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FACTORS AFFECTING THE GERMINATION AND HYPHAL ELONGATION OF GLOMUS FASCICULATUS ON AGAR MEDIUM

presented by

Karol Sue Elias

has been accepted towards fulfillment
of the requirements for

MASTER OF SCIENCE degree in Botany and Plant Pathology

Gene R. Safir
Major professor

Date 12/23/84

FACTORS AFFECTING THE GERMINATION AND HYPHAL ELONGATION OF *GLOMUS FASCICULATUS* ON AGAR MEDIUM

by

Karol Sue Elias

A THESIS

submitted to
Michigan State University
in partial fulfillment of the requirement
of the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1984

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

FACTORS AFFECTING GERMINATION AND HYPHAL ELONGATION OF GLOMUS FASCICULATUS ON AGAR MEDIUM

by

Karol Sue Elias

The spore germination rates on water agar of the vesicular-arbuscular mycorrhizal fungus Glomus fasciculatus were highest at water potentials of -4 to -6 bars. Root exudates or extracts from plants grown in a sterile nutrient solution, with or without phosphorus (P), did not affect germination. Root exudates collected from 2-week-old Trifolium repens cv `Ladino' seedlings, which were deprived of P, enabled hyphal growth from germinated Glomus fasciculatus spores of 21.4, 14.7, and 7.6 mm at 2, 4 and 6 weeks, respectively. Hyphal elongation in the presence of exudates from plants grown with P, or in the absence of exudates, was negligible (<1 mm). Root P at 2 weeks was not significantly different between plants grown with and without P. There were no significant differences between the conductivities of exudates from plants grown with or without P at 2, 4 and 6 weeks. The data suggest that the quality and the quantity of exudates from P-deprived seedlings stimulate hyphal elongation.

INTRODUCTION

In 1885, Frank coined the term "mycorrhiza" or fungus-root, to describe a symbiotic association between fungi and the roots of most higher plants. It wasn't until 1969 that Peyronnel et al. adopted the term `ectomycorrhizae' to describe mycorrhizal associations in which the fungi develop primarily on the root surface. He also used the term `endomycorrhizae' to describe a mycorrhizal association whereby the fungi develop extensively inside the root, and the term `ectendomycorrhizae,' which has characteristics of both types. My research is concerned with a type of endomycorrhiza called vesicular-arbuscular mycorrhizas (VAM). The VAM fungi involved are considered to be in the Zygomycetes.

In 1974, VAM fungi were reclassified by Gerdemann and Trappe into the family Endogonaceae which contains four genera known to form VAM: Glomus, Gigaspora, Acaulaspora and Sclerocystis. VAM fungi have very little host specificity, in fact most plants form VAM associations, except for members of the families Cruciferae, Chenopodiaceae, Fagaceae, Pinaceae and Betulaceae.

The specialized structures produced by VAM fungi have

been studied in great detail. Vesicles which are produced intra- and intercellularly, are sac-like swellings at the tip or the middle of hyphae. It is thought that vesicles are involved in both food storage and fungal reproduction because oil accumulates inside vesicles and thick-walled older vesicles can act as propagules. The arbuscule is similar to other fungal haustoria in that it is enclosed in a sac by the host plasma membrane and participates in an interaction whereby the host cell survives while the symbiont is gradually destroyed.

The beneficial effects of VAM infection on plant growth are well documented (Daft and Nicolson,1966; Hayman and Mosse,1971; Mosse,1974). Mycorrhizal fungi penetrate and ramify in host roots, and their hyphae branch out into the soil well beyond the area from which roots can take up nutrients. Mycorrhizal hyphae can absorb and transport nutrients, thus effectively increasing the soil volume from which plants absorb nutrients (Rhodes and Gerdemann,1980; Tinker,1975). Sanders and Tinker (1971) were the first to show that mycorrhizal roots obtain phosphorus from the same labile pool as do nonmycorrhizal roots. Moreover, no insoluble sources of nutrients have been found which mycorrhizae can exclusively tap. Nutrient transfer to the host plant occurs across hyphal and arbuscular walls in host roots and in return mycorrhizal fungi absorb carbon from host plant cells (Bevege et al.,1975).

Mycorrhizae can increase the uptake of immobile elements that slowly diffuse to roots. Phosphorus (P) is possibly the most important element involved since it is needed in large quantities by host plants but is highly immobile in soil. Mycorrhizae also increase the uptake of copper (Cu) and zinc (Zn), two highly immobile micronutrients (Rhodes and Gerdemann,1980). Since the diffusion rate of these nutrients through soil is slow, plants can deplete their soil sources within 1-2mm of the root surface rapidly. Thus, mycorrhizae provide an advantage to plants by enabling nutrient absorbtion from soils at large distances (as far as 7 cm) beyond their normal zone of depletion (Rhodes and Gerdemann,1980).

When other more mobile elements are available at very low concentrations in the soil, mycorrhizae also can be of benefit to the host plant. Manganese, magnesium, potassium, sulfur and strontium uptake has been shown to be increased by mycorrhizae in extremely deficient soils (Rhodes and Gerdemann,1980; Powell,1975). Mycorrhizae can improve nitrogen (N) nutrition of nodulating plants primarily through beneficial effects of P on nitrogen fixation activity (Daft and El-Giahmi,1974). In addition, mycorrhizae can insure translocation of nutrients during water stress and can hasten the recovery and increase the survival of host plants after a period of water stress (Safir et al.,1972; Nelsen and Safir,1982).

The infection process of VAM fungi has been studied by many scientists. In a recent review (1984) Bowen divided the process into three phases: preinfection growth to the root, initiation of the infection, and subsequent spread of the fungus in the host and in soil. Environmental factors such as the pH, temperature, nutrient and water availibility of the soil can affect any one of the individual steps within a phase of the infection process and thereby indirectly affect the ultimate degree of VAM root colonization.

The preinfection phase involves germination of spores, growth to the root and possibly growth in the rhizosphere (Bowen,1984). The plant's role in stimulation of VAM in soil is unclear. Spores can germinate in moistened soil in the absence of plant roots, but there have been no studies of whether roots or root exudates can influence this.

Detailed microscopic study has revealed much information concerning the penetration of the host and eventual development of vesicles and arbuscules. Since this has been reviewed recently by Carling and Brown (1982), it will not be discussed here.

There have been a few reports on the internal spread of VAM hyphae. Mathematical models have been developed to simulate the spread of infection (Smith and Walker,1981; Buwalda et al.,1982), however, the utility of these models is still limited by our lack of biological knowledge

concerning these systems. Physiological studies about the determinants of spread in the rhizosphere are also important. Bowen suggests that spread is probably affected both by translocation of substrates from existing arbuscules and, because of the nature of the rhizosphere, by exudates originating from the host plant.

I have chosen to study several aspects of the preinfection phase of VAM development. Chapter 1 covers my studies on the effect of several environmental and physiological factors on the germination of VAM spores. Chapter 2 reports my findings on the influence of root exudates, from plants exposed to two different nutritional regimes, on the growth of VAM hyphae *in vitro*.

Chapter 1. VAM SPORE GERMINATION STUDIES

Literature Review

The strategies by which fungal spore germination in soil is controlled vary considerably. Fungal pathogen propagules must have a mechanism whereby germination occurs in close proximity to a suitable host and under favorable environmental conditions (Cook and Baker,1983). Although specificity is generally not the case, some soilborne fungal pathogens depend on specific nutrients released from plant roots for propagule germination. Coley-Smith (1979) demonstrated this with Sclerotium cepivorum, the onion white rot fungus. Sclerotia of this fungus germinate in response to water-soluble alkyl cysteine sulfoxides, which are released only from roots of the host genus, Allium. Other fungal spores, such as Claviceps purpurea ascospores, have constitutive dormancy that is broken when the sclerotia are exposed to 0-10 C (Mitchell and Cooke,1968).

