of the medium used in the mycorrhizal studies of Mosse and Hepper (1975). A nutrient medium with the following composition in one liter of distilled water was utilized: 6 g agar (Difco-Bacto); 720 mg MgSO$_4$$\cdot$7H$_2$O, 65 mg KCl; 80 mg KNO$_3$; 4.9 mg MnCl$_2$$\cdot$4H$_2$O; 300 mg Ca(NO$_3$)$_2$$\cdot$4H$_2$O; 18 mg FeNaEDTA; 9.3 mg KH$_2$PO$_4$; 1.92 mg ZnSO$_4$$\cdot$7H$_2$O; 1.5 mg H$_3$BO$_3$; 1 µg CuSO$_4$$\cdot$5H$_2$O; 1.68 µg Na$_2$Mo$_4$$\cdot$2H$_2$O. The pH of this medium was adjusted to 7.0 before autoclaving. An autoclaved slurry of water agar and activated charcoal was then applied in a fine layer (ca. 2 ml) upon the top of the solidified nutrient medium. The water agar-charcoal matrix was prepared by combining 5 g of activated charcoal and 1 g agar with 100 ml of distilled water. The mixture was cooled to 38°C after autoclaving, and constantly agitated prior to
VAMF spores were obtained, stored, extracted, and surface-disinfested as described elsewhere. Approximately 10 spores were transferred into each tube. Spores were transferred within a few drops of water by a finely-drawn Pasteur pipette. Another layer (ca. 2 ml) of molten (38°C), charcoal-water agar mixture was applied over the spores within each tube. Ten tubes were prepared with Gl. etunicatum chlamydospores, and another five tubes were prepared containing azygospores of Gl. rosea. In an additional spore germination check, approximately 30 spores of each VAMF species were placed into separate sets of three petri plates containing 0.6% water agar. The plates containing the germination checks were placed inside light-tight containers and incubated in the growth chamber.

Seeds of A. glutinosa were surface-disinfested in 30% hydrogen peroxide as described elsewhere. Two seeds were added to each tube after the charcoal-water agar matrix had solidified. The seeds were placed into indentations within the solidified, charcoal-water agar matrix of each tube. A removable sleeve fashioned from aluminum foil was applied around each tube below the level of the seeds to exclude light from the root zone. The capped tubes were placed in a growth chamber on a 12-h light-dark cycle with a 18-29°C temperature range. Cool, white fluorescent tubes and incandescent lights provided approximately 100 µEm⁻²S⁻¹ at culture tube level.

Culture tubes were monitored periodically throughout the course of the study. Hyphal development in relation to root growth was observed.
in the nutrient medium below the charcoal–water agar matrix. The culture systems were examined with a dissecting microscope or with the 10X objective of a compound microscope. At 10 weeks, one culture system of each VAMF species was dismantled to check for VA mycorrhizal formation. The alder roots and VAMF hyphae were separated from the agar matrices and subjected to clearing and staining procedures. The remaining culture systems were dismantled in a similar manner, after at least five months.

VAM associations with root organs

Root organs were utilized in some of the attempts to establish an in vitro association between Glomus etunicatum and Alnus glutinosa. Root organs were excised from seedlings arising from the germination of surface-disinfested alder seeds in 1% water agar. The excised primary root organs, which averaged 9 mm in length, were transferred within a small block of water agar, and each placed in separate petri plates containing 20 ml of one of two different nutrient media. One medium that was utilized was a slightly modified version of the medium developed by Mosse and Hepper (1975). This medium contained the following components per liter of distilled water: 6 g agar (Becto-Difco); 720 mg MgSO₄·7H₂O; 65 mg KCl; 80 mg KNO₃; 4.9 mg MnCl₂·4H₂O; 300 mg Ca(NO₃)₂·4H₂O; 18 mg FeNaEDTA; 9.3 mg KH₂PO₄; 1.92 mg ZnSO₄·7H₂O; 1.5 mg H₃BO₃; 1 μg CuSO₄·5H₂O; 1.68 μg Na₂MoO₄·2H₂O; 3 mg glycine; 0.1 mg thiamine HCl; 0.5 mg nicotinic acid; 0.1 mg pyridoxine; 200 mg myo-inositol; 20 g sucrose. The other medium utilized was the same as the first except that it contained the following nutrients at half-strength:
MgSO₄; KCl; KNO₃; MnCl₂; and Ca(NO₃)₂. Both media were adjusted to pH 7.0 before autoclaving. Six plates of each nutrient medium were utilized in this study. Approximately 20 surface-disinfested chlamydospores were applied in proximity to each root organ. Chlamydospores were transferred within a few drops of water by a finely-drawn Pasteur pipette. In an additional germination check, approximately 15 chlamydospores were placed into each of eight petri plates containing 0.6% water agar. All petri plates were sealed with Parafilm®, and incubated in the dark at room temperature. Plates were periodically examined for contamination, spore germination, and root growth. Three months after inoculation, the root organs were cleared, stained, and observed under a compound microscope.

**Associations of VAMF with undifferentiated callus tissue**

Undifferentiated callus tissue of *A. glutinosa* also was utilized in examinations of *in vitro* interactions of host plant tissue with *G. rosea*. Callus tissue initially was derived from primary explants from the hypocotyl tissue of seedlings that were originated by germination of surface-disinfested alder seeds. The primary explants (approximately 3 mm³) were plated on petri dishes with a nutrient medium that contained agar, MS salts (Murashige and Skoog, 1962), sucrose, and B5 vitamins and hormone supplements (Gamborg et al., 1968). This medium contained the following components in each liter of distilled water: 6 g agar (Difco-Bacto); 30 g sucrose; 1.65 g NH₄NO₃; 1.9 g KNO₃; 0.44 g CaCl₂·2H₂O; 0.37 g MgSO₄·7H₂O; 0.17 g KH₂PO₄; 37.3 mg Na₂-EDTA; 27.8 mg FeSO₄·7H₂O; 6.2 mg H₃BO₃; 22.3 mg MnSO₄·4H₂O; 8.6 mg ZnSO₄·4H₂O; 0.83 mg KI; 0.25 mg
The medium was adjusted to pH 5.6 before autoclaving. All cultures were incubated in the dark at room temperature. The calli that developed were off-white in color and reached a diameter of approximately 1.5 cm after seven weeks. Calli were taken, split into several pieces (approximately 4 mm in diameter), and subcultured on a similar medium except that it contained 0.1 mg 2,4-D and 0.2 mg kinetin. The resulting calli which grew to over a centimeter in diameter, were subcultured again after 6 weeks. Callus pieces (approximately 5 mm across) were transferred to test tubes containing a new low-phosphate medium. The new medium was of the same composition as the root-organ culture medium (described elsewhere), except that it also contained 0.1 mg 2,4-D and 0.2 mg kinetin. A pre-germinated spore of *G. rosea* within a small block of water agar (approximately 5 mm square) was applied in proximity to each of five calli within five separate culture tubes. The culture tubes were incubated in the dark at room temperature and examined periodically. After 10 weeks, the culture tubes were dismantled. Calli were thinly sliced, cleared, and stained in a manner similar to that used to prepare roots. Stained callus slices were examined with a compound microscope in search of observable interactions between the VAMF and the host plant tissue.
Antibiotic Study

The effects of four antibiotics (chloramphenicol, gentamicin, penicillin G, and streptomycin) upon surface-sterilized spores of *Glomus etunicatum* and *Gigaspora rosea* were examined. Germination and hyphal growth in 1% water agar served as the critical variables that were monitored.

**Spore preparation**

Spores of *Gl. etunicatum* and *Gl. rosea* were maintained in pot culture (Gilmore, 1968) with *A. glutinosa*, and *Sorghum vulgare*. Spore-containing samples were taken from the pot cultures and stored at 4°C for at least two weeks before the initial spore extraction and surface-sterilization. Spores were extracted by decanting, wet sieving, and centrifugation-sugar flotation methods (Jenkins, 1964). Spores were separated from debris with stork-billed micro-dissecting forceps and placed in styrene capsules. The styrene capsules are used normally as specimen containers for use in critical point drying during SEM preparation.

**Surface sterilization**

Capsules containing 3-500 spores were fitted with a hard rubber collar to ensure submersion of the spores. The spores were first treated with two 2-min washes in the surfactant 0.05% (Vol/Vol) aqueous Tween 20 followed by a brief rinse in sterile distilled water (Mertz et al., 1979). These spores were then placed in 5% Chloramine T for 20 min, with 10 min of the treatment in vacuo (up to 600 mm Hg) (Mertz et
This was followed by several rinses in sterile distilled water. The spores were stored at 4°C in a filter-sterilized antibiotic solution containing 50 ppm chloramphenicol, 100 ppm gentamicin sulfate, 200 ppm streptomycin sulfate, and 20,000 units/L penicillin G potassium. After a minimum of three months storage, spores were prepared for these studies by an additional surface-sterilization procedure as described above, followed by a thorough rinse in sterile, distilled water. Potentially viable spores were aseptically placed into the agar in petri plates with stork-billed microdissecting forceps.

**Germination test**

Test plates consisted of 1% distilled water agar (Difco Bacto) and 1% distilled water agar with the following filter-sterilized antibiotics and respective concentrations incorporated into the medium: chloramphenicol (150, 75, and 35 ppm), gentamicin sulfate (100 and 50 ppm), streptomycin sulfate (50 and 25 ppm), and penicillin G potassium (50,000 and 25,000 units/L). The pH of the medium was adjusted to 6.6 before autoclaving. Final pH varied slightly from pH 6.5 to pH 6.8. Ten petri plates were used for each treatment with *G. etunicatum*, with an average of 23 spores in each petri plate. For *G. rosea*, four petri plates, averaging 12 spores per plate, were used for each treatment. Each petri plate was considered as an experimental unit for statistical analyses of treatment effects.

Spores which appeared to be injured during transfer to the petri plates were not included in the study. The petri plates for all
treatments were randomly placed and incubated in the dark in a growth chamber. A 12-h day-night cycle with temperatures ranging from 18-29°C was maintained in the growth chamber. Spores were examined periodically, under a dissecting microscope at 100X, for the production of germ tubes. The mortality rate also was monitored for _G. rosea_. Spores which turned brown and became plasmolyzed were classified as moribund.

**Hyphal elongation**

Images of hyphae from isolated single spores were traced onto acetate after all hyphal growth had stopped. The acetate tracings were placed over a grid with 20 squares per inch. This allowed for the number of grid-hyphal intersections to be converted directly to an estimation of hyphal length in mm (Marsh, 1971). Comparisons of hyphal length to spore diameter were made for 1% water agar controls. Spore diameters were estimated with an ocular micrometer on a compound microscope at 100X. The production of extra-matrical vesicles (emv) also was monitored for _G. rosea_.


RESULTS

Structural Studies

Spore viability and culture purity checks

Spore samples of *Glomus etunicatum* and *Gigaspora rosea* both contained viable spores after the surface-disinfestation treatments with Chloramine T. In 1% water agar, *Gl. etunicatum* chlamydospores began germinating 9 days after plating. At 36 days, over 82% (SE = 11.7, n = 6)\(^1\) of the chlamydospores had germinated. Azygospores of *Gi. rosea* began germinating after 18 days in 1% water agar; however, only 9% (SE = 3.8, n = 6) of the azygospores had germinated after 103 days. Nevertheless, 80% (SE = 11.6, n = 6) of these spores had germinated by 163 days. The *Gi. rosea* azygospores that had been placed upon dialysis tubing did not produce any germ tubes until 57 days; however, 68% (SE = 5.8, n = 7) of these spores did germinate by 163 days.

Checks of spore purity within the VAM *Alnus glutinosa* cultures revealed no evidence of contamination by other species of VAMF. The integrity of mycobiontic species within each culture was still intact several months after root samples were obtained for microscopic observation. Control cultures of *A. glutinosa* exhibited no signs of colonization by VAMF.

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\(^1\)SE is the standard error of the mean, and n is the number of plates. Each plate contained an average of 15 spores.
Gigaspora rosea

Azygospores produced by this isolate of Gi. rosea were typically spherical with a diameter ranging from 193\(\mu\) to 287\(\mu\) (Fig. 1, see Appendix. Newly-formed, mature azygospores were primarily white, cream, or pink. The predominance of rose-pink coloration for which this species was named was variable. With a majority of the spores, the rose-pink tint was barely visible or located only in the portion of the spore wall that was proximal to the attachment site of the parental hypha. A smaller proportion of these spores displayed a vivid pink coloration over the entire spore surface. The opacity of mature dormant spores ranged from nearly opaque to slightly translucent. Spores often became darker in color after several weeks in moist environments. Over this period, azygospores frequently yellowed, turned to overall brownish shades, or developed surface spots of dark brown.

The other wall layer of Gi. rosea azygospores appeared to be relatively smooth, and possessed no noticeable ornamentation. Detailed investigations of spore wall structure were hampered by sectioning difficulties with the resin-embedded spores. Nevertheless, limited information was obtained from scanning electron micrographs of freeze-fractured spores, and from light micrographs of 1\(\mu\) spore sections (Figs. 7 and 10). Azygospore walls apparently were composed, in most instances, of three major laminated wall layers. Occasionally, however, fractures of the inner-most wall layer revealed parallel striations that could be interpreted as additional lamina within this portion of the spore wall.
The combined thickness of all wall layers that encompassed these 
*Gi. rosea* azygospores was typically 4.0 to 8.4 μ. Widths of each 
individual major wall layer ranged from 0.4 to 2.8 μ for the outer 
layer, 0.7 to 3.3μ for the middle layer, and 0.9 to 6.8μ for the inner 
layer. Trends of comparative width differences among the three major 
wall layers were not always apparent. When a trend was observed, 
however, the inner wall layers tended to be thicker than the outer wall 
layers. Up to six minor striations were observed within the inner major 
wall layer. The striations within this layer measured from 0.2 to 0.7μ 
in thickness. After staining with methylene blue, the outer major wall 
layer was stained dark blue. Methylene blue produced only light 
staining of the middle wall layer, and an intermediate staining of the 
inner wall layer.