In the case of vesicular-arbuscular mycorrhizal (VAM) fungi, attempts to determine both physical and chemical germination requirements of chlamydospores in vitro, either in agar or in soil, have meet with varying degrees of success. Temperature of incubation (Schenck et al.,1975;

Coley and Safir,1980; Daniels and Trappe,1980; Koske,1981), pH (Mosse,1972; Green et al.,1976; Daniels and Trappe,1980; Siqueira et al.,1982), light (Godfrey,1957; Schenck et al.,1975), self-inhibitors (Watrud et al.,1978), growth inhibitors (Hepper,1979), nutrients (Mosse, 1962,1973a; Mosse and Phillips,1971; Daniels and Graham,1976; Hepper and Smith,1976; Hepper,1979; Daniels and Trappe,1980; Koske,1981; Siqueira et al.,1982), zearalenone(Stob et al., 1962; Wolf and Mirocha,1973; Mirocha and Christensen,1974; Mirocha et al.,1974), hyperparasites (Godfrey,1957; Schenck and Nicolson,1977; Daniels and Menge,1980; Koske,1981; Sylvia and Schenck,1983), spore dormancy (Godfrey, 1957; Mosse, 1959a,1959b; Coley and Safir,1980), water potential (Reid and Bowen,1979; Bowen,1981; Koske,1981; Sieverding,1981; Nelsen and Safir,1982; Sylvia and Schenck,1983), soil and root extracts (Newman and Watson,1977; Bowen,1981; Graham,1982), soil sterilization (Mosse,1959b; Daniels and Graham,1976; Daniels and Trappe,1980) and agricultural chemicals (Tommerup and Briggs,1981) have each been noted to affect germination of VAM fungi.

Temperature

Investigations by Schenck et al. (1975) using Mosse's medium No. 16 or soil extract agar, showed that VAM species have different optimum temperatures for germination. Maximum spore germination for Gigaspora coralloidea and G.

heterogama occurred at 34 C, whereas Glomus mosseae germinated best at 20 C. In 1980, Daniels and Trappe observed that optimum spore germination of Glomus epigaeus occurred in soil incubated between 18 and 25 C. In agreement with this, Koske (1981) observed that maximum germination of Gigaspora gigantea on water agar or sterile sand plates had occurred at 20-30 C. Coley and Safir in 1980 (unpublished) observed maximum germination of Glomus fasciculatus and G. mosseae at 25 C on water agar as well.

pH

The effect of pH on spore germination has not been extensively studied. The published results to date show great variability among fungal species. Green et al. (1976) observed germination of three VAM species on soil extract agar. Although germination occurred over a broad pH range, Glomus mosseae collected in Washington, germinated best at pH 7, whereas Gigaspora coralloidea and G. heterogama, both collected in Florida, germinated best at pH 5 and pH 6, respectively. The results are consistent with the pH ranges from which the three species of VAM fungi were originally derived. G. mosseae was from a soil with a neutral to alkaline pH. G. coralloidea and G. heterogama were from soils with an acid pH. Green et al. suggest that it is likely soil pH in conjuction with soil temperature are major factors which limit distribution of VAM species. Daniels and Trappe (1980) had very similar results with Glomus

epigaeus, which germinated best between pH 7.0 and 7.4 on nonsterilized soil. They concluded that soil pH significantly influences VAM germination. Siqueira et al. (1982) have now complicated the issue further. They found an interaction between pH and medium composition in that pH optimum differed between media. The ability of certain species of VAM fungi to become established in low pH soils while others become established only when low pH soils are limed (Mosse,1972) may be related not only to the soil pH but also to a pH-nutrient availability interaction for the soil under study. Siqueira et al. suggests that laboratory germination assays using soil extract agar which contains soil nutrients may provide more realistic and useful information than water agar for studying the potential for VAM introduction into a specific soil or area.

Light

Judging from the literature, very little attention has been paid to the effects of light or absence of light on germination of chlamydospores of VAM fungi. Frequently authors neglect to mention whether incubation is done with or without light. Godfrey (1957) germinated spores of Endogone microcarpa at 20 C, and while she mentioned trying both light and darkness she does not describe the effects on germination. Schenck et al. (1975) observed that germination of Gigaspora coralloidea and G. heterogama chlamydospores on soil extract agar was affected by light or

darkness over a range in temperatures. Spore germination in the dark was significantly superior to that in the light for G. coralloidea at 20 and 25 C, and G. heterogama at 34 C. This is the first report of improved spore germination by VAM fungi in the absence of light.

Self Inhibitors

Watrud et al. (1978) have stated that failure to subculture VAM fungi in the laboratory has been assumed to be due to a lack of essential nutrients in the growth media. However, work with the obligate parasite Ustilago maydis and various other cultured plant species (Anagnostakis,1964; Ernst,1974) suggested that adsorption of endogenous or exogenous inhibitory substances by media components may enhance growth and development. They incubated Gigaspora margarita spores on agar medium containing activated charcoal and found an increased rate of hyphal growth. Since pretreatment of spores with activated charcoal did not enhance fungal growth, they concluded that a fungal growth inhibitor is produced or released during and/or after germination.

Growth Inhibitors

Hepper (1979) observed the response of ungerminated and germinated spores to inhibitors of protein and nucleic acid synthesis. Cycloheximide, a specific inhibitor of protein synthesis, drastically decreased germination of Glomus caledonius spores which suggests that protein synthesis is

required for germination and subsequent hyphal development. Actinomycin D, which is thought to specifically inhibit messenger RNA synthesis, severely retarded hyphal development of pregerminated G. caledonius spores. This suggests that protein synthesis required for germination is programmed by stored m-RNA and that the synthesis of new m-RNA is required before hyphal growth can take place. Proflavine hemisulphate, which inhibits RNA synthesis, had deleterious effects on germination and subsequent growth of G. caledonius. This suggests that synthesis of new RNA is obligatory for both processes. 5-fluorouracil, which specifically affects the synthesis of ribosomal and soluble RNA, significantly affects both germination and subsequent growth of G. caledonius, again suggesting that RNA is necessary. Ethidium bromide, which has been shown to inhibit the synthesis of mitochondrial DNA, decreased the germination and hyphal growth of G. caledonius spores. This suggests that new mitochondrial material is formed in G. caledonius spores at the start of germination. Hepper concluded that there is no serious limitation to the synthesis of either nucleic acid or protein during the germination and subsequent hyphal development of G. caledonius and that in this respect the endophyte resembles saprophytic fungi more than other obligate biotrophs.

Nutrients

A. Mineral Nutrients

It is well documented that the mineral contents of the growth medium affect VAM fungal associations, however, few scientists have examined their effects on germination and/or hyphal development *in vitro*. Mosse and Phillips (1971) attributed the poor spore germination and fungal root colonization of plants grown in a balanced nutrient solution to high salt contents. In support of this, Hirrel (1981) showed that both sodium and chloride salts reduced germination of *Gigaspora margarita* on agar. To overcome this inhibition, Mosse (1962) used spores pregerminated in nutrient-free medium as inoculum. She also reported the necessity of a pseudomonad being present to obtain penetration and infection of seedlings by *Endogone* sp. She speculated that the presence of contaminant microorganisms is needed to deplete certain nutrients excreted from the roots of the plant host in 2-member cultures. In later experiments, Mosse and Phillips (1971) were able to obtain typical root colonization in complete salts medium after most of the nutrients from the medium had been removed by growing seedlings. In 1976, Daniels and Graham found that all but the lowest concentrations of nutrients added to water agar inhibited the germination of surface-sterilized spores of *Glomus mosseae*. They suggested that spores in the soil are unable to germinate until the levels of certain

compounds are lowered by the activities of other microorganisms. In support of this idea, Powell (1976) reported that spore germination of four VAM fungi which were embedded in agar on glass slides buried in soil occurred in the presence or absence of roots. After further studies of the effects of several physical and biological factors on germination of G. epigaeus, Daniels and Trappe (1980) concluded that spore germination is not directly stimulated by the presence of the host plant, but that optimum conditions for spore germination of some VAM fungi may also be conditions most suited to growth of many host plants.

It is clear that nutrients affect the establishment of mycorrhizal associations, but it is not clear if their effect is on spore germination or on the ability of germinated spores to colonize the roots. Siqueira et al. (1982) added several concentrations of NPK salts to agar media and saw no decrease in germination of Gigaspora margarita. In fact good germination was obtained in all treatments. They also found that CaH_2PO_4 alone or in combination with N and K increased germination, whereas, nitrogen did not affect germination, but did reduce germ tube growth. This report agrees with recent work by Daniels and Trappe (1980) who reported that various N and K levels had no significant influence on germination of G. epigaeus spores whereas P levels of up to 100ppm caused a slightly significant increase in spore germination. However, Koske

(1981) found no effect of P level on germination of Gigaspora gigantea spores and concluded that the reduction and eventual elimination of VAM infection in plants receiving high levels of P, as observed by Daft and Nicolson (1969) and Mosse (1973b) was related to changes in the physiology of the host plant, rather than a direct effect of phosphorus on spore germination.

In 1979, Hepper reported the effect of heavy metals on VAM spore germination. She found that Glomus caledonius spore germination was decreased by manganese, copper and zinc but the inhibition was not permanent. Spores removed from agar containing the above heavy metals germinated normally when transferred to water agar. Similar results (Hepper and Smith,1976) were obtained with G. mosseae except that a higher concentration of zinc was required to completely stop germination. The levels of manganese and zinc which inhibit germination (0.136 and 0.70 ppm respectively) are in the range commonly found in the soil solution of normal and even deficient soils. Hepper and Smith suggest that this may have a bearing on the establishment of VAM infections with G. mosseae spores, especially because liming and water-logging of soils both cause great increases in the concentration of divalent ions (Mn^{2+}, Zn^{2+} and Cu^{2+}) in the soil solution. In support of this, Samuel (1926) reported greater mycorrhizal infection of plants grown from soils known to be deficient in

manganese than in those from normal sites.

B. Carbon Compounds

In 1978, MacDonald and Lewis reported that VAM fungi possess the enzymes required for carbon metabolism, which suggests that resting spores might be able to respond to exogenous sources of carbon such as root exudates present in the rhizosphere. Siqueira et al. (1982) examined the effect of four sugars and four organic acids on germination and germ tube growth of Gigaspora margarita. In all cases both germination and germ tube growth on agar was inhibited. These results support previous studies (Mosse,1959b; Daniels and Graham,1976; Hepper and Smith,1976; Hepper, 1979; Koske,1981). For example Koske observed a significant reduction in spore germination of G. gigantea in sand plates in response to glucose. Germ tube length and number per spore was also reduced. Mosse suggested that the strong inhibitory effect of sugars could possibly provide clues to the mechanism that confines VAM colonization to the root cortex.

C. Vitamins

Siqueira et al. (1982) observed that thiamine significantly increased germination and germ tube growth of Gigaspora margarita spores on agar. This has been found by Hepper (1979) and Hepper and Smith (1976) for other VAM fungal species, and the fungi appear to require different concentrations for germination than for growth. Thiamine

and many other vitamins are components of root exudates (Rovira,1969) and may play an important role in the early events of mycorrhizal root colonization.

D. Plant Growth Substances

All or most compounds found in individual plant cells have been found in root exudates (Hale et al.,1971), which suggests that very small quantities of plant growth substances may be lost to the rhizosphere in this manner (Rovira,1965). There have been many reports on the effects of plant growth substances produced by soil microorganisms on plants but the reverse of this, the effect of plant growth substances produced by plants on soil microorganisms, has received very little attention. Azcon et al. (1981) observed an inhibitory effect of ethylene supplimented agar on germination of Glomus mosseae spores. The presence of auxins, gibberellins and cytokinins enhanced hyphal growth but did not affect spore germination. Powell (1976) argued that plants stimulated mycorrhizal fungal growth in soil by a nutrient or hormone present in root exudates. However, he also suggested that plant growth substances produced by active populations of microorganisms in the rhizosphere influence VAM fungal associations.

Zearalenone

Originally, in the mid-19th century deBary brought forth the hypothesis that sex hormones are involved in the sexual reproduction of fungi (Wolf and Mirocha,1973).

However, it was not until the 1930's that others, working with Sapromyces reinschii and Achlya bisexualis, convincingly demonstrated the function of sex hormones. Since then, sexual reproduction in many other fungal genera has been shown to be controlled by hormonal systems (Wolf and Mirocha,1973).

Zearalenone, an estrogenic secondary metabolite produced by several species of Fusarium colonizing corn and other cereals, was first studied by Stob et al. (1962). Zearalenone is of practical importance because it causes hypoestrogenism when contaminated feed is ingested by animals (Mirocha and Christensen,1974). Is has also been shown that zearalenone is physiologically active in plant as well as animal systems. Later studies (Mirocha et al.,1974) revealed that zearalenone might influence sexual reproduction in Fusarium roseum "Graminearum," the organism which synthesizes it. Other scientists noted that zearalenone is always present in Fusarium isolates that form perithecia. Further work by Mirocha et al. (1974) demonstrated that zearalenone influenced sexual reproduction in other genera of the Ascomycetes. It would be interesting to investigate the effect zearalenone has on VAM fungi since other sex hormones have been reported to affect spore germination and/or hyphal growth, as mentioned previously (Azcon et al.,1981).

Hyperparasites

Hyperparasitism of VAM fungi by chytridiaceous fungi has been observed (Ross and Ruttencutter,1977; Schenck and Nicolson,1977; Daniels and Menge, 1980; Sylvia and Schenck,1983). It is apparently a widespread phenomenon and may limit mycorrhizal populations especially in wet soils.

Germination studies involving VAM fungal spores often show that microbial contamination is negatively correlated with germination (Sylvia and Schenck,1983). Contaminating fungi are predominately common, fast-growing zymogenous soil saprobes, many of which may produce antimicrobial metabolites. Hence, both competition and antibiosis are possible mechanisms for inhibition of germination. Fruiting structures or hyphae of these fungi were not observed within VAM spores (Sylvia and Schenck,1983). They concluded that these fungi must have a different role than do more specialized hyperparasites (Godfrey,1957; Ross and Ruttencutter,1977; Daniels and Menge,1980). The effect of these contaminating microflora on VAM fungi differ widely. Koske (1981) reported that contaminants of Gigaspora gigantea did not effect spore germination. In contrast to this, Sylvia and Schenck (1983) observed that contaminants had an inhibitory effect on spore germination of G. etunicatum but did not influence germination of G. macrocarpum. They suggested that a greater understanding of the influence of soil microflora on VAM fungi may help

explain the variability observed in their germination.