Large lipid droplets appeared to predominate the interior of 
dormant *Gi. rosea* azygospores. Many nuclei and a network of cytoplasmic 
components were interspersed throughout the lipid droplets. All of 
these internal components of the azygospore were apparently distributed 
in nonuniform manners.

Azygospores of *Gi. rosea* are formed on the terminus of a bulbous, 
suspensor—like structure that is a characteristic of *Gigaspora* species 
(Figs. 2 and 3). Remnants of this structure and additional short 
segments of parental hyphae frequently remained attached to the mature 
spore during spore extraction procedures for this isolate. The bulbous, 
suspensor—like structure was spherical or subglobose with a diameter of 
30 to 60μ. At least two wall layers were apparent within the bulbous
structure at the point of spore attachment (Fig. 5). The inner wall of the bulbous attachment appeared to be continuous with an inner wall layer within the azygospore. This wall layer constricted at the attachment site to form a narrow pore with a diameter of 1 to 3 μ (Figs. 4 and 5). Conclusive information about structures that may function in occluding these pores was not obtained. Limited evidence did suggest, however, that the pore was blocked on at least the spore side by wall-like material that also appeared to be continuous with the inner layer of the spore wall (Fig. 6).

A branching hyphal segment, 5 to 10 μ in diameter, that emerged from the suspensor-like structure also was observed frequently (Fig. 2). This hypha extended to the spore surface, but apparently was not connected intimately to the spore. The subtending parental hypha gradually enlarged as it approached the bulbous, suspensor-like structure, and thus was not always readily distinguishable from the attachment structure. Main trunks of parental hyphae were typically 6 to 15 μ wide before they enlarged. Numerous lateral branches were also present on the parental hyphae. The lateral branches ranged from 1 to 8 μ in diameter at their point of origin.

Germination of Gi. rosea azygospores occurred with the emergence of a germ tube directly through the spore wall (Figs. 8, 9, 18, and 19). Each azygospore typically produced only one germ tube; however, up to seven germ tubes from one spore were occasionally observed (Fig. 11). Germ tubes arose most frequently from the portion of the spore that was proximal to the bulbous, suspensor-like structure.
Germ tubes of *Gi. rosea* were constricted as they passed through the spore wall with diameters from 6 to 9μ (Figs. 8 and 9). External examination of the spore surface did not reveal any obvious signs of mechanical penetration of the spore wall by the germ tube. Occasionally, however, a slightly raised collar (0.3 to 1.1μ wide) was observed on the spore surface surrounding the germ tube at the point of emergence. Some germ tubes gradually expanded to their normal growing diameter (7.7 to 12.3μ; Fig. 8) after emerging, while other germ tubes became swollen (10.1 to 17.6μ in diameter; Fig. 9) in the hyphal portion adjacent to the spore surface before returning to their usual size range. After germination in water agar, the main hyphal trunk grew with a negative geotropic orientation in most instances. Geotropic influences upon secondary hyphal branches were not apparent.

Hyphal growth from germinated spores in water agar was variable in form (Figs. 12 and 13). The diameter of newly formed hyphae was diminished as the total hyphal length increased. Segments of main hyphae frequently grew in a bending sigmoid fashion with occasional lateral branches growing away from some apices within the sigmoid curve. Some spores generated hyphae which consisted of a series of connected curved branches with similar orientation. As independent hyphal growth slowed, some hyphae branched perpendicularly into two smaller hyphae which grew in opposite directions. Many other azygospores produced hyphae that grew and branched in a seemingly irregular manner. Hyphae of *Gi. rosea* also exhibited the wound-healing response that was first noted by Gerdemann (1955b) (Fig. 15). No fusion of hyphae produced by
separate spores of this species was observed.

Clusters of echinulate extramatrical vesicles (emv) were sometimes produced upon Gli. rosea hyphae in water agar (Figs. 20, 25-29). The emv clusters were formed frequently on smaller lateral branches of main hyphae. Up to 14 vesicles were observed within a single cluster. Extramatrical vesicles appeared to form from coiled hyphae that emerged from a swollen central structure. This central structure consisted of a short hyphal base with terminal subglobose swelling approximately 25 μ in diameter. Newly-formed extramatrical vesicles lack the echinulate ornamentation that is possessed by mature vesicles. Individual echinulate vesicles were 18 to 37 μ wide and approximately 42 μ long. Spines of various size and distribution covered the portion of the mature vesicle surface that was distal to the parental hypha. Each spine was roughly conical with a rounded apex. Individual spines ranged up to 5.5 μ wide and 4.5 μ long. Two main wall layers appeared to encompass each vesicle. Spines were formed by an extension of the outer wall layer. The inner wall layer was positioned across the base of the spines.

A detailed investigation upon the possible function of extramatrical vesicles was not performed; however, a few observations were noted for emv's produced by germinated azygospores in water agar. When emv's were produced in water agar they were typically formed within a few days after spore germination, but occasionally they were not generated until four weeks after germination. Clusters of emv's were usually formed within 12 mm of the parent spore. Up to six emv clusters
were formed on hyphae from a single spore of *Gi. rosea*. At the time of
emv formation, each vesicle was filled with cellular contents. In most
instances, the connections between the emv's and other portions of the
growing hyphae appeared to remain open until the vesicles were emptied
of their contents (Figs. 28 and 29). In additional studies reported
elsewhere (Antibiotic Study; p. 63), it was determined that the growth
of *Gi. rosea* hyphae in water agar was highly correlated to the number of
emv clusters formed on the hyphae. Occasionally the contents within
emv's became separated from the main hyphae by the formation of a
septum. When this occurred a new radial mass of thin hyphae
(approximately 3 μ in diameter) eventually emerged from within the emv
cluster, and thereby utilized the residual contents of the vesicles
(Figs. 26 and 27). Efforts to localize the site of origin for each new
hypha were unsuccessful. The number of new hyphae was, however, at
least similar to the number of individual vesicles that were contained
within the emv cluster. Nevertheless, conclusive evidence on the
capacity of emv's to serve as spores by producing separate germ tubes
has not thus far been obtained.

Senescence of *Gi. rosea* hyphae in water agar was characterized by
the sequential withdrawal of cytoplasm and concurrent formation of septa
(Fig. 14). Retraction of hyphal cytoplasm usually began at hyphal tips
distal to the parent spore, then progressed systematically toward the
spore until all hyphae were evacuated. After cytoplasmic evacuation,
hyphal septa were evident in variable spacing patterns. Spacing
intervals between septa were often irregular and sometimes relatively
constant, depending upon each individual hyphal segment. Lengths between adjacent septa within major hyphae were typically between 15 and 150 μ. Cytoplasm was occasionally confined within the hypha by the formation of bordering septa, especially if the parent spore was physically separated from the hypha.

Hyphae of *G. rosea* appeared to penetrate roots of *A. glutinosa* in various manners. Frequently, major hyphae penetrated directly through outer epidermal cell walls after forming an appressorium (Fig. 23). Other hyphae penetrated intercellularly by growing between the epidermal cells (Fig. 22). Considerable hyphal growth was noted on the surface of root segments that were heavily colonized by the mycobiont (Fig. 21). These surface hyphae were capable of penetrating additional sites within the root.

Hyphae of *G. rosea* grew intercellularly (Fig. 37) and intracellularly (Fig. 36) within the root cortex of *A. glutinosa*. Hyphal growth seemingly was unobstructed by host cell walls within the cortex; however, no endophyte structures were observed within the stele. Penetration into host cortical cells was generally accomplished by larger hyphae with diameters between 0.7 and 4.4 μ. Penetrating hyphae were usually constricted at the point of cell wall penetration. After gaining entry into the host cortical cell, additional portions of *G. rosea* hyphae frequently demonstrated the capacity to egress from the cell.

The arbuscule was the predominant structure formed by *G. rosea* within the roots of *A. glutinosa* (Figs. 30–35, 38–42). Although
arbuscules were formed within every layer of the cortical parenchyma, they were more predominant in the inner layers adjacent to the endodermis. Intracellular hyphal growth following host cell penetration generate the main arbuscular trunks. The main trunks of the arbuscules were composed of large hyphae with diameters between 0.7 and 10.0 \mu. Growth of the arbuscular trunk varied from a nearly symmetrical balanced orientation to random coiling. The arbuscular trunk continues to grow by forming dichotomous branches which diminish in diameter with each successive branch. Continued dichotomous branching produced arbuscules with a multitude of terminal arbuscular tips with typical diameters between 0.1 and 1.0 \mu. Although total arbuscule size was variable, fully formed arbuscules frequently occupied nearly all regions within the host cortical cell.

Collapsing of the terminal arbuscular tips was the first evidence of \textit{Gi. rosea} arbuscule degeneration (Figs. 33 and 34). Continued arbuscule deterioration appeared to proceed with progressive arbuscule collapse from the terminal tips toward the main arbuscule trunk. Sequential aggregation of collapsed arbuscule portions was observed as evidence of succeeding stages of arbuscule decline. In the latter stages of arbuscule breakdown, the arbuscule remnants were clumped together into an amorphous mass located at the origin of the main arbuscular trunk (Fig. 35). Aggregation of entire arbuscules produced vestigial structures which typically ranged from 1 to 10 \mu across.

Various stages of arbuscular development were frequently observed within a single host cell. Diverse developmental phases were
occasionally displayed by separate portions within a single arbuscule that was generated by one penetrating hypha. Multiple penetrations of a host cell by more than one hypha could also produce arbuscules with contrasting developmental stages.

_Glomus etunicatum_

Chlamydospores generated by this isolate of _Glomus etunicatum_ were globose to subglobose, and possessed diameters between 75 and 138 μ (Fig. 57). Newly formed, mature spores were creamy yellow, but older spores developed a brownish cast. The outer surface of newly formed spores was only slightly textured; however, deterioration of the outer spore wall produced a roughened and irregular spore surface. A funnel-shaped basal protrusion was present on chlamydospores at the attachment site of the parental hypha. The portion of the basal protrusion that was proximal to the spore was typically between 8 and 15 μ in width. Remnants of parental hyphae ranged from 4 to 7 μ in diameter before expansion toward the attachment site occurred.

Chlamydospores from this _Gl. etunicatum_ isolate are encompassed by two major wall layers. The outer surface of the hyaline outer wall layer was frequently irregular. This was apparently the result of external degradative processes. The outer wall layers ranged from 0.8 to 2.2 μ in width. The inner wall layer of the chlamydospores was quite variable in thickness and was composed of multiple indiscrete fused lamina. Thickness of the inner wall layer typically ranged from 2.5 to 11 μ. The thickening in the subtending hypha was composed of an extension of the inner spore wall layer into the basal protrusion.
Staining with methylene blue produced a lightly stained outer wall layer and a dark blue inner wall layer.

The interior of *G1. etunicatum* chlamydomspores was nearly filled with large lipid droplets that ranged up to 20 \( \mu \) in diameter (Fig. 43). A fine network of cytoplasmic components and numerous nuclei was interspersed throughout the lipid droplets. Internal structures within the chlamydomspores were not always distributed uniformly. Nuclei and cytoplasm were sometimes more concentrated within a small area toward the periphery of the spore interior. A more detailed examination of the structural composition within the chlamydomspore was not performed.

Germination of *G1. etunicatum* chlamydomspores typically occurred by germ tube emergence through the remnants of the subtending hypha (Fig. 44). On rare occasions germination proceeded directly through the spore wall (Fig. 45). Spores that germinated through the spore wall produced more than one germ tube in some instances. Germ tubes were constricted when they emerged from the spore wall. Diameters of *G1. etunicatum* germ tubes were usually between 5.8 and 15.6 \( \mu \) at the point of emergence. There was no evidence of a geotropic influence upon the orientation of *G1. etunicatum* germ tube growth in water agar. In addition, hyphae from separate *G1. etunicatum* chlamydomspores in close proximity frequently anastomosed (Fig. 47).

Roots of *A. glutinosa* were approached and penetrated by *G1. etunicatum* hyphae in various manners. In some instances, the hyphae branched repeatedly upon approach to the root. This process resulted in the formation of an "infection fan" (Fig. 58). As branching continued
within the "infection fan," hyphal tips became progressively smaller. Persistent growth and branching of *Gl. etunicatum* hyphae occasionally produced hyphae that were small enough to penetrate root hairs (Fig. 59). Larger *Gl. etunicatum* hyphae also were common in the rhizosphere of *A. glutinosa* (Figs. 49 and 60). In general, larger hyphae were associated more closely to a source of growth-promoting nutrients. The growth-promoting nutrients presumably were derived from the parent spore, and from root colonization by a connected hyphal network within the same root system or within a nearby root system of a separate plant. Larger hyphae produced in these situations also were capable of root penetration. These hyphae could produce appressoria and ingress directly into cells of the root epidermis, or they could gain entry into the root by passing between the epidermal cells (Fig. 61).

Within *A. glutinosa* roots, the intercellular hyphae of *Gl. etunicatum* grew with an orientation that was primarily longitudinal. Diameters of these intercellular hyphae were variable, ranging from 0.6 to 11.5 μ (Fig. 50). Hyphae were capable of penetrating into root cortical cells from intercellular spaces or from adjacent cells (Fig. 51). Penetrating hyphae, which ranged from 0.2 to 5.5 μ in diameter, often noticeably became constricted as they passed through the host cell wall. Upon entry into a cell the intracellular hypha passed through the cell without differentiation, branched repeatedly to generate an arbuscule, or supported the formation of a vesicle.

The production of arbuscules by *Gl. etunicatum* within the root of *A. glutinosa* was localized primarily in the two cortical cell layers.
that bordered the endodermis (Figs. 52, 53, and 64-68). Arbuscules were formed by repeated dichotomous branching of a main hypha after it had ingressed into a host cortical cell. As branching continued the hyphal tips of the arbuscule became smaller, but the cell volume that the arbuscule occupied became larger. Initial arbuscular trunks were variable in size, ranging from 0.6 to 12.5 μ in diameter. Arbuscular tip diameters were usually smaller than 0.8 μ, and were occasionally as narrow as 0.1 μ. Arbuscules of Gl. etunicatum frequently occupied a major proportion of the space between the cell walls of an A. glutinosa cortical cell.