Spore Dormancy

There have been reports (Godfrey,1957; Mosse,1959b) of increased germination of various VAM fungi following cold storage of spores in the soil at 5-10 C for several weeks to several months. While the practice is accepted, there are very few quantitative data on the effects of cold storage on the germination of spores. Coley and Safir (1980) did a study to provide quantitative evidence of the effects of cold treatments on increased rates of germination of Glomus fasciculatus and G. mosseae spores. They found that storage at -10 C was more effective than 4 C in producing increased germination on agar over the time periods tested. However, after further experimentation and communication with other researchers, Coley and Safir (1980) suggested that there is a possible degree-day requirement of some kind to break dormancy.

Water Potential

There have been several reports on the effects of water stress on VAM development and response in the plant. Readhead (1975) found that soil watering regimes optimal for plant growth were also optimal for mycorrhizal colonization of Khaya grandifoliola seedlings and production of fungal spores. Similarly, Saif et al. (1975) reported that the size of the Endogone spore population increased as soil water content approached field capacity. More recently,

Nelsen and Safir (1982) found a correlation between reduction in onion fresh weight and reduction of spore production of Glomus etunicatum in response to drought stress at both low and high soil P levels. Trinick (1977) observed that mycorrhizal infection of lupins was reduced by high soil moisture. Sieverding (1981) showed that excessive soil moisture had a considerable inhibitory influence on development and function of VAM, particularly in a plant with a weak root system.

Even though the effects of different soil moisture regimes on VAM infection had been suggested from field and greenhouse observations, Reid and Bowen (1979) were the first to experimentally study this in the laboratory. Their data showed that moderate moisture changes had extremely large effects on infection. Bowen (1981) later showed that these slight changes in soil moisture had drastic effects on germination of Glomus mosseae spores as well. This agrees with a previous study in which Daniels and Trappe (1980) observed optimal spore germination when soil moisture was at or above field capacity. This was the first study of the effects of soil moisture on the development of VAM fungi apart from the host plant. Koske (1981) and Sylvia and Schenck (1983) also observed this stimulatory effect of high soil moisture on germination of VAM spores on agar, sand and membrane filters.

Root Exudates

The substances which are constituents of root exudates (self inhibitors, mineral nutrients, carbon compounds, vitamins and plant growth substances) have been previously discussed individually. These compounds do affect germination and hyphal growth of VAM fungi. However, most studies are on the effect of an individual compound at a range of concentrations, whereas VAM spores are exposed to an ever fluctuating ratio of these compounds as affected by all the environmental factors discussed previously.

Substances leak out of plant roots into the rhizosphere via water films around roots and soil particles. Hence, soil moisture has great influence on this path of diffusion and the distance which substances are able to travel from the root. High molecular weight substances generally have low diffusivities so they accumulate near the root and there is a steep concentration gradient away from the root. In contrast, low molecular weight substances capable of more rapid diffusion from the root surface have a much more shallow concentration gradient in a soil with similar soil moisture (Bowen,1981). A microbial growth model (Newman and Watson,1977) based on these diffusion properties, suggested that in most conditions substances affecting biological activity beyond 1-2mm from the root will need to be effective at very low concentrations. Hence, these substances are likely to have a triggering effect on spore

germination rather than be an important substrate for growth.

Graham (1982) was the first scientist to study the role of citrus root exudates on germination of Glomus epigaeum spores on water agar. After seven days exposure of spores to exudates, germination increased from 10% to 27% and germ tube length increased more than 400%. Exudates from sudangrass had effects on germination similar to those from citrus roots. He concluded that root exudates that stimulate spore germination are non-specific and are produced by unrelated plant types. The root exudates were collected under non-sterile conditions so the exudate solution was a mixture of root and microbial metabolites. His results supported earlier studies (Graham et al.,1981, 1982 and Johnson et al.,1982) wherein it was proposed that exudation affects germination and growth of VAM fungi and thereby influences subsequent root colonization and VAM formation.

Although in situ VAM spores are exposed to root exudates in combination with microbial metabolites, conclusions about the importance of root exudates can be made only after experiments are conducted with exudates collected aseptically from plants grown under sterile conditions.

Soil Sterilization

Mosse (1959b) demonstrated stimulation of VAM spore germination by a dialysable substance produced in soil only in the presence of actively growing microorganisms. There was no germination on media prepared from autoclaved soil or from soil to which Rose Bengal was added. Mosse suggested that the absence of a stimulatory compound in the absence of microorganisms or destruction of the compound during autoclaving could explain this germination inhibition. Her results can also be explained (Daniels and Graham,1976) on the basis of excessive amounts of solutes which are absent from soil containing living organisms but present in autoclaved soil. Hence, if a stimulatory compound is destroyed by autoclaving, a similar loss should occur when soil extracts are autoclaved. Daniels and Graham (1976) did further research on this possibity and found that autoclaving did not destroy stimulatory effects of compounds isolated from nonsterile soil on the germination of Glomus mosseae spores. Moreover, they also found that germination was suppressed when a dialysate from a chemically sterilized soil was tested in combination with a dialysate from nonsterile soil. This clearly suggests that lack of germination with extracts from fumigated soil is due to the presence of inhibitory compounds rather than the loss of stimulatory compounds. In 1980, Daniels and Trappe reported evidence to further clarify previous findings. They again

found that G. epigaeus spores would not germinate on autoclaved soil. Toxin production from autoclaving could not explain this inhibition because germination was inhibited in irradiated and steamed soil as well. High levels of nutrients released by autoclaving, irradiating or steaming soil is unlikely to be the cause of inhibition of germination since germination did not occur even when small portions of autoclaved soil were added to water, thus greatly diluting any excess nutrients. They concluded that it is more likely that the lowering of microbial populations in some way inhibits germination. VAM spores could possibly contain self inhibitors which are normally metabolized by other soil microflora.

Agricultural Chemicals

Most investigations on the effects of agricultural chemicals on VAM fungi have assessed changes in root colonization (percent root infected) and/or spore production (Jalali and Domsch,1975; Bailey and Safir,1978). Tommerup and Briggs (1981) divided development of the VAM symbiotic relationship into four stages (1) spore germination or initiation of hyphal growth from infected root inoculum; (2) growth of hyphae through the soil to the root; (3) penetration and successful initiation of infection in roots; (4) spread of infection, development of a mycorrhizal relationship with roots and spore production. They tested the response of three VAM species in stages 1 and 2 to five

chemicals (Bavistin, Difolatan, Frampropisopropyl, Metoxuron and Trifluralin). They observed that neither germination or growth of hyphae was affected by the chemicals. Previous studies (Sutton and Sheppard,1976; Bailey and Safir,1978) with some of the same chemicals showed decreased root colonization, hence, this led them to conclude that these chemicals tested do significantly effect VAM fungi but have little or no effect on stages 1 and 2 in the development of VAM fungus/host associations.

The purpose of this research was to determine the effects of method of storage, water potential, root exudates from phosphorus-deprived plants, and zearalenone, on the germination of VAM chlamydospores in axenic culture.

Materials and Methods

A. Influence of storage solution over time on germination

Three VAM species, Glomus fasciculatus (Thaxt. sensu Gerd.)Gerd. and Trappe, G. mosseae (Nichol. & Gerd.)Gerd. and Trappe, and Gigaspora margarita Becker & Hall were grown in sorghum (Sorghum vulgare) pot cultures (Mosse and Phillips,1971) in the greenhouse for 4 months and stored at 4C for at least 4 months before use. This VAM infested soil was wet sieved (Appendix 1) to eliminate most of the soil and organic debri which was collected on the sieve. A modified centrifugation-flotation technique involving both 30% sucrose and several Ficoll solutions of various densities (Appendix 1) was used. Organic debris was carefully removed from the spore suspension by hand with a pasteur pipet under a dissecting microscope. Spores were then surface-sterilized with a solution containing 2% Chloramine-T (w/v), 0.02% streptomycin sulfate(w/v) and sodium lauryl sulfate (SLS) followed by washing with sterile distilled water (Appendix 2). At this time the spore suspension was divided into three subsets and placed in one of the three treatment solutions (streptomycin/gentamicin, Ringer's solution or sterile distilled water (Appendix 3). Four replicate aliquots of spores were taken weekly from treatment solutions at 0-12 weeks and plated out on an agar medium (Hepper,1981;Appendix 4). Plates were incubated at 25-27C in the dark. Germination percentage was determined

after 7-10 days. This experiment was performed twice.

B. Influence of zearalenone on germination

A medium designed for root organ culture (ROC) (Hepper and Mosse,1980;Appendix 5) but modified to contain phosphorus (W/P), contain no phosphorus (W/OP) or contain only water agar (N-FERT) was used in this experiment. To study the influence of zearalenone on VAM spore germination and subsequent hyphal growth, I added zearalenone, (International Minerals and Chemical Corporation, Terra Haute,Indiana), dissolved in ethanol, to the ROC agar at a range of concentrations from 0-5000 ppm. Six replicate plates of each treatment at each concentration were made. One plate was subsampled to obtain the water potential of the agar using a Wescor dewpoint hygrometer and C-52 sample chambers following a modified procedure of Nelsen et al. (1978). One cm diameter agar plugs were taken from petri plates with a cork borer and placed into shallow sample chambers. Water potential was measured after at least a 2 hour incubation period. The other five replicate plates were inoculated with 20 surface-sterilized Glomus fasciculatus spores isolated from sorghum pot cultures as described previously. Plates were incubated at 25-27C in the dark and percentage germination was determined after 7-10 days. This experiment was performed twice.

C. Influence of water potential on germination

A medium designed for root organ culture (ROC) (Hepper and Mosse,1980;Appendix 5) but modified to contain phosphorus (+P), contain no phosphorus (-P) or contain only water agar (-FERT) was used in this set of experiments. To study the influence of water potential on VAM spore germination, I attempted to adjust the osmotic potential of the media using various concentrations of Bacto agar, polyethylene glycol (PEG) or sorbitol.

Bacto agar was added to the ROC agar at a range of concentrations from 8-40gms/l water. Surface sterilized (Appendix 6) white clover (Trifolium repens L. cv `Ladino') seeds were placed on the agar (4 seeds/tube) in two replicate test tubes of each treatment to determine if roots could penetrate the agar.

A 60% w/v PEG solution using distilled water was made. To eliminate all contaminant ions commonly present in PEG, a procedure by Reid et al. (1978) was followed. AG1-X2, a strong anion exchanger, was added to the PEG and put on a shaker for at least 3 hours. This resin was filtered out and then AG50W-X2, a strong cation exchanger, was added and the mixture was again put on a shaker for at least 3 hours. The resin was filtered out and the resulting PEG solution, free of contaminants, was autoclaved and then incorporated into ROC agar at a range of concentrations from 0-30gms PEG/100ml water. Sorbitol was also added to ROC agar at a

range of concentrations from 0-30gms/100ml water.

Six replicate plates of each treatment at each concentration were made. One plate was used to measure the water potential of the agar with the Wescor dewpoint hygrometer and C-52 sample chambers as described previously. The other five replicate plates were inoculated with 10-15 surface-sterilized Glomus fasciculatus spores (20/plate) isolated from sorghum pot cultures as described previously. Plates were incubated at 25-27C in the dark and germination observations were made after 7-10 days. This experiment was performed twice.

D. Influence of root exudates on germination

White clover (Trifolium repens cv `Ladino') seeds were surface sterilized with 70% ethyl alcohol for 30 seconds followed by 0.1% $HgCl_2$ in 1mM HCl for 5-7 minutes followed by exhaustive washing with sterile distilled water (Appendix 6). The seeds were then placed on moist filter paper in a sterile petri dish incubated at 23 C in the dark for 2 days to allow for germination. 50 seedlings were then transferred from the petri dish to moist cheesecloth in a sterilized square glass staining dish (Figure 1) containing 100ml of sterilized Hoagland's nutrient solution, with phosphorus (W/P) or without phosphorus (W/OP) (Appendix 7). The dishes were enclosed in sterile clear plastic bags, tightly sealed and the seedlings grown under 16 hour daylength fluorescent lights (4.5 Klux). The Hoagland's

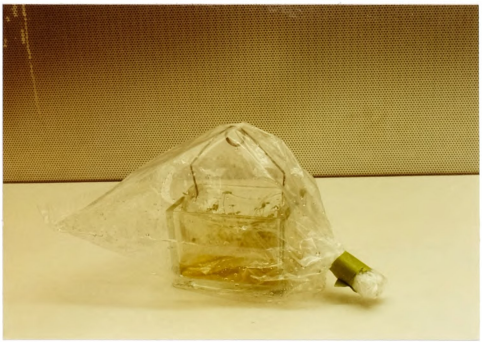

Figure 1. Glass staining dish apparatus which enables collection of exudates from aseptically grown plants.

solution was replaced every 7 days. Treatments consisted of 5 replicate staining dishes each containing 50 seedlings.

At 2, 4 and 6 weeks exudates were collected from the roots of these clover seedlings. Briefly, at the end of each 2 week period, all plants were taken out of the nutrient solution, rinsed with sterile distilled water several times and then placed in sterile distilled water for a 24 hour period. The exudate solutions from all dishes within a treatment were pooled, filter sterilized (0.45um), rotory evaporated at 40 C to 1/10 original volume, filter sterilized again and stored at 4 C. This was repeated at 4 and 6 weeks. Contamination checks were done once a week by plating out spent nutrient solution on water agar and PDA at the time of nutrient solution replacement. No contamination was observed.

Root exudates collected from phosphorus deficient and non-deficient plants at 2, 4 and 6 weeks were added to root organ culture (ROC) agar at a range of concentrations from 0.0 to 8.0ml exudate solution per 10ml total volume of media. Sorbitol was added to attain the optimum water potential of -3 bars for germination of G. fasciculatus spores, as determined in a previous experiment. G. fasciculatus spores (30 per plate) were placed on the agar plates and then incubated at 25-27 C in the dark. Percent germination was monitored at five-day intervals. This experiment was performed twice.

Statistical analysis by analysis of variance (ANOVA) or standard error of the mean (SEM) was conducted where appropriate and stated in the results.

Results

A. Influence of storage solution over time on germination

All three VAM species, Glomus mosseae, G. fasciculatus and Gigaspora margarita, germinated on nutrient-amended agar plates, (Figure 2-4 and Appendix 8) and did so within five to ten days. Each point on Figures 2-4 represents the mean of four replicate plates. Appendix 8 contains all raw data.

Germination of G. mosseae spores (Figure 2) was the lowest (10% or less) and G. margarita spores (Figure 3) the highest (48% or less) from the outset of the experiment. In addition, after 2 weeks in storage no G. mosseae spores had remained germinable on agar. Even though there were no significant differences (ANOVA, $P=0.05$) in germination rates after storage in the storage solutions the longer the time in storage the lower the germination for both fungal species ($P=0.05$).