Primary vesicle generation by Gl. etunicatum within A. glutinosa roots appeared to follow initial arbuscule formation (Figs. 54, 55, 68, and 69). Nevertheless, vesicles and arbuscules often existed simultaneously in adjacent regions within the root cortex. Vesicles were produced intercellularly and intracellularly, within the root cortex. Vesicles were spherical to subglobose with diameters up to 60 μ. Hyphae that supported the typically terminal vesicle formation possessed diameters from 0.5 to 6.5 μ. Extensive enlargement of vesicles frequently disrupted the root cortical cells of A. glutinosa. Although a study of vesicle longevity was not performed, it did appear that vesicles could persist for extended periods within the root. Intact vesicles were frequently observed in older root segments that contained no other obvious signs of colonization by Gl. etunicatum.

Developing spores of Gl. etunicatum were occasionally observed. These spores were formed upon hyphae that were associated with A.
glutinosa roots or upon independent hyphae that originated from germinated chlamydospor in water agar (Figs. 48, 56, 60, 62). Spores that were formed in water agar never achieved the full dimensions that are exhibited by typical, mature chlamydospores of Gl. etunicatum (Fig. 48). The production of these smaller spores was frequently associated with other contaminant fungi or bacteria which were occasionally present within the water agar. Some small, thin walled spores with diameters between 20 and 50 µ resembled intercalary spores since a short hyphal segment continued beyond the terminal end of the spore. The small spores ranged from hyaline to various shades of yellow and brown.

Attempts Toward In Vitro Culture of VAM

VAM associations with intact alder seedlings

Over 85% of the alder seeds germinated within one week. This high germination percentage ensured the development of at least one, and usually two, alder seedlings in each tube (Fig. 63). The rate of germination of the VAMF spores in the tubes was not observed, since the spores were sandwiched between opaque layers of the charcoal-water agar mixture. Of the spores placed in the 0.6% water agar for germination checks, germination rates averaged 34% (S.E. = 13.2, n = 3)\(^1\) for the Glomus etunicatum chlamydospores and 30% (S.E. = 5.3, n = 3) for the

\(^1\)S.E. is the standard error of the mean and the n is the number of plates. Each plate contained an average of 10 spores.
Gigaspora rosea azygospores after 14 days. Roots typically emerged below the charcoal-water agar layer within a few weeks. At 10 weeks, only one of the tubes containing alder seedlings and VAM fungi exhibited any sign of contamination by extraneous microorganisms. Nevertheless, nearly all of these in vitro endomycorrhizal culture systems were eventually contaminated when the growth chamber became plagued with an outbreak of mites. After four to five months, growth of A. glutinosa seedlings within these test tubes ceased and overall vitality began to decline. Contaminating microorganisms, nutrient depletion, moisture stress, or other environmental conditions were all possible factors that could have contributed to the decline of seedling vitality. The aerial portions of the alder seedlings ranged from 3.7 to 7.0 cm in height at the onset of degeneration. Each seedling shoot produced at least nine leaves, and the branching root systems had extended down at least 2 cm into the nutrient medium. Most seedlings that were involved in potential VAM associations were allowed to proceed into a degenerative state before harvest, in hopes of attaining in vitro sporulation of the VAMF. The deteriorating condition of the alder seedlings at the time of harvest precluded a meaningful assessment of quantified VAMF colonization levels within the root systems. Thus, results of this study are based primarily upon the monitoring of the external development of the root systems and VAMF within the clear nutrient medium. Microscopic examination of cleared and stained roots served mainly to confirm root colonization by the VAMF.
Gigaspora rosea in culture with alder seedlings

At three weeks, hyphae with clusters of extramatrical vesicles were apparent in the nutrient media of all five mycorrhizal culture tubes containing *G. rosea* in conjunction with *A. glutinosa*. The branching hyphal growth within the nutrient medium did not exhibit any exclusive patterns of attraction toward the alder root system; however, many associations between hyphal growth and host roots were noted. Primary "runner" hyphae, which were oriented parallel to the root, were frequently observed growing within the root hair zone. Up to five branches of a "runner" hypha contacted the root epidermis within a root length interval of one mm. Main branches from the "runner" hypha typically produced appressorium-like structures at the point of root contact. In other instances, hyphae branched repeatedly upon approach to the root in the manner of an "infection fan." The fine hyphal portions of an "infection fan" that did not contact the root frequently became septate. Other portions of the *Gigaspora* hyphal network grew through the nutrient medium in a manner that seemingly was unaffected by host roots. In certain situations, repeated branching of lateral hyphae produced "rhizoid-like" structures similar to those noted by Hepper and Mosse (1975) with *Glomus mosseae* (Figs. 16 and 17). The "rhizoid-like" structures were typically formed toward the periphery of the culture tube when the nutrient medium had begun to shrink because of desiccation. Hyphal growth by *G. rosea* appeared to diminish over time as host plant vigor declined. Hyphae produced lateral branches with progressively decreasing diameters until the onset of septation. Extramatrical
vesicles were usually emptied of their cellular contents before the cessation of hyphal growth (Figs. 28 and 29). In at least some instances, the deterioration of hyphal growth by *Gi. rosea* seemed to be associated with the appearance of contaminating microorganisms. During the later declining stages of these endomycorrhizal culture systems, some segments of residual *Gi. rosea* hyphae were structurally degraded. This decomposition apparently resulted from the activities of various microbial contaminants; however, no attempts were made to identify the extraneous microorganisms that were potentially involved in these degradative processes. Throughout the course of this study, no developmental stages of azygospore formation were observed within any in vitro culture system that contained *Gi. rosea*.

Of the five in vitro mycorrhizal culture systems involving *Gi. rosea*, only one was dismantled and examined before observable deterioration in host-plant vigor had occurred. Alder root samples from this endomycorrhizal culture system were collected after 10 weeks of growth. There was no visual evidence of contaminating microorganisms within the culture tube at the time of harvest. In addition, no microbial growth was observed after samples from the growth medium were plated on nutrient agar. Light microscopic examinations of the cleared and stained root samples from this culture system confirmed the establishment of an in vitro VAM association between *Gi. rosea* and *A. glutinosa*. Intercellular and intracellular hyphae were the predominant structures of *G. rosea* that were observed within the colonized portions of the alder root system. Rows of intracellular arbuscules also were
observed occasionally within the inner cortical cell layers of young lateral roots. The remaining four endomycorrhizal culture systems involving *Gl. rosea* were left intact for five months before they were dismantled for microscopic examinations of the alder root systems. At the time of harvest, the alder seedlings within each remaining tube were in a state of decline, and all four tubes had become contaminated with a wide array of microorganisms. Nevertheless, some evidence of root colonization by *Gl. rosea* was apparent within alder root systems from two of the culture tubes. Although intact, intracellular arbuscules were not observed within these deteriorating root systems, segments of intercellular hyphae were occasionally noted.

*Glomus etunicatum* in culture with alder seedlings

In the *in vitro* associations of *Gl. etunicatum* and *A. glutinosa* seedlings, root growth into the nutrient medium typically preceded the appearance of VAMF hyphae. Within the nutrient medium, the fine hyphae of *Gl. etunicatum* formed an intricate network which extended several millimeters away from the seedling roots. These interconnecting hyphal networks possibly were comprised of hyphae from separate parental chlamydospores; however, this occurrence could not be confirmed since the chlamydospores were embedded in the opaque charcoal-water agar layer. The hyphal network of *Gl. etunicatum* appeared to establish frequent contacts with the seedling root systems of *A. glutinosa*. With larger hyphae, an appressorium-like swelling was occasionally observed at the point of root contact. In other situations, the alder roots were approached by a progressive series of dichotomously branched hyphae.
These branching hyphae frequently formed a framework that resembled an "infection fan" in structure (Fig. 58). In these instances, hyphal diameters were progressively reduced as the hyphae continued to branch and grow toward the host root. Some of the narrower hyphae (2 to 8 μ in diameter) approached and contacted the outer surfaces of alder root hairs in a manner that possibly resulted in host penetration (Fig. 59). Nevertheless, this mode of ingress by *Gl. etunicatum* remains only tentative since an internal confirmation of root hair penetration was not obtained. The networks of *Gl. etunicatum* hyphae also contained hyphal segments that grew longitudinally along the root epidermal surface. In some instances, this form of hyphal growth appeared to precede intercellular root penetrations by the mycobiontic hyphae. As noted previously, however, in situ verifications of genuine root penetrations by specific hyphal segments were unattainable. Some components of the *Gl. etunicatum* hyphal system did assume the characteristics of "runner" hyphae. These hyphal segments developed in a manner that closely followed the growth of host roots. In addition, these hyphae also continually produced lateral branches that established relatively steady contacts with the host roots and with other adjacent areas of the nutrient medium. The characteristics of "runner" hyphae were, however, seldom attributable to distinct units of developing hyphae. In most cases, root associations involved continuous extensions of an interconnecting hyphal network. These hyphal networks seemed to form numerous connections between separate portions of the seedling root system. In other situations, continuous frameworks of *Gl. etunicatum*
hyphae established consistent contacts with separate lateral roots from independent root systems.

Developmental stages of spore production were observed in two in vitro culture systems that contained *Gl. etunicatum*. After 16 weeks in culture, evidence of the sporulation process first was observed in the area just beneath the charcoal-water agar layer. Microscopic observation was restricted severely within this area of the culture tube; however, limited aspects of the *Gl. etunicatum* sporulation process remained visible. The sporulation processes continued very gradually during the subsequent four weeks. Shortly after this time, growth of both mycobionts and host seedlings had ceased within the deteriorating in vitro culture systems.

All of the visible spores were produced within a two-millimeter radius of a host root. In addition, hyphal traverses between the spores and the nearest apparent root contact seldom involved distances over a few millimeters. Early stages of spore formation first were apparent as translucent, spherical swellings on the hyphae. In some situations, spores were formed upon short lateral branches extending from a primary hyphal. Other spores were produced by parental hyphae that originated from interconnecting hyphal networks. The diameters of the parental hyphae ranged from 2 to 8\(\mu\). Spore formation was frequently initiated at points up to 75 \(\mu\) away from the terminus of the parental hypha. Only four visible spores developed the appearance of mature chlamydospores (Fig. 62). These spores were light brown and ranged from 80 to 100\(\mu\) in diameter. Several other observable spores that were produced in vitro
remained relatively small (20 to 35 μ in diameter) as the conditions within the culture systems continued to deteriorate. Thus, no evidence was obtained to firmly link these smaller spores with the development of larger chlamydospores.

Intraradical structures of *Gl. etunicatum* were infrequent within alder roots from the culture tube that was harvested at 10 weeks. Mycobiontic colonization was observed in the form of intercellular and intracellular hyphae at localized points within these roots. In addition, visual inspection and nutrient agar culture indicated that this endomycorrhizal culture system was free of extraneous microorganisms at the time of harvest. The remaining nine *in vitro* culture systems of this type proceeded into a degenerative state before the host roots were collected for clearing and staining procedures. One of the mycorrizal culture systems that contained stages of spore development was apparently still gnotobiotic at the time of harvest; however, a wide array of contaminating microbes was evident within all of the other remaining culture tubes. Remnants of VA mycorrhizal colonization were observed within deteriorating roots from three culture systems. Intercellular and intracellular hyphae of *Gl. etunicatum* were present at sporadic locations within these alder roots. In addition, spherical swellings which apparently represented early stages of vesicle formation also were observed within roots from one of the sporulating culture systems.

**VAM associations with root organs**

Of the *Gl. etunicatum* chlamydospores that were placed near root organs of *A. glutinosa* on the full-strength nutrient medium, 24% (S.E. =
had germinated after 30 days. This germination rate was similar to that achieved by spores with root organs on the medium with certain nutrients (Ca, Cl, K, Mn, Mg, N, S,) at half-strength, of which 22% (S.E. = 7.5, n = 6) germinated. In contrast, only 3% (S.E. 1.4, n = 8) of the chlamydospores germinated in plates containing 0.6% water agar. Two plates of each nutrient medium with root organs became contaminated with bacteria; however, in each case the bacterial contamination did not seem to affect germination rates. No contamination was evident on the plates containing water agar.

Root organs of *A. glutinosa* did not grow well on either nutrient medium. Within a few weeks, roots typically became necrotic, especially in the older portions of the root. Root growth typically occurred from the root apices and ranged from 5 to 15 mm in length. Lateral roots were initiated on only four root organs. In addition, no root organ produced more than two lateral roots, and no lateral root exceeded 6 mm in length.

Clearing and staining procedures revealed that *Gl. etunicatum* had colonized at least four *A. glutinosa* root organs on each nutrient medium. Similar patterns of root colonization were produced upon both types of nutrient media. Intraradical structures of the mycobiont were usually restricted to an older portion of the root organ. This zone of endomycorrhizal colonization was situated between the severed hypocotyl and the region of new longitudinal growth. Root organ colonization by *Gl. etunicatum* typically appeared as a continuous unit which was

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1S.E. is the standard error of the mean, and n is the number of petri plates.
localized within root segments of a few millimeters in length. Each mycorrhizal root organ contained only one or two discernible units of mycobiontic colonization. Intraradical structures of Gl. etunicatum were never observed within slender, newly-grown segments that were located within several millimeters from the root organ tip. At harvest, most root organs exhibited various stages of tissue necrosis within older root portions which included regions of mycorrhizal colonization.

The degree of mycorrhizal development varied among separate root organs, and among localized sites within individual root organs. Intercellular and intracellular hyphae were observed in all mycorrhizal root organs of A. glutinosa. A majority of these root organs also contained areas where remnants of degraded arbuscules were the prevalent mycobiontic feature. Stages of vesicle development also were observed sporadically within the degraded arbuscular regions of four root organs. The developing vesicles were relatively small, with diameters less than 25 µ. No evidence of extraradical chlamydospore formation by Gl. etunicatum was observed.