G. fasciculatus spores germinated (Figure 4) at a moderate level (20% or less). Interestingly enough, both the storage solution used and the length of time in the solution had significant effects on spore germination levels ($P<0.001$). Similar results were obtained in a replicate experiment.

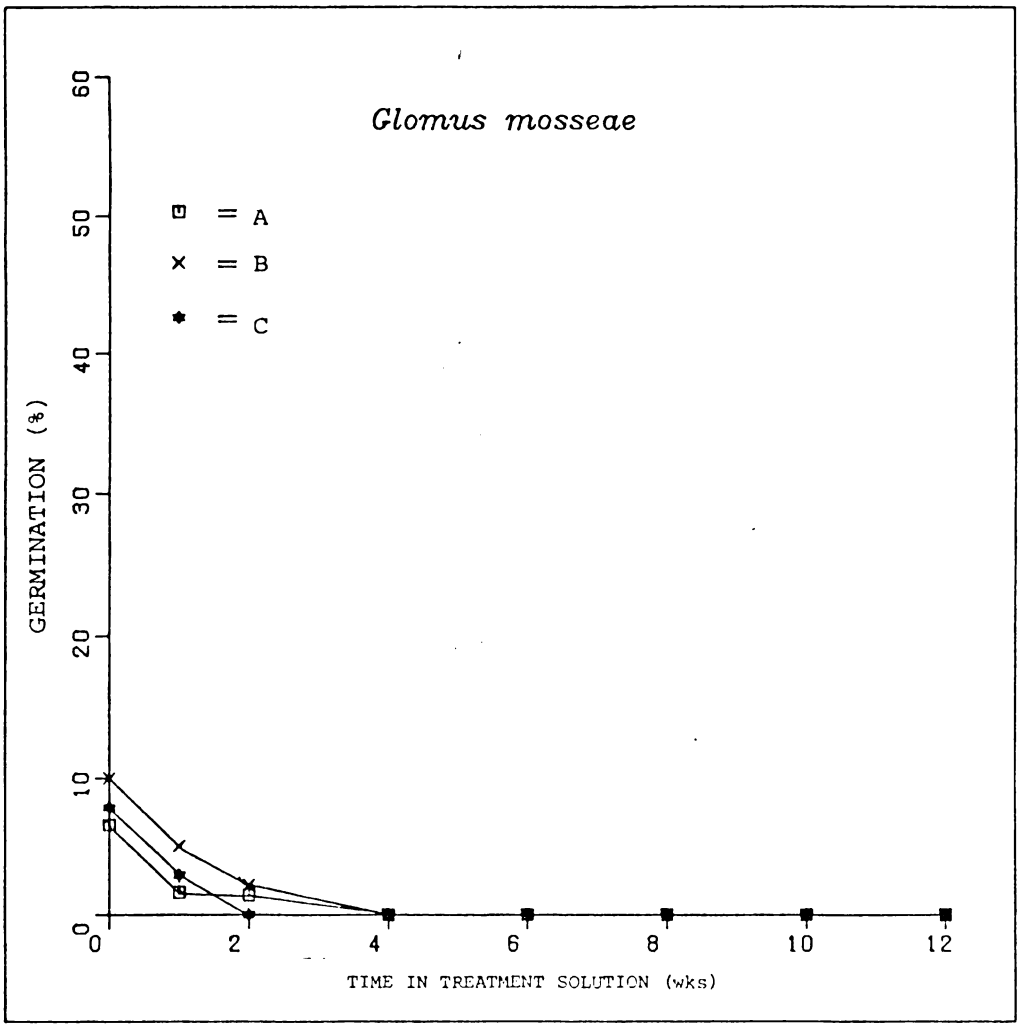

Figure 2. The effect of time in storage, in three treatment solutions, on germination of Glomus mosseae spores on agar. A = storage solution containing streptomycin and gentamicin, B = storage solution containing Ringer's salt solution, C = storage solution containing sterile glass distilled water. Each point is the mean of 4 replicate plates each containing 30 spores.

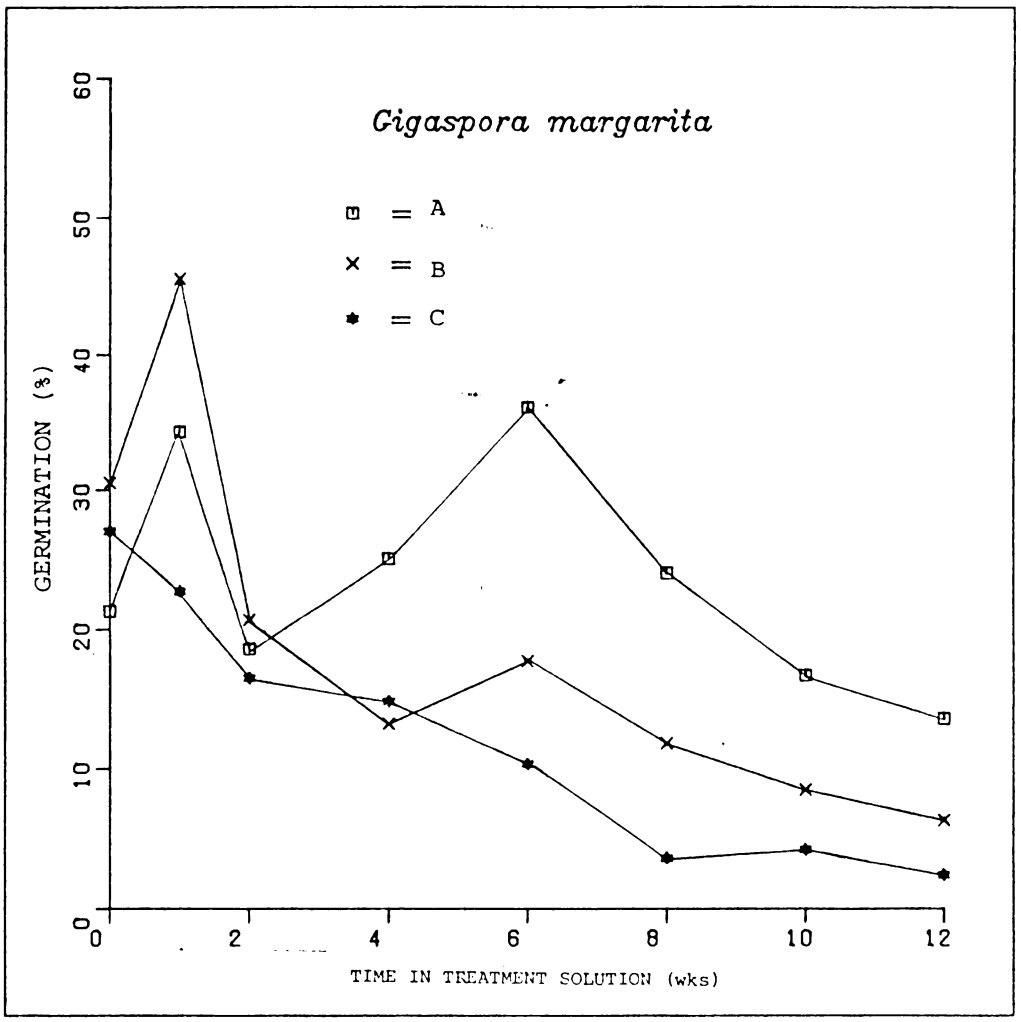

Figure 3. The effect of time in storage, in three treatment solutions, on germination of Gigaspora margarita spores on agar. A = storage containing streptomycin and gentamicin, B = storage solution containing Ringer's salt solution, C = storage solution containing sterile glass distilled water. Each point is the mean of 4 replicate plates each containing 30 spores.