**Associations of VAMP with undifferentiated callus tissue**

Undifferentiated callus tissue from A. glutinosa grew only minimally on the low-phosphate medium that was used to examine interactions with Gi. rosea. Nevertheless, the callus tissue remained viable during the interaction study. Three of the five germinated spores of Gi. rosea produced hyphae that were less than 4 cm in length. Signs of growth interactions were not apparent in the hyphae from these three spores. Hyphae from the two remaining spores did, however, exhibit growth
characteristics that indicated a possible interaction with the callus tissue and (or) the nutrient medium. Hyphal growth from each of these spores exceeded 35 cm. Branches of these hyphae grew into loosely-aggregated callus and also grew several centimeters down into the nutrient medium. In addition, at least two clusters of extramatrical vesicles also were produced upon the hyphal branches that arose from each spore. When cleared sections of the calli were examined under a compound microscope, intercellular hyphal growth between the callus cells was occasionally observed. No signs of callus cell penetration by *Gi. rosea* hyphae were noted.

**Antibiotic Study**

There was no sign of contamination associated with any of the spores placed in 1% distilled water agar with antibiotics incorporated. With *Gl. etunicatum*, one spore out of 200 in the water agar controls became contaminated with bacteria, and it failed to germinate. Three of 50 *Gi. rosea* spores in the controls became overgrown with bacteria, of which two germinated. However, it is assumed that other microbes, that could not grow on the nutrients provided, may have been present.

The germination rates for each treatment of *Gl. etunicatum* and *Gi. rosea* were determined (Figs. 70 and 71, see Appendix). Spores of *Gl. etunicatum* germinated primarily by regrowth through the parental hyphal attachment, although occasional germination through the spore wall also was noted (Figs. 44 and 45). With *Gi. rosea*, germination occurred exclusively through the spore wall (Figs. 12 and 13). Spore germination occurred predominantly between 5 and 36 days with *Gl. etunicatum*, and
between 9 to 27 days with *Gi. rosea*. Gentamicin appeared to be the only antibiotic that significantly inhibited germination of *Gl. etunicatum* at the test concentrations (see Table 1). At 36 days, *Gl. etunicatum* had 85% or better germination for each of the following treatments: 1% water agar (control), 1% water agar with penicillin (50,000 and 25,000 units/L), 1% water agar with chloramphenicol (150, 75, and 35 ppm), and 1% water agar with streptomycin (50 and 25 ppm). In contrast, treatments with higher concentrations of chloramphenicol (150 and 75 ppm) most significantly inhibited the germination rate of *Gi. rosea* (Table 2). All levels of chloramphenicol that were tested significantly increased the mortality rate of *Gi. rosea* spores at 77 days. The remaining eight treatment groups ranged from 53 to 74% germination at 27 days, and from 14 to 44% mortality at 77 days.

Hyphae from *Gl. etunicatum* spores germinating within 36 days were measured after elongation had ceased. The effect of the various antibiotic treatments upon maximum hyphal elongation was determined (Table 3). The mean maximum hyphal length for the 1% water agar controls was 13.4 mm. The mean control length was exceeded significantly by both penicillin treatments (25,000 and 50,000 units/L) and by the half-strength streptomycin treatment (25 ppm). Higher concentrations of gentamicin (100 ppm) and chloramphenicol (150 ppm) appeared to have a deleterious effect upon the hyphal growth of *Gl. etunicatum* (Fig. 46).

The antibiotic effects upon hyphal growth of *Gi. rosea* were summarized (Table 4). Hyphae from germinated spores in the 1% water agar controls grew to an average maximum length of 80.3 mm. The mean maximum
Table 1. Effect of antibiotics on germination rates of *Glomus etunicatum*

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<th>Treatment</th>
<th>% Germination</th>
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<tbody>
<tr>
<td>1% water agar (control)</td>
<td>97.3 A¹</td>
</tr>
<tr>
<td>Penicillin G (50,000 units/L)</td>
<td>94.1 AB</td>
</tr>
<tr>
<td>1/2 Chloramphenicol (75 ppm)</td>
<td>93.7 AB</td>
</tr>
<tr>
<td>1/4 Chloramphenicol (35 ppm)</td>
<td>91.7 ABC</td>
</tr>
<tr>
<td>Streptomycin (50 ppm)</td>
<td>90.2 BC</td>
</tr>
<tr>
<td>1/2 Penicillin G (25,000 units/L)</td>
<td>90.0 BC</td>
</tr>
<tr>
<td>Chloramphenicol (150 ppm)</td>
<td>88.4 BC</td>
</tr>
<tr>
<td>1/2 Streptomycin (25 ppm)</td>
<td>85.7 C</td>
</tr>
<tr>
<td>1/2 Gentamicin (50 ppm)</td>
<td>65.1 D</td>
</tr>
<tr>
<td>Gentamicin (100 ppm)</td>
<td>14.5 E</td>
</tr>
</tbody>
</table>

¹Means within each major grouping with the same letter are not significantly different at the probability level of .05, Duncan's multiple range test (analysis performed on arcsin percentage germination).
Table 2. Effect of antibiotics on germination and mortality rates of *Gigaspora rosea*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Germination</th>
<th>% Mortality&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2 Gentamicin (50 ppm)</td>
<td>74.3 A&lt;sup&gt;2&lt;/sup&gt;</td>
<td>21.8 A</td>
</tr>
<tr>
<td>1/2 Water agar (control)</td>
<td>69.6 A</td>
<td>36.3 A</td>
</tr>
<tr>
<td>1/2 Streptomycin (25 ppm)</td>
<td>66.4 A</td>
<td>26.3 A</td>
</tr>
<tr>
<td>Penicillin G (50,000 units/L)</td>
<td>63.1 AB</td>
<td>13.5 A</td>
</tr>
<tr>
<td>Streptomycin (50 ppm)</td>
<td>58.5 AB</td>
<td>32.3 A</td>
</tr>
<tr>
<td>Gentamicin (100 ppm)</td>
<td>55.4 AB</td>
<td>44.1 A</td>
</tr>
<tr>
<td>1/2 Penicillin G (25,000 units/L)</td>
<td>53.5 AB</td>
<td>27.3 A</td>
</tr>
<tr>
<td>1/4 Chloramphenicol (35 ppm)</td>
<td>53.0 AB</td>
<td>97.7 B</td>
</tr>
<tr>
<td>1/2 Chloramphenicol (75 ppm)</td>
<td>41.1 BC</td>
<td>89.2 B</td>
</tr>
<tr>
<td>Chloramphenicol (150 ppm)</td>
<td>18.4 C</td>
<td>96.4 B</td>
</tr>
</tbody>
</table>

<sup>1</sup>Mortality = % of spores and geminated spores dead at 77 days.

<sup>2</sup>Means within each major grouping with the same letter are not significantly different at the probability level of .05, Duncan's multiple range test (analysis performed on arcsine percentage).
Table 3. Effect of antibiotics on hyphal length of *Glomus etunicatum*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2 Penicillin G (25,000 units/L)</td>
<td>23.4 A¹</td>
</tr>
<tr>
<td>Penicillin G (50,000 units/L)</td>
<td>19.7 B</td>
</tr>
<tr>
<td>1/2 Streptomycin (25 ppm)</td>
<td>18.9 B</td>
</tr>
<tr>
<td>1/2 Gentamicin (50 ppm)</td>
<td>13.6 C</td>
</tr>
<tr>
<td>1% Water agar (control)</td>
<td>13.4 C</td>
</tr>
<tr>
<td>Streptomycin (50 ppm)</td>
<td>12.9 C</td>
</tr>
<tr>
<td>1/4 Chloramphenicol (35 ppm)</td>
<td>10.0 CD</td>
</tr>
<tr>
<td>1/2 Chloramphenicol (75 ppm)</td>
<td>8.0 DE</td>
</tr>
<tr>
<td>Chloramphenicol (150 ppm)</td>
<td>5.7 E</td>
</tr>
<tr>
<td>Gentamicin (100 ppm)</td>
<td>5.3 E</td>
</tr>
</tbody>
</table>

¹Means within each major grouping with the same letter are not significantly different at the probability level of .05, Duncan's multiple range test.
Table 4. Effect of antibiotics on hyphal length of *Gigaspora rosea*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2 Gentamicin (50 ppm)</td>
<td>126.8 A&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>1/2 Penicillin G (25,000 units/L)</td>
<td>105.0 AB</td>
</tr>
<tr>
<td>Gentamicin (100 ppm)</td>
<td>99.4 AB</td>
</tr>
<tr>
<td>1/2 Streptomycin (25 ppm)</td>
<td>88.3 AB</td>
</tr>
<tr>
<td>Penicillin G (50,000 units/L)</td>
<td>82.4 AB</td>
</tr>
<tr>
<td>1% Water agar (control)</td>
<td>80.3 B</td>
</tr>
<tr>
<td>Streptomycin (50 ppm)</td>
<td>65.8 B</td>
</tr>
<tr>
<td>Chloramphenicol (150 ppm)</td>
<td>5.9 C</td>
</tr>
<tr>
<td>1/2 Chloramphenicol (75 ppm)</td>
<td>3.8 C</td>
</tr>
<tr>
<td>1/4 Chloramphenicol (35 ppm)</td>
<td>2.9 C</td>
</tr>
</tbody>
</table>

<sup>1</sup>Means within each major grouping with the same letter are not significantly different at the probability level of .05, Duncan's multiple range test.
hyphal length from spores in the 50 ppm gentamicin treatment significantly exceeded that of the control. All test concentrations of chloramphenicol appeared to inhibit hyphal growth of *G. rosea*.

The diameter of *G. etunicatum* chlamydospores ranged from 75 to 138 μ with a mean diameter 109 μ. With the 1% water agar controls, there was no apparent relationship between spore diameter and maximum hyphal length. However, in agar with 100 ppm gentamicin the larger spores exhibited a significantly greater tendency to germinate. The mean diameter of the spores that germinated in gentamicin (100 ppm) was 116μ, whereas 103 μ was the mean diameter of the spores that failed to germinate (*F = 57, df = 1, 205, P=0.0001*). Observations on the direction of germ tube growth revealed no evidence of geotropism in the germination of growth of *G. etunicatum*. It is also of interest to note that hyphae growing from separate *G. etunicatum* spores in close proximity often became fused (Fig. 47). There was no production of vegetative spores on any of the *G. etunicatum* spores observed in this study.

In contrast to that of *G. etunicatum*, the direction of germ tube growth of *G. rosea* exhibited a very strong negative geotropic orientation. The diameter of *G. rosea* spores used in this study ranged from 193 to 287 μ with a mean of 241μ. There was no significant correlation between spore diameter and the length of hyphae produced by a spore. However, there was a strong positive correlation between hyphal length and the number of extra-matrical vesicle clusters that were produced on the hypha (see Table 5). One to six extra-matrical vesicle clusters often were produced on *G. rosea* hyphae, except those grown in
Table 5. Correlation coefficients and levels of significance for variables of *Gigaspora rosea* (control)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Correlation Coefficient</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length vs. diameter</td>
<td>0.06</td>
<td>0.76</td>
</tr>
<tr>
<td>Length vs. #emv₁</td>
<td>0.87</td>
<td>0.0001</td>
</tr>
<tr>
<td>Length vs. germination time</td>
<td>-0.42</td>
<td>0.02</td>
</tr>
<tr>
<td>#emv₁ vs. germination time</td>
<td>-0.31</td>
<td>0.07</td>
</tr>
<tr>
<td>#emv₁ vs. diameter</td>
<td>-0.03</td>
<td>0.86</td>
</tr>
</tbody>
</table>

¹Number of extra-matrical vesicle groups.
agar in which chloramphenicol was incorporated. The formation of emv's began 3 to 29 days after spore germination. The extra-matrical vesicles were formed 1 to 12 mm from the parent spore, usually on a side branch of the main hypha. The apparent function of the emv's seemed to be as an energy reserve for the growing main hypha (Gerdemann and Trappe, 1974; Nicolson and Gerdemann, 1968). If the pathway to the growing hypha remained open, the emv clusters were eventually emptied of their contents (Figs. 28 and 29). Occasionally a septum formed to separate the emv food reserve from the senescing, evacuating main hypha. In these instances, the emv contents were utilized by a new radial mass of thin hyphae which emerged from within the emv clusters (Figs. 26 and 27).
DISCUSSION

Structural Studies

Examinations of spore structure in this study were hampered by the difficulty in obtaining adequate sections of resin-embedded spores. The utilization of various measures to improve resin-infiltration was not sufficient to overcome the problems that were encountered. While working with dormant azygosporas of *Gigaspora margarita*, Sward (1981a) overcame infiltration problems with a 20-sec pretreatment with 0.5% sodium hypochlorite. In this study, it was hoped that treatment with Chloramine T would fulfill the same purpose. Rupturing of the spore wall also has been successful in previous attempts to facilitate infiltration of VAMF spores (Macdonald et al., 1982; Mosse, 1970b; Sward, 1981a). Other efforts which were employed in this study to improve resin-infiltration of spores included: using loose spores which were not surrounded by agar, performing all infiltration steps under conditions with constant rotation and low humidity, extending the infiltration process to several days, and employing different kinds of resin with low viscosities. When all of the aforementioned techniques were applied, resin-infiltration into the spores improved. Nevertheless, sectioning difficulties still persisted even though spore interiors appeared to be embedded adequately with resin. The primary cause of sectioning problems was related apparently to the spore walls. Structural and(or) compositional properties of the spore walls may be somewhat incompatible with infiltration by the resins. During microtomy, the spore wall or layers of the spore wall tended to separate from the rest of the resin section.
Similar problems were noted during sectioning of vesicles that were produced intraradically by *Glomus etunicatum*. Hopefully, solutions to these hinderances will be obtained so that the internal structure of these spores and vesicles can be examined in greater detail.

Roots were cleared adequately and stained by all of the methods that were utilized in preparation for light microscopic observation. However, clearing techniques that did not involve high temperatures generally left roots in better condition for physical manipulations. PVL was more convenient to use for specimen mounting than was Permount® since roots could be mounted directly in PVL after clearing and staining. The disadvantage of PVL as a mounting medium is its tendency to shrink over time, which can sometimes distort specimens. For this reason, Permount® seems preferable as a long-term mounting medium.

Host root cytoplasm was removed sufficiently by both techniques that were used in preparation for SEM observation of mycobiontic structures within the root. However, the method derived from King et al. (1981) which utilized soluene® instead of KOH is much less complicated. Therefore, the soluene® clearing method is preferable with compatible specimens. After fixation for SEM, dormant spores that were air-dried from absolute ethanol appeared to have the same satisfactory morphological preservation as those that were subjected to critical point drying. Nevertheless, the critical point drying procedure is still useful in preserving more delicate structures, such as hyphae. The process of freeze-fracturing spores is frequently quite arduous, but this technique did provide worthwhile results. After the fracturing process,
internal spore structure was observed more clearly with SEM. Freeze-fracturing was especially useful in defining spore wall layers.

The structural characterization of the *Gigaspora rosea* azygospores that were used in this study was reasonably comparable to the original description of this species (Nicolson and Schenck, 1979). Some spores used in this study were slightly smaller than the minimum diameter of 230 μ that was listed in the species description. Nicolson and Schenck (1979) also state that *Gi. rosea* azygospores possess two to five inseparable wall layers 1-2 μ thick. Although spores in this study possessed three major wall layers, some major inner wall layers did possess parallel striations that could be interpreted as additional laminated wall layers (Fig. 7). A number of factors could contribute to differences in spore structure. These factors include the following: 1) Genetic variation among spore isolates may contribute to several structural differences. 2) Some spore structures most likely will be altered during the maturation process. For instance, the inner wall layers of maturing *Gi. rosea* azygospores seemed to become thicker with time. 3) Growth conditions under which spores are produced may affect spore morphology. 4) Spore anatomy may change during extraction and isolation procedures. 5) Methods utilized to prepare spores for observation may influence certain anatomical aspects of the spores.

There are apparently no previous reports on the morphology of *Gi. rosea* azygospores other than light microscopic examinations of intact or crushed spores. Nevertheless, detailed structural and ultrastructural investigations of *Gigaspora margarita* azygospores have been performed by
Sward (1981a,b,c). *G. rosea* and *G. margarita* appear to share enough similar features to allow for meaningful comparisons between these two species. In histochemical and TEM studies upon dormant azygospore sections of *G. margarita*, Sward (1981a) found these spores to contain four major wall layers. It is difficult to compare the four spore wall layers of *G. margarita* to the three spore wall layers of *G. rosea* without additional histochemical and TEM examinations of *G. rosea* azygospores. Nevertheless, both species possessed outer spore wall layers that readily separated from the other wall layers. In addition, the third major wall layer exhibited a laminated appearance in the azygospores of both *G. rosea* and *G. margarita*. The structure of the bulbous, suspensor-like attachment of *G. rosea* also possessed similarities with that found by Sward (1981a) for *G. margarita*. With both species, the outer wall layer of the bulbous, suspensor-like structure appears to be continuous with the outer spore wall layer. Continuity also exists between inner wall layer organization within the attachment structure and the azygospore for both species. Sward (1981a) also described a double walled plug or occlusion which separated *G. margarita* spore contents from the subtending hyphal lumen. Limited SEM evidence supported the existence of an occlusion in these specimens of *G. rosea* (Fig. 6).

Some differences also exist among the spore walls of *G. margarita* which were studied by Sward (1981a) and those of *G. rosea* which were examined in this investigation. A fourth major wall layer, which was present in the *G. margarita* azygospores examined by Sward, was not
apparent in the *G. rosea* azygospores of this study. Since the *G. rosea* spores were newly formed, it is conceivable that a fourth wall layer could have been formed later during the prolonged period that preceded germination. However, evidence to support this hypothesis is yet to be obtained. Variation in the thickness of spore wall layers could provide a supporting basis for distinguishing between representatives of *G. rosea* and *G. margarita*. Further studies on the composition and fine structure of the spore wall layers of *G. rosea* are necessary to determine if additional analogies exist with the wall layers of *G. margarita* azygospores.

The *G. rosea* azygospores germinated in a manner that is characteristic of *Gigaspora* species, because germ tubes emerged directly through the spore wall (Gerdemann and Trappe, 1974) (Figs. 8, 9, 11-13, 18, and 19). Germ tube constriction at the point of emergence seemed to indicate that at least some mechanical forces may be involved in the germination process of *G. rosea*. The occasional presence of a slightly raised collar on the spore wall around the germ tube could be the result of enzymatic degradative processes, secretory processes, or physical pressure produced during germination. Sward (1981b,c) found evidence that two different processes were involved in spore wall penetration by germ tubes of *G. margarita*. In these studies, the inner spore wall layers apparently were degraded by enzymatic activity during germination. In addition, a "flap" of outer wall layer material at the point of germ tube emergence suggested that this wall layer was penetrated by physical pressure from the germ tube. No "flaps" were observed on the germinated
cells" to avoid confusion with other uses of "vesicle" in VAM terminology. Although this suggestion has merit, the more established term of "extramatrical vesicle" is used in this report because these structures usually were not soil-borne.

The external structure of the extramatrical vesicles produced by *G. rosea* in this study (Figs. 20, and 25-29) was similar to that entailed in the original description of this species (Nicolson and Schenck, 1979). *G. margarita* also produces emv clusters of comparable structure (Becker and Hall, 1976; Sward, 1978). Extramatrical vesicles appear to function in some manner as compartments for temporary food storage. Decreases in stored materials within emv's were typically accompanied by increases in the growth of hyphae that were connected to the emv cluster. Additional studies on the composition of the stored food reserves within emv's could provide valuable information on the metabolism and nutritional requirements of the mycobiont. It also would be of interest to compare emv food reserves to those that are contained within the parent spore. Continued investigation of principles that are responsible for positive correlations between emv production and hyphal growth also could provide insight into the mycobiont physiology. Such studies could elucidate relationships between hyphal growth and intrahyphal transport of energy sources. Additional information about mechanisms involved in triggering emv formation also should prove useful. The apparent ability of emv's to support limited independent hyphal growth (Figs. 26 and 27) suggests that these structures may potentially act as infective propagules; however, emv's of *G. margarita* did not fulfill this role in other studies.
(Biermann and Linderman, 1983). Nevertheless, additional studies are required before the overall significance of env's as a specialized structural stage within the life cycle of these VAMF is fully understood.

Hyphae of *Gi. rosea* have expressed the capacity for both intercellular and intracellular modes of penetration into the roots of *A. glutinosa*. This compares to studies of *Gi. margarita* by Sward (1978). In Sward's investigation, the mycobiont produced appressoria for direct penetration into root epidermal cells of *Trachymene anisocarpa* (Turcz) B. L. Burtt and *Leptospermum juniperinum* Sm. Variation in the mycobiont, host roots, and the environment of the rhizosphere could all influence the manner in which a mycobiont penetrates into host roots. Variation in VAMF hyphae could arise from inherent genetic differences and from differences in the nature of the nutrient source for the growing hyphae. These factors would determine the ability of hyphae to produce the appropriate enzymes and(or) sufficient mechanical force for various modes of root penetration. Root properties that influence mycobiont penetration are also likely to vary, even between different portions of the same root system. Other physical and biological components of the rhizosphere also may influence root penetration by VAMF.

The basic structure of *Gi. rosea* within *A. glutinosa* roots was similar to that typified by most VAM associations. As is common with most VA mycorrhizas formed by species of *Gigaspora*, vesicles were not formed within roots colonized by *Gi. rosea*. Abundant intracellular and intercellular growth of *Gi. rosea* hyphae seemed to indicate that cortical cell walls and the middle lamella within *A. glutinosa* roots did not
impede seriously the spread of this endophyte. This was in contrast to the study of Sward (1978), in which *Gi. margarita* was found to spread by predominately intracellular growth within colonized root sites of *Leptospermum juniperinum* and *Trachymene anisocarpa*. *Gi. rosea* hyphae occasionally formed coil-like structures within cells of the outer cortex; however, the coiled hyphae frequently continued to grow and branch, thereby producing arbuscules. For this reason, coils cannot be considered as a structure that was readily separable from arbuscules in this particular VAM. This fact does, however, raise questions about what conditions determine whether intracellular hyphae will develop into arbuscules or remain relatively undifferentiated. The ability of *Gi. rosea* hyphae to egress from cortical cell of *A. glutinosa* is also of interest. Little is known about the timing of hyphal egress from a cortical cell in relation to arbuscular development on hyphal branches within the same cell. The capacity of *Gi. rosea* to grow from cortical cells also implies that the failure of the endophyte to penetrate the stele is dependent upon other factors than the possible mechanical barrier that is imposed by the Casparian strip. Observations of various stages of arbuscular development within a single cortical cell raised additional questions about conditions that contribute to arbuscular decline. This occurrence seemed to imply that factors responsible for arbuscular decline are extremely localized within subcellular regions and(or) over time.

The formation of azygospores by *Gi. rosea* was not examined in this study. On rare occasions, however, the hyphae of *Gi. rosea* formed
structures that resembled the bulbous, suspensor-like appendage on which azygospores are typically produced (Fig. 24). In some instances, cytoplasm began to collect in a saclike structure that was produced on the terminal end of the bulbous, suspensor-like structure. Lateral hyphal branches that arise near the base of the bulbous structure were of additional interest. Two of the hyphal branches frequently grew and curved around so that the bulbous structure was encircled distantly from two different directions. All of the forementioned structural features resembled early stages in the sporulation process of *G. margarita* that were recently observed by Miller-Wideman and Watrud (1984) on root cultures of tomato. It therefore seems likely that *G. rosea* did begin to initiate the sporulation process in water agar on a few rare occasions. In these instances, the sporulation process probably was not completed because the required nutrients and(or) conditions were not supplied by the water agar medium. Since the complete life cycle of *Gigaspora* spp. is still poorly understood, the overall significance of the forementioned structures and events in the sporulation process of *G. rosea* remains undetermined. Nonetheless, this phenomenon provided encouragement about the possibility of achieving *in vitro* sporulation of *Gigaspora* spp. in independent culture.

Dormant chlamydospores from this *G. etunicatum* isolate possessed a morphology that is similar to that entailed in the original description of this species by Becker and Gerdemann (1977). It was not unexpected that most of the *G. etunicatum* chlamydospores germinated by the extension of a germ tube from the parental hyphal attachment (Fig. 44),
because most chlamydosporic species of VAMF germinate in this manner (Trappe and Schenck, 1982). The occasional germination directly through the spore wall of *Gl. etunicatum*, and the rare production of multiple germ tubes were interesting exceptions (Fig. 45). These exceptions perhaps were attributable to changes in spore wall properties. An alteration of the spore wall could be caused by microbial activity or by the oxidative treatment used during surface sterilization procedures. Other studies indicated that surface-sterilization treatments and the age of the spore influenced the production of multiple germ tubes by *Gl. margarita* azygospores (Sward, 1981c).

This was the first report of hyphal anastomosis within *Gl. etunicatum* (Fig. 47); however, hyphal anastomosis has been reported for other chlamydosporic species within the Endogonaceae (Godfrey, 1955; Godfrey, 1957; Hepper and Mosse, 1975; Mosse, 1961; Mosse, 1973). Continued investigation of this phenomenon should help delineate phylogenetic relationships among these mycobionts. Additional studies also are needed before the significance of hyphal anastomosis within the natural life cycle of these VAMF is fully understood.

Many intricacies of a VA mycorrhizal relationship were exemplified by the various modes of penetration that were utilized by *Gl. etunicatum* to gain entry into roots of *A. glutinosa*. Detailed information about root penetration by species of *Glomus* remains scarce, even though most morphological studies of VAM involve mycobionts from this genus (Scannerini and Bonfante-Fasolo, 1983). Nevertheless, root penetration by other *Glomus* spp. apparently can occur with or without the formation
of structures that resemble appressoria (Carling and Brown, 1982; Cox and Sanders, 1974; Scannerini and Bonfante-Fasolo, 1983). Other factors besides the genetic makeup of the host and endophyte also may influence the mode of root penetration which is utilized by the mycobiont. These factors may involve the inoculum source and the infective vigor of the VAMF (Hayman, 1983). Numerous environmental factors that influence root penetration by VAMF also are discussed in the review by Hayman (1983).

The host plant apparently plays an important role in determining the extent of intercellular and intracellular hyphal growth by the mycobiont within the root. When onion and maize were colonized by Glomus spp., intercellular hyphae were predominant (Cox and Sanders, 1974; Gerdemann, 1965). In contrast, growth of Glomus hyphae within roots of yellow poplar was mainly intracellular (Gerdemann, 1965). Colonization of soybean by Gl. mosseae was comparable to that of A. glutinosa and Gl. etunicatum in that both intercellular and intracellular hyphae were common (Carling and Brown, 1982).