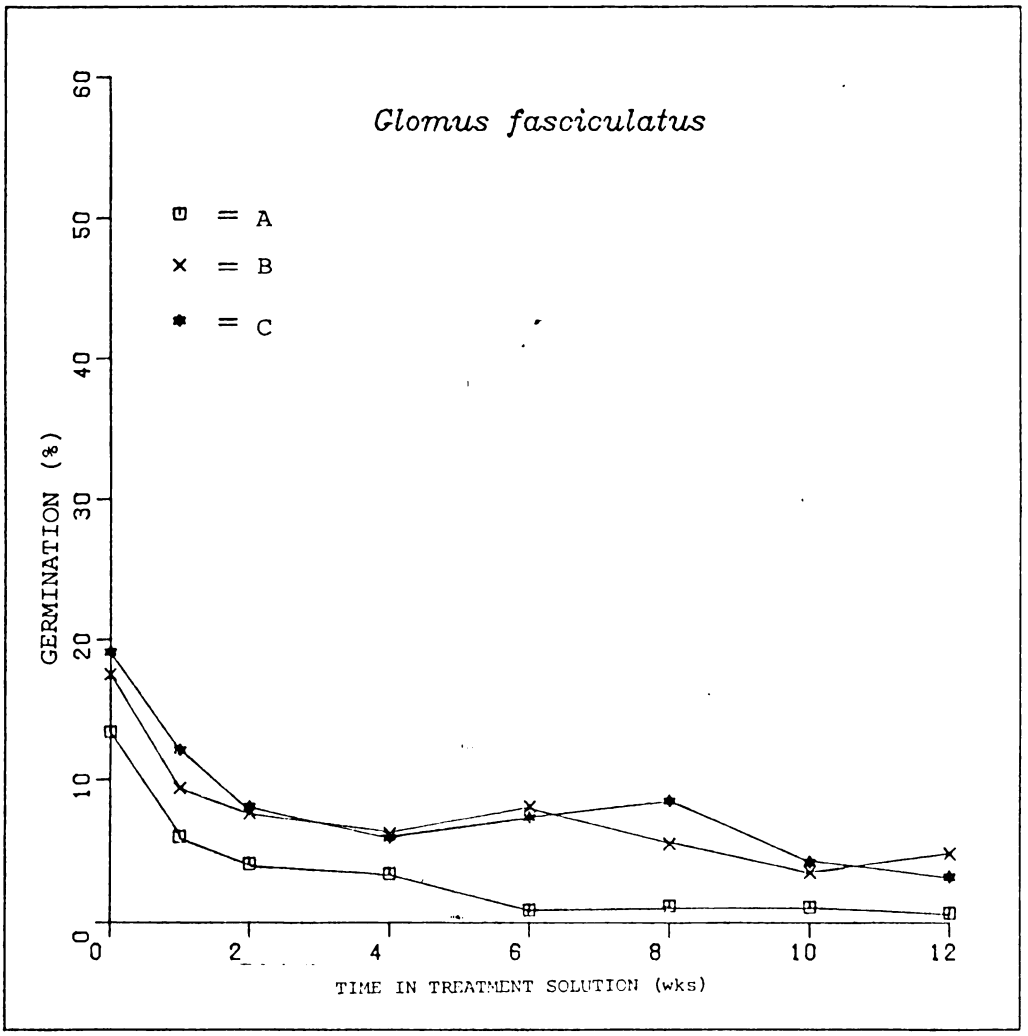

Figure 4. The effect of time in storage, in three treatment solutions, on germination of Glomus fasciculatus spores on agar. A = storage solution containing streptomycin and gentamicin, B = storage solution containing Ringer's salt solution, C = storage solution containing sterile glass distilled water. Each point is the mean of 4 replicate plates each containing 30 spores.

B. Influence of water potential on germination

The addition of bacto agar to ROC agar caused no significant change in water potential (Figure 5) even though the agar was so hard that seed germination was decreased and seedling roots could not penetrate the agar surface (Table 1). Water potential did not change as concentration of Bacto agar was increased (Figure 5) and germination and growth decreased with the addition of Bacto agar (Table 1). Both PEG and sorbitol decreased the water potential in a linear fashion (Figures 6 and 7). Sorbitol caused greater changes than did PEG (-60.53 bars versus -17.33 bars, respectively). It should be noted that ROC agar, amended with greater than 10gms PEG/100ml water, would not solidify.

Germination of G. fasciculatus spores on ROC agar were affected in a similar manner by water potentials adjusted with the two osmotica (Appendix 9 and 10). In the presence of PEG or sorbitol the germination rate peaked at from -4.0 to -6.0 bars, and declined rapidly to almost no germination above and below this range (Figure 8 and 9). This peak in germination was not observed with Bacto agar, probably because the water potential never reached -4 to -6 bars (Table 2). Similar results were obtained in a replicate experiment.

C. Influence of root exudates on germination

The addition of root exudates, from plants grown in P deficient or non-deficient solutions, to ROC agar had no