Arbuscular development by Glomus spp. was a primary concern of previous ultrastructural studies of VAM. Arbuscules generally were considered to be the site of the most dynamically active interface between host plant cells and the mycobiont. A site of nutrient exchange between the host and mycobiont is most likely located at the arbuscular interface. Previous ultrastructural investigations of arbuscules formed by Glomus spp. within various hosts have revealed that the arbuscules are surrounded continually by the host plasmalemma and host cytoplasm throughout their developmental cycle (Brown and King 1982a; Cox and
Sanders, 1974; Holley and Peterson, 1979; Kariya and Toth, 1981; Kaspari, 1973; Kaspari, 1975; Kinden and Brown 1975c; Kinden and Brown, 1976; Scannerini and Bellando, 1968; Scannerini and Bonfante-Fasolo, 1979; Scannerini and Bonfante-Fasolo, 1983; Strullu, 1978). The interfacial zone between the host plasmalemma and the arbuscular cell wall has received considerable attention since this area may provide important clues about the dynamic nature of VA mycorrhizal relationships. Appearance and composition of the interfacial matrix appears to vary according to the host-endophyte combination, the developmental stage of the symbiosis, or the fixation procedures used to prepare specimens for observation (Bonfante-Fasolo et al., 1981; Brown and King, 1982a; Carling and Brown, 1982; Carling et al., 1977; Cox and Sanders, 1974; Dexheimer et al., 1979; Holley and Peterson, 1979; Kariya and Toth, 1981; Kaspari, 1975; Kinden and Brown, 1975b,c; Kinden and Brown, 1976; Scannerini and Bellando, 1968; Scannerini and Bonfante-Fasolo, 1979; Scannerini and Bonfante-Fasolo, 1983). Evidence from examinations of the interfacial zone indicates that at least some, if not most, of the interfacial matrix is composed of wall-like materials that are produced by the host cell. Other morphological alterations within the cytoplasm of host cortical cells during colonization by Glomus spp. also have been observed (Brown and King, 1982a; Carling and Brown, 1982; Cox and Sanders, 1974; Cox and Tinker, 1976; Holley and Peterson, 1979; Kariya and Toth, 1981; Kaspari, 1975; Kinden and Brown, 1975b,c; Scannerini and Bonfante-Fasolo, 1977; Scannerini and Bonfante-Fasolo, 1979; Scannerini and Bonfante-Fasolo, 1983). It commonly is believed that the ultrastructural changes within
host cells reflect modifications in metabolic activities in response to the mycobiont. These metabolic modifications might be related to nutrient exchange between the host plant and the fungus, or (and) to a host defense reaction in response to invasion by the mycobiont.

Arbuscules of *Glomus* spp. are apparently short-lived, and probably begin to deteriorate as soon as their morphological development is complete (Carling and Brown, 1982). Because of this, most arbuscular structures that are observed within a root represent various stages of arbuscular decline. Arbuscule life-span from formation through decline may last only 4 to 5 days (Bevage and Bowen, 1975; Cox and Tinker, 1976). It remains yet to be determined whether arbuscular decline is the result of autolytic processes or if it is because of physiological activities within the host cells. Arbuscular deterioration processes appear quite similar within a variety of host plants (Carling and Brown, 1982; Cox and Sanders, 1974; Holley and Peterson, 1979; Kariya and Toth, 1981; Kaspari, 1975; Kinden and Brown, 1976; Scannerini et al., 1975). Decline of arbuscules usually begins at the hyphal tips in one or more portions of the arbuscule. After breakdown of the fungal membrane, the smaller arbuscular branches begin to collapse. Fungal wall remnants start to aggregate as the deterioration process proceeds toward the base of the arbuscular trunk. Arbuscular decline is frequently accompanied by the formation of transverse septa which serve to isolate viable segments of hyphae from the degenerating portions. In the final stages of arbuscular deterioration, the residual arbuscule is reduced into a condensed clump of aggregated fungal walls that usually is encased with interfacial
material. The arbuscule remains surrounded by the host plasmalemma throughout its formation and decline. Following complete degeneration of the arbuscule, the host cell reestablishes cytological characteristics that are similar to those of an uncolonized cortical cell.

The comparison of this study's examination of the arbuscular development of *G. etunicatum* with most other ultrastructural investigations of VAM involving *Glomus* spp. was difficult. Additional TEM and histochemical examinations of *A. glutinosa* colonized with *G. etunicatum* would provide additional information to allow more meaningful comparisons with other studies. Nevertheless, SEM investigations revealed that arbuscular structures produced by *G. etunicatum* within roots of *A. glutinosa* (Figs. 52 and 53) did not appear to possess any major differences from those of *Glomus* species in bean and yellow poplar (Holley and Peterson, 1979; Kinden and Brown, 1975a,c, 1976).

Detailed ultrastructural examinations of the vesicles which are produced intraradically by *G. etunicatum* in association with *A. glutinosa* were not completed in this study. Examinations of this type were hampered by difficulties encountered during the resin-embedding and sectioning processes. Nevertheless, some relatively thorough structural investigations of the vesicles produced by other *Glomus* spp. within roots of various host plants have been performed in spite of the technical difficulties that impede these studies (Brown and King, 1982a; Holley and Peterson, 1979; Kinden and Brown, 1975b; Scannerini and Bonfante-Fasolo, 1983). Vesicles produced by other *Glomus* spp. within various host roots also may be formed intercellularly or intracellularly. Developing
vesicles are multinucleate with moderately dense cytoplasm that also contains small lipid droplets and glycogen particles (Brown and King, 1982a; Holley and Peterson, 1979). As the vesicles mature, lipids accumulate until distinct lipid globules nearly fill the entire volume of the vesicle. Vesicles of _Glomus_ spp. that have been examined thus far possess thickened three-layered walls (Holley and Peterson, 1979; Kinden and Brown, 1975b; Scannerini and Bonfante-Fasolo, 1983). The walls of some vesicles are so thick and regular that the vesicles resemble chlamydospores. Other vesicles of _Glomus_ spp. that are thin-walled and irregularly shaped bear only limited similarity to chlamydospores. Mature vesicles may function as food storage organs or as infectious propagules (Gerdemann, 1968). The limited observations of vesicles formed by _Gl. etunicatum_ did not reveal any major differences from vesicles produced by other species of _Glomus_; however, additional studies are required before more accurate comparisons of vesicle development can be achieved.

Various stages of spore production have been observed with other species of _Glomus_ (Godfrey, 1957; Hepper, 1979, 1981; Hepper and Mosse, 1975; Mosse and Hepper, 1975; Powell, 1976; Warner and Mosse, 1980). Surface examinations of _Gl. etunicatum_ spore formation (Figs. 48, 56, 57, 60, and 62) revealed many similarities with other _Glomus_ spp. Of particular interest were the small (20-50 μ in diameter), thin-walled spores that frequently resemble intercalary spores (Fig. 48). These spores were apparently analogous to structures produced by _Gl. caledonius_ and _Gl. mosseae_ that have been termed "vegetative" spores (Hepper, 1979,
It was clearly established that *Gi. rosea* and *Gl. etunicatum* could both form a VA mycorrhizal relationship with *A. glutinosa*. These two endogonaceous fungi possessed many similarities in their colonization of *A. glutinosa*. Both mycobiontic species possessed the capacity for intercellular and intracellular modes of penetration into, or growth within the host root system. Hyphae from the two species also were constricted noticeably as they passed through host cell walls. Neither mycobiont produced readily distinguishable coils, or penetrated the stele. The basic patterns of arbuscular development also were somewhat similar for both VAMF species.

Both *Gl. etunicatum* and *Gl. rosea* produced intraradical structures with wide variations in size and form. Because of these morphologic variations, it would be difficult to identify these mycobiontic species on the basis of intraradical structure. Vesicles within the roots of *A. glutinosa* were formed by *Gl. etunicatum*, but not by *Gi. rosea*. These vesicles represent the primary difference in intraradical structure between these two VAMF species. Hopefully, continued studies will determine additional structural characteristics that will facilitate the identification of VAM endophytes in the absence of spores.

Colonization of specific host plant by a known, taxonomically
identifiable fungal endophyte is a fundamental prerequisite toward understanding anatomical aspects that are characteristic of particular VAM. Achieving this initial step helps to define further the structural characteristics that develop from hyphae arising from specific spore types. Continued investigation should yield additional insight about the influence of various host plants upon the structural development of VAM endophytes. A variety of specific host-VAMF combinations must be evaluated before morphological variations in mycobionitic development are better understood. It also must be considered, however, that studies of specific host-mycobiont combinations require the maintenance of controlled conditions which may not reflect a natural situation. Physical and biotic components of the natural environment also may exert a direct or indirect influence upon the anatomical development of VAMF in the field. Factors of special interest in the study of VAM A. glutinosa include the effects of additional root symbioses upon the development of VA mycobionts. Nitrogen-fixing root nodules formed by an actinorrhizal relationship of Alnus spp. with Frankia spp. usually occur on field grown alders. In addition, Alnus spp. are capable of forming various other ectomycorrhizal relationships (Hall et al., 1979). Thus, it also may be necessary to consider the interactions of various other root symbioses of Alnus glutinosa with VAM before the development of the VAMF in natural situations is more fully understood.
Attempts Toward In Vitro Culture of VAMF

VAMF associations with intact alder seedlings

In this investigation, VAMF colonization of A. glutinosa seedlings was obtained in vitro within a chemically-defined agar growth medium (Fig. 63). Resting spores of Gi. rosea or Gl. etunicatum were both effective as mycorrhizal fungal inoculants. Limited evidence also suggested that both of these VAMF were capable of endomycorrhizal formation in the absence of other microorganisms. The clear nutrient medium within these culture systems permitted limited observation of extraradical mycobiontic development. Various stages of sporulation occasionally were produced by Gl. etunicatum in culture with A. glutinosa seedlings (Fig. 62), but spore formation by Gi. rosea was never observed. Quantitative assessments of in vitro endomycorrhizal development were restricted, however, because of delayed microbial contamination and general deterioration within the culture systems at harvest time. Nevertheless, these culture systems seemed to support only sporadic mycorrhizal colonization of alder seedlings by either mycobiont.

The formation of VAM with intact seedlings within the in vitro culture systems of this study was encouraging; however, these culture systems did not support optimal growth and development of the mycobionts. Improving the rate, prevalence, and longevity of in vitro mycobiontic development will likely require additional modifications in culture methods and conditions. Culture modifications could influence mycobiontic development by direct actions upon VAMF, and(or) through indirect mechanisms that involve alterations of host plant metabolism and
the subsequent endomycorrhizal interactions. Thus, the in vitro culture conditions that promote optimal VAM development may vary with each specific host-mycobiont combination.

Test tube cultures of VA mycorrhizal alder seedlings would perhaps respond favorably to a higher light intensity within the growth chamber. Allen and St. John (1982) recommended a minimum light intensity of 500 to 700 μE m⁻² s⁻¹ for in vitro culturing of endomycorrhizal hosts. Higher light levels can, however, cause internal heating within test tube culture systems. Thus, the successful culturing of VAM hosts within test tubes under high light perhaps also requires a means to maintain internal culture system temperatures within a range suitable for VAM development.

Formation and maintenance of VAM also seems to require adequate aeration (Allen and St. John, 1982; Tacon et al., 1983; Saif, 1981). In addition, previous investigations have determined various effects of nutrient medium composition upon in vitro development of specific VAM associations with intact host plants (Allen and St. John, 1982; Allen et al., 1981; Hepper, 1981; Mosse, 1962; Mosse and Phillips, 1971). Concentration and source of phosphate within VAM culture media, the primary concern of previous nutritional studies, are both capable of dramatic impacts upon VAM development.

Activated charcoal was incorporated into the water agar germination matrix to reduce effects of potential inhibitors of germination and(or) hyphal growth of VAMF (Daniels and Trappe, 1980; Watrud et al., 1978b). In addition, the opaque charcoal layer also excluded light that also could inhibit formation and growth of VAMF germ tubes (Schenck et al.,
This opacity of the germination matrix, however, also prevented observations of spore germination within these culture systems. Thus, precise influences of activated charcoal upon early mycobiontic development could not be determined in situ. The charcoal layer was of potential value to in vitro culture systems of this study, since this layer did not prevent spore germination and root colonization by Gl. etunicatum or Gi. rosea. In any event, formulation of an efficacious germination matrix without charcoal remains preferable if such a medium permitted observation of VAMF development outside host roots.

Other workers have also established axenic VAM cultures upon agar-based nutrient media with Bouteloua gracilis (H.B.K.) Lag. ex Steud., Trifolium parviflorum Ehrh., and Trifolium repens L. serving as intact host plants which were associated with Glomus spp. (Allen et al., 1979; Allen et al., 1980; Allen et al., 1981; Allen et al., 1982; Hepper, 1981; Mosse, 1962; Mosse and Phillips, 1971). VAM culture systems that utilize an agar substrate can be successfully established without employing highly-specialized culture apparatus. Agar-based media also provide a solid substrate that holds the biological constituents of VAM in place. These particular culture systems can therefore be designed to permit in situ observational monitoring of various VAM developmental aspects within the clear nutrient medium. The defined composition of agar culture substrates also is well-suited for refined physiological studies of the VAM interaction. In addition, an agar growth medium can facilitate collection of VAM culture components for postharvest analysis.
Successful axenic cultures of VAM also can be established with whole plants within systems that utilize nutrients in solution (Hepper, 1981; Macdonald, 1981; St. John et al., 1981). In additional investigations, nutrient solutions were employed within various VAM culture systems under nonaxenic conditions (Crush and Hay, 1981; Elmes et al., 1984; Elmes and Mosse, 1984; Howeler et al., 1982; Mosse, 1962; Mosse and Thompson, 1984; Ojala and Jarrell, 1980). Supplying VAM culture systems with liquid nutrients can minimize potential problems caused by nutrient and(or) moisture depletion. This feature should contribute to increases in the longevity of certain VAM cultures. Liquid nutrients are applicable within a wide range of VAM culture systems that perform diverse functions. Culture systems of this type can be designed to allow direct microscopic monitoring of VAM development, and(or) permit subsampling from such cultures (Hepper, 1981; Macdonald, 1981). Nutrient solutions also are useful in physiological studies of VAM (Elmes and Mosse, 1984; Howeler et al., 1982; Mosse, 1962; Mosse and Thompson, 1984; Ojala and Jarrell, 1980; St. John et al., 1983). In addition, nutrient solutions also can support effectively culture systems designed to maintain VAMF isolates and(or) to produce VAMF inoculum (Elmes et al., 1984; Elmes and Mosse, 1984; Hepper, 1981; Macdonald, 1981; Mosse and Thompson, 1984).

VAM associations with root organs

In the root organ study, Gl. etunicatum chlamydomes exhibited an uncharacteristically low germination rate in water agar. The association of higher spore germination levels with root organs upon nutrient media also was unexpected, since Mosse and Hepper (1975) found that a similar
nutrient medium caused a dramatic reduction in germination by *Gl. mosseae* chlamydospores. In addition, their study demonstrated that root organs of tomato or clover did not enhance spore germination. In later investigations, Hepper and Smith (1976) determined that concentrations of both zinc and manganese within the root organ medium were responsible for differential inhibitions of germination that varied among spore isolates of *Gl. mosseae*. Germination rates of these *Gl. mosseae* spores were improved with the addition of thiamine HCl or nicotinic acid, and by storage at 6°C for several weeks. The *Gl. etunicatum* chlamydospores of this study were utilized within a few days of their collection. Thus, the poor germination of these spores possibly was related to innate factors that contribute to spore quiescence and (or) dormancy. Newly-formed spores of four VAMF species have been shown to possess various innate dormancy periods, during which germination could not occur (Tommerup, 1983a). In most instances, host plant roots have no apparent influence upon the germination of VAMF spores (Daniels and Trappe, 1980; Mosse and Hepper, 1975; Powell, 1976; Tommerup, 1983 a,b); however, citrus root exudates stimulated *Glomus epigaenum* spore germination under sterile conditions (Graham, 1982). Soils containing higher densities of soybean roots also were associated with increased germination by various VAMF species including *Gl. etunicatum* (van Nuffelen and Schenck, 1984). Applications of various other nutrient amendments also can promote VAMF spore germination (Daniels and Duff, 1978; Daniels and Graham, 1976; Daniels and Trappe, 1980; Hepper and Smith, 1976; Mosse, 1959; Siqueira et al., 1982). Differences in moisture or pH also can contribute to
variation in VAMF spore germination rates (Daniels and Trappe, 1980; Green et al., 1976; Hepper, 1984; Koske, 1981a; Siqueira et al., 1982; Sylvia and Schenck, 1983). Medium composition can influence the effects of pH upon spore germination (Siqueira et al., 1982). Since additional germination tests were not performed, the factors that contributed to variation in Gl. etunicatum germination rates remain unidentified. It is noteworthy, however, that direct inoculations with ungerminated Gl. etunicatum chlamydospores did result in endomycorrhizal colonization of root organs upon both nutrient media. This contrasts with the study of Mosse and Hepper (1975), where successful root organ inoculations required pregermination of VAMF spores upon a separate germination medium. In that study, pregermination procedures also helped to ensure surface-sterility of the VAMF inoculum.

The 50% reduction in concentration of certain nutrients (Ca, Cl, K, Mg, Mn, N, S) within the root-organ medium produced no apparent effects upon spore germination or root colonization by Gl. etunicatum. Results of previous investigations indicate that germination processes of at least some VAM fungi can tolerate relatively diverse concentrations of various macronutrients, including N, P, and K (Daniels and Trappe, 1980; Hepper, 1983a; Koske, 1981a; Siqueira et al., 1982). In contrast, VAMF germination is potentially very sensitive to even low levels of certain micronutrients such as Cu, Mn, or Zn (Hepper, 1979; Hepper and Smith, 1976). Within the culture systems of this study, a wider variation in test concentrations of the specific nutrient combination apparently was required before observable influences upon endomycorrhizal development
were determinable. Potential influences of these nutrient combinations possibly were masked by root metabolism, interactions between separate nutrients, mode of inoculation, or other experimental conditions.

Effective culturing of VA mycorrhizal root organs has potential value in physiological or developmental studies of VAM, or in the propagation of mycobiontic lines. In other studies, VAM have been successfully established with root organs of tomato and clover (Hepper and Mosse, 1975; Miller-Wideman and Watrud, 1984; Mosse and Hepper, 1975). Root organ cultures of Gl. mosseae have characteristically produced many small "vegetative" spores (Hepper and Mosse, 1975; Mosse and Hepper, 1975). In addition, mature azygosporules were developed by cultures of Gl. margarita in association with root organs of tomato (Miller-Wideman and Watrud, 1984). Abundant growth and lateral branching by the root organs were characteristic in all of the aforementioned VAM cultures. Subsequent penetrations of extraradical VAMF hyphae into newly-grown root organ segments resulted in continued endomycorrhizal colonization of additional regions behind the meristematic areas. Thus, continued development of VAM mycobionts within root organ cultures may possibly require a progression of dynamic interactions with an actively growing root system.

This study demonstrates that VAM associations between Gl. etunicatum and root organs of A. glutinosa could be established upon a chemically-defined growth medium. These in vitro culture systems did not, however, support adequate growth by either mycorrhizal partner. Extraradical VAMF hyphae did not produce any observable stages of spore formation.
Intraradical structures of *Gli. etunicatum* remained localized within an older portion of the alder root organ. Decline of the host and the mycobiont proceeded within this localized area, without successive endomycorrhizal colonization of additional regions within the root organ. Thus, viable mycorrhizal interactions between alder root organs and *Gli. etunicatum* apparently were short-lived within these culture systems. Restrictions on endomycorrhizal development probably were related to poor root growth, but also could involve other factors that limited extraradical growth or penetration by the mycobiont. Establishing more vigorous endomycorrhizal cultures of this type will probably require improvements in the nutrient medium and(or) culture methods. A desirable growth medium for these cultures must provide conditions that promote root growth without inhibiting mycobiontic development.

**Associations of VAMF with undifferentiated callus tissue**

Whether undifferentiated callus tissue exerted any interacting influences upon the hyphal growth of *Gli. rosea* was difficult to determine. This prospect however, appeared promising in two instances where *Gli. rosea* produced extensive hyphal growth along with clusters of extramatrical vesicles. Nevertheless, this issue remains clouded in light of later studies (reported on p. 63) that demonstrated a high correlation between hyphal growth and emv production. Even though these other studies were conducted in water agar, *Gli. rosea* hyphae that generated emv clusters typically grew considerably longer than those that did not produce emv's. Factors that contribute to emv formation remain undetermined. Thus, the extended hyphal growth observed in the two
callus tissue cultures could be a function of env production which may or may not involve a promoting influence derived from the callus cells. Intercellular hyphal growth through the callus tissue provided additional evidence of a possible interaction between Gi. rosea and the callus cells. This evidence also was questionable, however, since these particular calli were composed of loosely aggregated cells with large intercellular spaces that probably offered little restriction to hyphal growth. Since hyphal penetration of callus cells was not observed, it remained possible that Gi. rosea hyphae were growing through the large intercellular spaces in a noninteracting manner. Thus, results from the study of the interaction between Gi. rosea and callus tissue of A. glutinosa remain inconclusive, but hold a degree of promise. The callus tissue and nutrient medium were apparently not inhibitory to the hyphal growth of Gi. rosea, and were possibly beneficial in certain instances. This apparently is the first reported interaction study involving a VAMF and undifferentiated callus tissue. Additional investigations that utilize this novel approach possibly could contribute new information about the physiological mechanisms involved in a VAM symbiosis.

Antibiotic Study

The necessity for separate tests to evaluate the individual effects of each antibiotic upon spore germination and hyphal growth became apparent after a preliminary study. In the preliminary study, the germination of Gi. etunicatum chlamydospores in 1% water agar was compared to that in 1% water agar that also contained a combination of
four antibiotics (100 ppm gentamicin sulfate, 50 ppm streptomycin sulfate, 150 ppm chloramphenicol, and 50,000 units/L penicillin G potassium). After 18 days at room temperature, none of the spores in the antibiotic treatment had germinated; however, 72% (SE = 8.6, n = 11) of the spores in the water agar controls had germinated. At 62 days, the rate of spore germination had reached 57% (SE = 3.3, n = 10) in the antibiotic combination treatment, compared to 93% (SE = 3.8, n = 11) in the controls. The incorporation of the four antibiotics into the water agar medium also resulted in a severe inhibition of hyphal growth. In the presence of the antibiotics, all hyphal growth by Gl. etunicatum was suspended shortly after germ tube emergence. This effect was not observed within the water agar controls. Thus, the aforementioned preliminary study established a need for an initial examination of the independent effects of each separate antibiotic upon spore germination and hyphal growth.

The attainment and maintenance of gnotobiotic endomycorrhizal cultures is greatly facilitated by the utilization of compatible antibiotics. The efficacy of antibiotics for use in endomycorrhizal culture is based upon their possession of the following characteristics: stability of anti-microbial action in long term culture; lack of detrimental effects upon spore germination; ability to allow for normal hyphal elongation processes; capacity to permit root colonization by VAMF; and, capability for noninterference with other normal developmental

\(^1\)SE is the standard error of the mean, and \(n\) is the number of petri plates. Each plate contained an average of 11 spores.
life processes of both host tissue and the mycobiont.

The preliminary results of this study and others (Hepper, 1979; Mertz et al., 1979; Tommerup and Kidby, 1980) indicated that different types of endomycorrhizal fungi do in fact react dissimilarly to diverse antibiotics at various concentrations. At this time, one can only speculate on the nature of this response. Further studies are necessary to determine if the response of these endophytes is species specific, specific to certain biotypes, or perhaps a reaction to the environmental conditions. It would be ideal if continued investigations could obtain the information necessary to determine which antibiotics and their respective levels are consistently effective for use with specific types of endomycorrhizal fungi.

The results of this study demonstrated the complex nature of antibiotic interaction with VAMF. Chloramphenicol (150, 75, and 35 ppm) did not seem to affect adversely the germination of *Gl. etunicatum* spores. However, higher concentrations of chloramphenicol appeared to have a deleterious effect upon hyphal elongation. Hyphal growth of *Gi. rosea* also seemed to be more sensitive to chloramphenicol than was germination. The *Gl. etunicatum* germination results were comparable to those of Tommerup and Kidby (1980) with spores of *Glomus caledonicum* (Nicol. & Gerd.) Trappe & Gerd. and *Acaulospora laevis* Gerd. & Trappe; however, the germination of *Gi. rosea* seems to be much more sensitive to chloramphenicol. Tommerup and Kidby (1980) proposed a two-phase growth response (germination and germ tube growth) based upon the sensitivity of the mycobiont to various chemical treatments. It also was suggested that
spores possessing germ tubes might allow some chemicals to enter the cytoplasm at a faster rate, thereby increasing the sensitivity response of the VAMF. A similar reaction by *G. caledonicum* was noted by Hepper (1979) in response to cycloheximide and actinomycin D. The two-phase growth response by the two species in the investigation of Tommerup and Kidby (1980) did not appear to be as apparent with chloramphenicol. However, the action of chloramphenicol upon *G. etunicatum* and *G. rosea* in this study tended to support the idea of a two-phase growth response. Of these species, *G. rosea* was the most sensitive to chloramphenicol since concentrations above 35 ppm significantly repressed spore germination and hyphal growth, while increasing the mortality rate. In light of this evidence, higher concentrations of chloramphenicol only may be useful in surface-sterilization and (or) short term storage procedures for this spore type of *G. rosea*. With *G. etunicatum*, higher concentrations of chloramphenicol could be utilized in surface-sterilization or storage solutions; whereas, lower concentrations could possibly be of use for incorporation into agar media.

The inhibition of *G. etunicatum* germination by both concentrations of gentamicin (100 and 50 ppm), and the inhibition of hyphal elongation by the higher concentration of gentamicin (100 ppm) would seem to preclude this antimicrobial agent from use in agar cultures designed for this species. Since the inhibitory effects of this antibiotic appeared to be reversible, gentamicin could possibly be employed for surface-sterilization and storage of *G. etunicatum* spores. The higher tendency for larger spores to germinate in 1% water agar incorporated with
gentamicin could be because of less antibiotic reaching the cytoplasm of larger spores. The spores which did germinate in half-strength gentamicin exhibited normal hyphal growth. This could represent physiological differences between germination and hyphal growth processes. Another possibility is that Gl. etunicatum may have some capacity for gentamicin tolerance for which selection is possible. Previous studies have shown that gentamicin (100 ppm), or gentamicin (100 ppm) in combination with streptomycin (250 ppm), did not affect adversely the germination and growth of Gigaspora margarita Becker and Hall or the growth of Glomus fasciculatum (Thaxter sensu Gerdemann) Gerd. & Trappe (Mertz et al., 1979). These antibiotics were effective when used as a rinse culminating surface-sterilization procedures, incorporated into culture media, or used in a storage solution at 4°C. These authors found high levels of surface-sterility to be associated with high rates of germination for Gi. margarita. Surface-sterility did not affect the germination rate of Gigaspora gigantea Nicol. and Gerd (Koske, 1981a). The results of the present study indicated that gentamicin (100 and 50 ppm), and streptomycin (50 and 25 ppm) caused no severe adverse effects upon the germination or hyphal growth of Gi. rosea. This seems to imply that Gi. rosea and Gi. margarita may respond similarly to these two antibiotics. Germination and hyphal growth of Gi. rosea also was relatively unaffected by the presence of penicillin (50,000 and 25,000 units/L). Gentamicin, streptomycin, and penicillin G all appear to be well suited for diverse use in Gi. rosea culture systems.

Streptomycin (50 and 25 ppm) and penicillin G (50,000 and 25,000
units/L) also appeared to be useful antibiotics for incorporation into culture media intended for *Gl. etunicatum*. Streptomycin has frequently been utilized successfully in surface-sterilization procedures for spores of various endomycorrhizal fungi (Hepper, 1979; Hepper and Smith, 1976; Macdonald, 1981; Mertz et al., 1979; Mosse, 1959, 1961, 1962, 1973; Mosse and Hepper, 1975; Sylvia and Schenck, 1983; Tommerup and Kidby, 1980; Watrud, 1982; Watrud et al., 1978a, 1978b). It was somewhat surprising that with penicillin G (50,000 and 25,000 units/L) incorporated into 1% water agar, the hyphae of *Gl. etunicatum* grew to a significantly greater average length than that of the controls. This occurrence might, in part, be attributable to the low levels of sodium citrate that was present as a buffer in the penicillin G potassium preparations. Several organic acids, including citric acid, have been reported to be potentially beneficial to growth, as carbon sources, for an endogonaceous fungal species (Mosse, 1959). The results of this study would tend to indirectly support these findings. There seemed to be no obvious explanation for the increased growth of *Gl. etunicatum* in 25 ppm streptomycin sulfate, or for that of *Gi. rosea* in 50 ppm gentamicin sulfate.

The possible presence of bacterium-like organelles (or organisms), spiraplasma-like organisms, or other microorganisms within the cell walls of VAMF represents an additional factor that must be considered when evaluating the effects of antibiotics upon VAM mycobionts. Electron microscopic examinations have demonstrated the presence of various BLOs (bacterium-like organelles or organisms) within the cytoplasm of a
variety of VAMF (Macdonald and Chandler, 1981; Macdonald et al., 1982; Mosse, 1970c; Sward, 1981a,b,c). Helical organisms that resemble spiroplasms also have been found within the cytoplasm of Glomus spp. (Tzean et al., 1983). Additional studies revealed the existence of a variety of bacteria within chlamydospore lumens of Glomus macrocarpum Tul. and Tul (Varma et al., 1981). However, the exact nature of the relationships of these organelles and(or) organisms with VAMF remains unknown. It seems plausible that similar biological entities could occur within the spore types used in this study, although this has not yet been confirmed. Antibiotics that affect the growth of such microorganisms and(or) microorganism-like organelles could either enhance or inhibit the growth of VAMF, depending upon the relationship of these entities with VAM.

The antibiotic action of gentamicin, streptomycin, and chloramphenicol is based upon their abilities to interfere with protein synthesis on the 70S ribosome (Avers, 1976; Davis, 1973). The apparent inhibition of Gl. etunicatum by gentamicin, and Gi. rosea by chloramphenicol seemed to indicate that mitochondrial protein translation is a necessary prerequisite for germination and growth of these endogonaceous fungi. Inhibitor studies on Gl. caledonicum spores by Beilby (1983) suggested that the germination process requires early expression on the mitochondrial genes. In these studies, various treatments with chloramphenicol inhibited protein synthesis by the chlamydospores. It was further implied that a significant amount of protein synthesis may be necessary within ungerminated spores before the
mitochondria become fully functional. Unpublished fine structure studies by Mosse (Hepper, 1979) revealed that the spore mitochondria of some VAM endophytes become increasingly developed during the germination process. The variation in sensitivity to different antibiotics may reflect differences in selective permeabilities among diverse types of VAM endophytes.

These studies will hopefully be continued so that suitable antibiotic combinations are found for use in in vitro studies of Gl. etunicatum and Gi. rosea. The four-antibiotic combination used in this study as a storage solution appeared to be well-suited for spores of Gl. etunicatum. However, Gi. rosea spores seemed to lose viability over time in this antibiotic solution, with many spores turning brown and becoming vacuolated. It was not known whether this was an adverse response to the antibiotics (particularly chloramphenicol), the limitation of oxygen, the water potential of the storage medium, or to other conditions such as pH. A similar loss of viability was found during storage of Gi. margarita spores, irregardless of surface-sterilization (Sward et al., 1978). The viability of Gi. gigantea decreased proportionately with the water potential of the surrounding medium (Koske, 1981a). However, no adverse effects were reported with Gi. margarita spores that were stored for one year in a gentamicin-streptomycin storage solution (Mertz et al., 1979).

The fact that larger spores of Gl. etunicatum or Gi. rosea did not produce proportionately longer hyphae was somewhat puzzling if one assumes that larger spores contain more lipid reserves that could fuel hyphal elongation. Perhaps some other nutritive factor was limiting,
became unstable, or was lost after germination, and thus must be supplied by another source. Work by Hepper (1979) with Gl. caledonicum tends to support this principle. The fungus itself also might contain a mechanism that restricts hyphal growth unless a host plant or other source of nutrition is contacted. Evidence was found for the production of an inhibitor by Gi. margarita which limited the hyphal growth of that fungus (Watrud et al., 1978b). Hyphal elongation would result in an increase in the surface area to volume ratio for the VAMF, thereby changing the rate of influx or efflux of certain materials in the organism. These events also could function in the limitation of hyphal growth.

The correlation between the length of a Gi. rosea hypha and the number of extramatrical vesicle clusters produced on the hypha was also noteworthy. Do vigorously growing hypha contribute to the production of more emv clusters, or do emv clusters contribute to the vigor of hyphal growth? The formation of emv's and their contribution to the growth of Gigaspora species deserves further investigation.

Anastomosis between hyphae from separate parent spores has been previously reported with other endogonaceous species (Godfrey, 1955, 1957; Hepper and Mosse, 1975; Macdonald, 1981; Mosse, 1956, 1961, 1973; Tommerup, 1981, 1984) but still raises important questions about the role and delimitations of this event in nature. As techniques for studying endogonaceous fungi in vitro become more refined, the process of hyphal anastomosis could be utilized in the delineation of species, types, or races within the Endogonaceae. This type of asexual genetic exchange also could function possibly as a means by which hybrids between
different ecotypes of compatible mycobionts could be produced and developed to fulfill specialized roles as research tools or be a selection criteria in future attempts to optimize individual endomycorrhizal symbioses.

The volume of soil occupied by a single VAM endophyte also could be extended by hyphal anastomosis. Heap and Newman (1980) recently demonstrated the presence of VAMF hyphal links between separate root systems. Additional investigations by other workers suggest that nutrients are transferred between plants by way of VAM interconnections (Francis and Read, 1984; Whittingham and Read, 1982). These events seem to at least raise the possibility of vast networks of interconnecting hyphae and root systems existing in undisturbed soils. If this is the case, the extent of endomycorrhizal involvement in the dynamics of the soil ecosystem has been greatly underestimated.

The negative geotropic orientation of *Gi. rosea* spore germination is similar to that found by Watrud et al. (1978a) with *Gi. margarita*. Aerial germ tubes of *Gi. gigantea* also were negatively geotropic; however, this response could be altered by an attractant that was produced by plant roots (Koske, 1982). The bidirectional growth of secondary hyphal branches of *Gi. margarita* that was observed previously (Watrud et al., 1978a) also was also noted in the present study with *Gi. rosea*. It is interesting that *Gl. etunicatum* did not exhibit a similar response. However, more studies are needed to determine the exact function and prevalence of this response in nature.

Hyphal growth originating from separate extra-matrical vesicle
clusters (Figs. 26 and 27) is unreported for *Gigaspora* species (Gerdemann, 1955a). This occurrence seemed to raise the possibility that extra-matrical vesicles could function as infectious propagules, as well as energy reserve storage structures. This possibility remains questionable, however, since Biermann and Linderman (1983) were unsuccessful in their attempts to establish VAM by using extra-matrical vesicles of *Gl. margarita* and *Gl. gigantea* as inoculum. Nevertheless, the role of these structures in nature is still poorly understood and warrants further investigation.

Bacteria in the soil play a major role in the ecosystem of the rhizosphere. It, therefore, seems very probable that interactions of bacteria, as well as other components of the soil microflora, with VAM and VAMF are a prevalent factor in the dynamics of the rhizospheral ecosystem. Investigations with VAMF in vitro, under gnotobiotic conditions are, therefore, not necessarily representative of endomycorrhizae found in natural soil ecosystems. What these investigations do provide, is a method by which the interacting role of microorganisms and various other factors in the rhizosphere can be evaluated under controlled conditions.
Vesicular-arbuscular mycorrhizal associations were established in vitro with intact seedlings and root organs of *Alnus glutinosa* grown upon an agar-based nutrient medium. Spores of *Gigaspora rosea* or *Glomus etunicatum* were utilized as the VAM fungus inoculum. Gnotobiotic conditions did not prevent the formation of VAM. Various stages in the extraradical development of VA mycobionts could be observed in situ within successful VAM culture systems. Limited sporulation occurred within certain VAM cultures that contained whole seedlings colonized with *G. etunicatum*.

In an attempt to facilitate the establishment of axenic cultures of endogonaceous fungi, the effects of four antibiotics upon surface-sterilized spores of *Gl. etunicatum* and *Gi. rosea* were investigated. The antibiotics and their respective concentrations were chloramphenicol (150, 70, and 35 ppm), gentamicin (100 and 50 ppm), penicillin G (50,000 and 25,000 units/L), and streptomycin (50 and 25 ppm). Rates of spore germination and hyphal growth were monitored.

With *Gl. etunicatum*, germination exceeded 85% on control plates and on agar into which chloramphenicol, penicillin, or streptomycin were incorporated. Germination of spores on agar containing 100 and 50 ppm gentamicin was significantly inhibited, with larger spores showing a greater tendency to germinate. Hyphae from *Gl. etunicatum* spores grew to an average maximum length of 13 mm on the 1% water agar controls. Hyphal growth was enhanced with penicillin, and seemed to decrease with increasing concentrations of chloramphenicol. The effects of
streptomycin and gentamicin were dependent upon their concentrations.

The 1% water agar controls produced a germination rate of 70% with *Gi. rosea*. The gentamicin, streptomycin, and penicillin treatments were not significantly different from the controls. Higher concentrations of chloramphenicol seemed to inhibit the germination, and increase the mortality rate of *Gi. rosea* spores. The mean maximum hyphal length from *Gi. rosea* spores in the 1% water agar controls was 80 mm. Gentamicin (50 ppm) appeared to enhance hyphal growth of this species. Of the remaining treatments, only chloramphenicol significantly inhibited hyphal growth.

Dormant azygospores of *Gi. rosea* were encompassed by a wall that typically possessed three major layers. In some instances, the inner layer of the spore wall was laminated in appearance. *Gi. rosea* azygospores were formed upon a bulbous, suspensor-like structure similar to that of other *Gigaspora* species.

Limited evidence suggests that newly-formed azygospores of *Gi. rosea* may require a several week dormancy period before germination processes can be initiated. Each azygospore ordinarily produced only one germ tube; however, up to seven germ tubes from one spore were occasionally observed. Germ tubes emerged directly through the azygospore wall, and frequently were constricted at the point of emergence. Primary germ tube growth by *Gi. rosea* usually was directed with a strong negative geotropic orientation.

Length of *Gi. rosea* hyphal growth in water agar was not significantly correlated to the diameter of the parent azygospore; however, hyphal growth was highly correlated with the number of extra-
matrical vesicle (emv) clusters that were produced upon the growing hypha. The emv clusters contained up to 14 echinulated vesicles which were each encased by two main wall layers. In water agar, emv's usually were emptied of their cellular contents as the connected hyphal segments continued to grow. When filled emv clusters became separated (by a septum) from their parental hypha, they occasionally supported a new radial growth of thin hyphae. Other *Gi. rosea* hyphae exhibited a wound-healing response, and also produced "rhizoid-like" structures within the nutrient medium of *in vitro* culture systems. Hyphal diameter tended to diminish with continued growth away from a food source. Senescence of *Gi. rosea* hyphae was characterized by the sequential withdrawal of cytoplasm, and the subsequent formation of irregularly spaced septa.

*Gi. rosea* hyphae penetrated roots of *A. glutinosa* in various fashions. Direct penetration through the epidermal cell wall was accomplished with appressorial formation by the VAMF hyphae. Other hyphae penetrated host roots by intercellular growth between epidermal cells.

*Gi. rosea* hyphae also were capable of intercellular and intracellular growth within host roots. In addition, hyphae apparently could egress from host cortical cells, but hyphal growth into the stele was never observed. The arbuscule was the predominant structure formed by *Gi. rosea* within *A. glutinosa* roots. Arbuscules were most common within cortical cells that were near the endodermis, and frequently occupied large volumes within these host cells. Continued dichotomous branching within arbuscules produced terminal tips with diameters between
Arbuscular decline was evident as progressive stages ranging from collapsed terminal branches to complete aggregation into an amorphous mass near the base of the original arbuscular trunk. Various stages of arbuscular development occasionally were present within a single arbuscule. In additional situations, a single host cell was penetrated by more than one arbuscular trunk. Continuous stages of extraradical azygospore formation were not observed; however, bulbous, suspensor-like structures were infrequently formed upon *Gl. rosea* hyphae in water agar.

Mature chlamydospores of *Gl. etunicatum* possessed two major wall layers with variable laminations in the inner wall. Chlamydospore germination typically occurred by germ tube emergence through the remnants of the subtending hypha. In rare instances, germination proceeded directly through the spore wall. This unusual germination process occasionally produced more than one germ tube. The direction of germ tube growth by *Gl. etunicatum* revealed no signs of geotropic influence. Hyphae from separate chlamydospores in close proximity frequently anastomosed. No significant correlation was found between chlamydospore diameter and the length of hyphae produced by *Gl. etunicatum* in water agar.

Roots of *A. glutinoso* were approached and penetrated in various manners by *Gl. etunicatum* hyphae. Within *in vitro* cultures, growing hyphae were not attracted noticeably toward host roots that were located more than a few millimeters away. An intercellular mode of root ingress was utilized by *Gl. etunicatum* hyphae in some instances. Direct
intrapcellular penetration by major hyphae was accomplished by
appressorial formation. In certain situations within in vitro culture,
very narrow Gl. etunicatum hyphae contacted root hairs in a manner that
possibly preceded host penetration. In vitro cultured roots frequently
were approached by branching Gl. etunicatum hyphae contained within
frameworks that resembled "infection fans," "runner" hyphae, or
interconnecting hyphal networks. In some in vitro cultures, continuous
hyphal networks appeared to maintain consistent connections between
separate root systems of A. glutinosa.

Gl. etunicatum hyphae grew in both intercellular and intracellular
manners within roots of A. glutinosa, but stelar penetration was not
observed. Characteristic arbuscules typically were formed within inner
cortical cell layers. Intracellular and intercellular vesicles with
diameters up to 60μ also were produced by Gl. etunicatum within the root
cortex.

Vegetative spores (20 to 50 μ in diameter) occasionally were formed
upon independently-grown hyphae of Gl. etunicatum, and upon extraradical
hyphae that were associated with host roots within in vitro cultures. In
some instances, mature chlamydospores were formed within in vitro
cultures of VA mycorrhizal A. glutinosa.


Mosse, B. 1961. Experimental techniques for obtaining a pure inoculum of an Endogone sp., and some observations on the vesicular-arbuscular infection caused by it and by other fungi. Recent Advances in Botany 2:1728-1732.


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Germination rates for spores of *Glomus etunicatum* in the presence of antibiotics

![Germination rates graph](image)

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