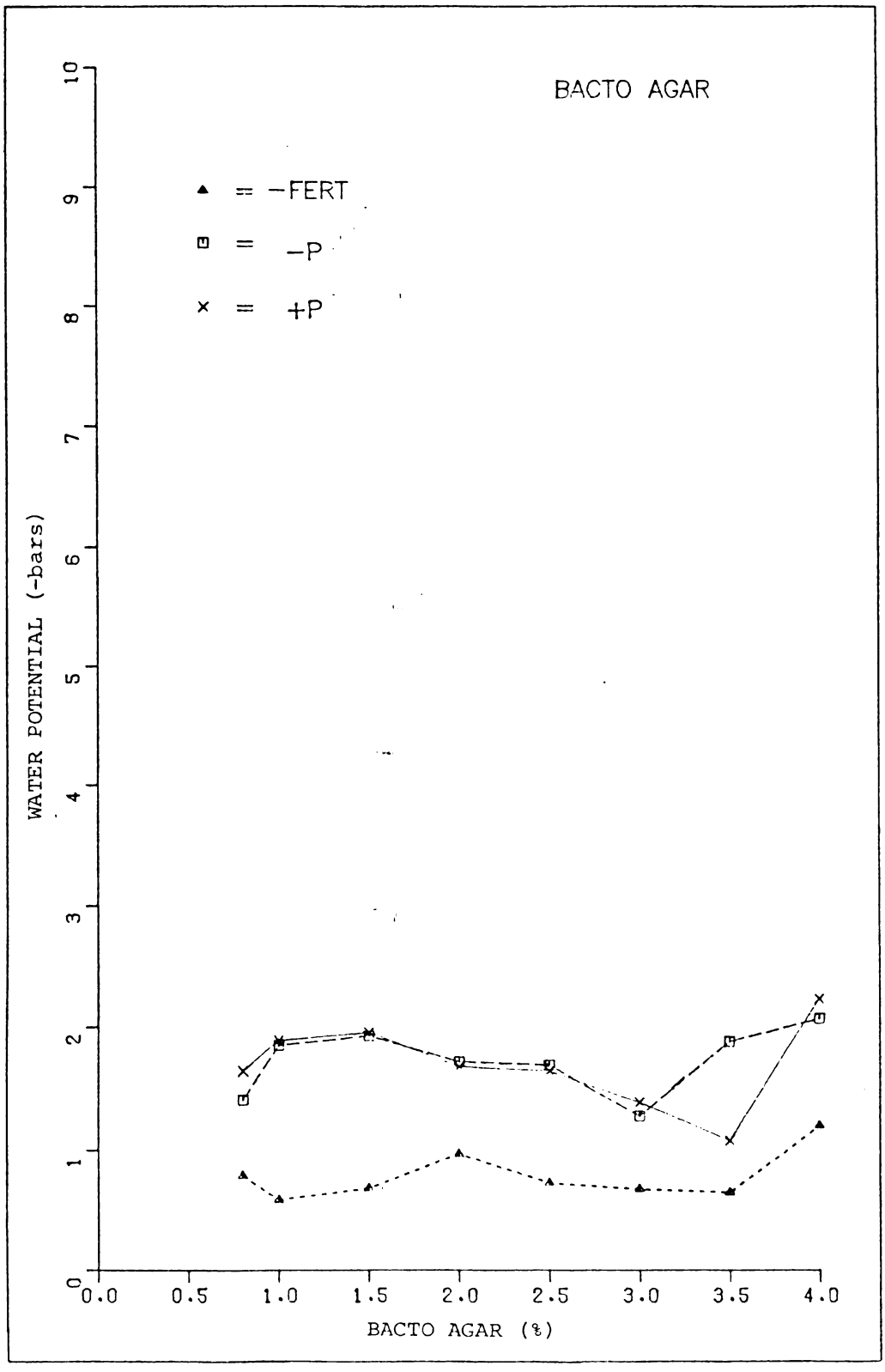

Figure 5. The effect of Bacto agar on the water potential of a VAM growth medium. -FERT=water agar, -P=VAM growth medium without P, +P=VAM growth medium with P.

Table 1. The influence of Bacto agar concentration on growth of Trifolium repens seedlings.

Bacto Agar Added (%)	Media Used					
	A(-FERT)		B(-P)		C(+P)	
	% Germination[a]	Growth Rating[b]	% Germination	Growth Rating	% Germination	Growth Rating
0.8	100.0	3.9 ± 0.1[c]	58.4	1.3 ± 0.0	100.0	3.5 ± 0.1
1.0	87.5	3.4 ± 0.4	33.3	1.3 ± 0.0	33.4	1.4 ± 1.0
1.5	83.4	3.0 ± 0.5	33.3	1.3 ± 0.0	50.0	1.2 ± 0.4
2.0	41.7	1.5 ± 0.4	33.3	0.8 ± 0.4	33.4	0.9 ± 0.6
2.5	50.0	1.7 ± 0.3	66.7	2.3 ± 0.7	37.5	1.4 ± 1.0
3.0	58.4	1.9 ± 0.1	33.3	0.8 ± 0.4	50.0	1.7 ± 0.3
3.5	45.9	1.1 ± 0.4	16.7	0.5 ± 0.4	0.0	0.0 ± 0.0
4.0	0.0	0.0 ± 0.0	16.7	0.7 ± 0.5	16.7	0.5 ± 0.4

[a] Values represent the mean of 2 tubes, each containing 4 seedlings.

[b] Seedling Growth Rating Index
- 4 = roots grew to bottom of tube, appear normal
- 3 = roots reach half way down tube
- 2 = roots just enter top few cms of agar
- 1 = germination but roots could not penetrate agar
- 0 = no germination

[c] Standard Error of the Mean

-FERT = water agar

-P = without phosphorus

+P = with phosphorus

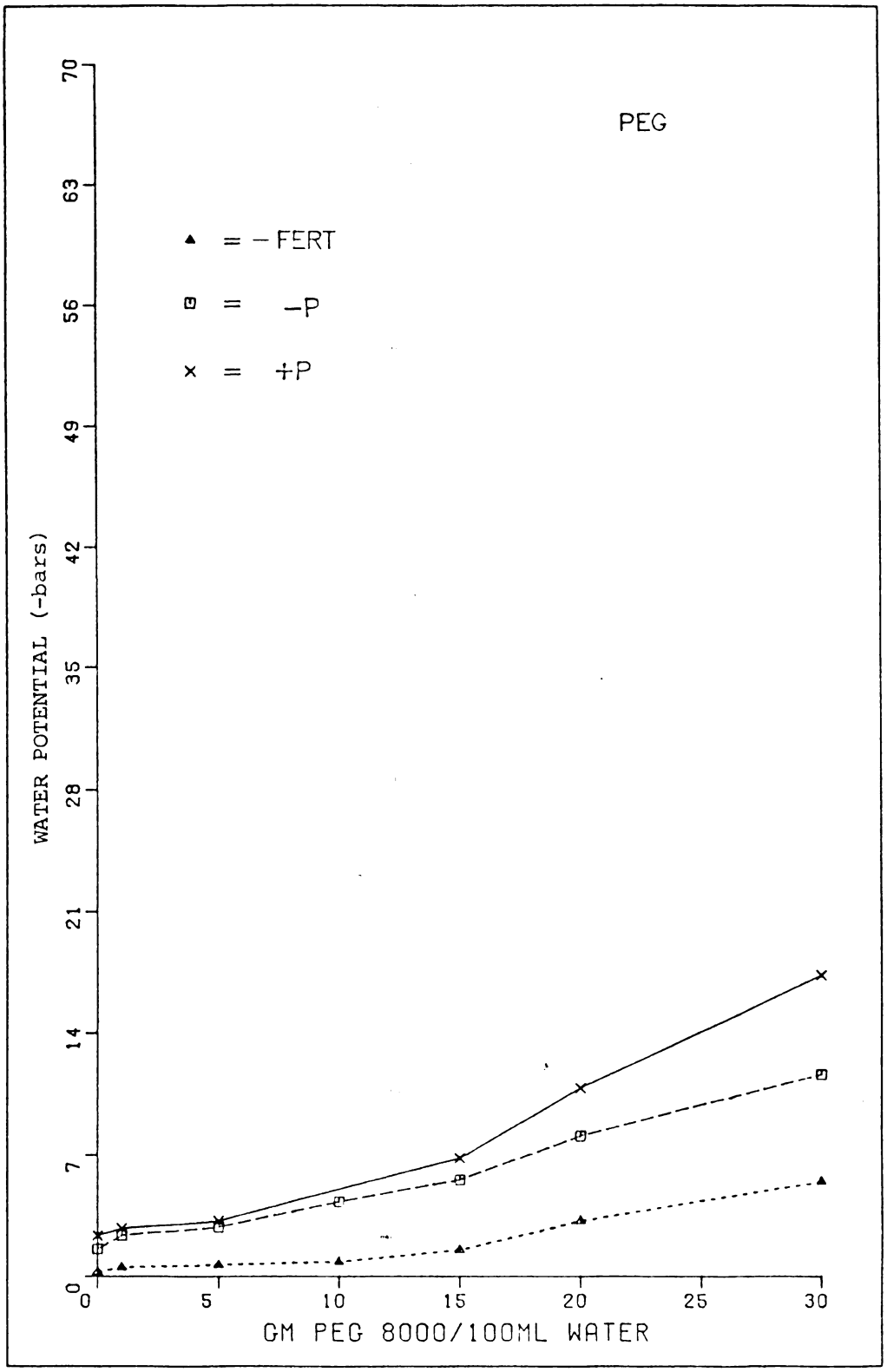

Figure 6. The effect of PEG on the water potential of a VAM growth medium. -FERT = water agar, -P = VAM growth medium without P, +P = VAM growth medium with P.

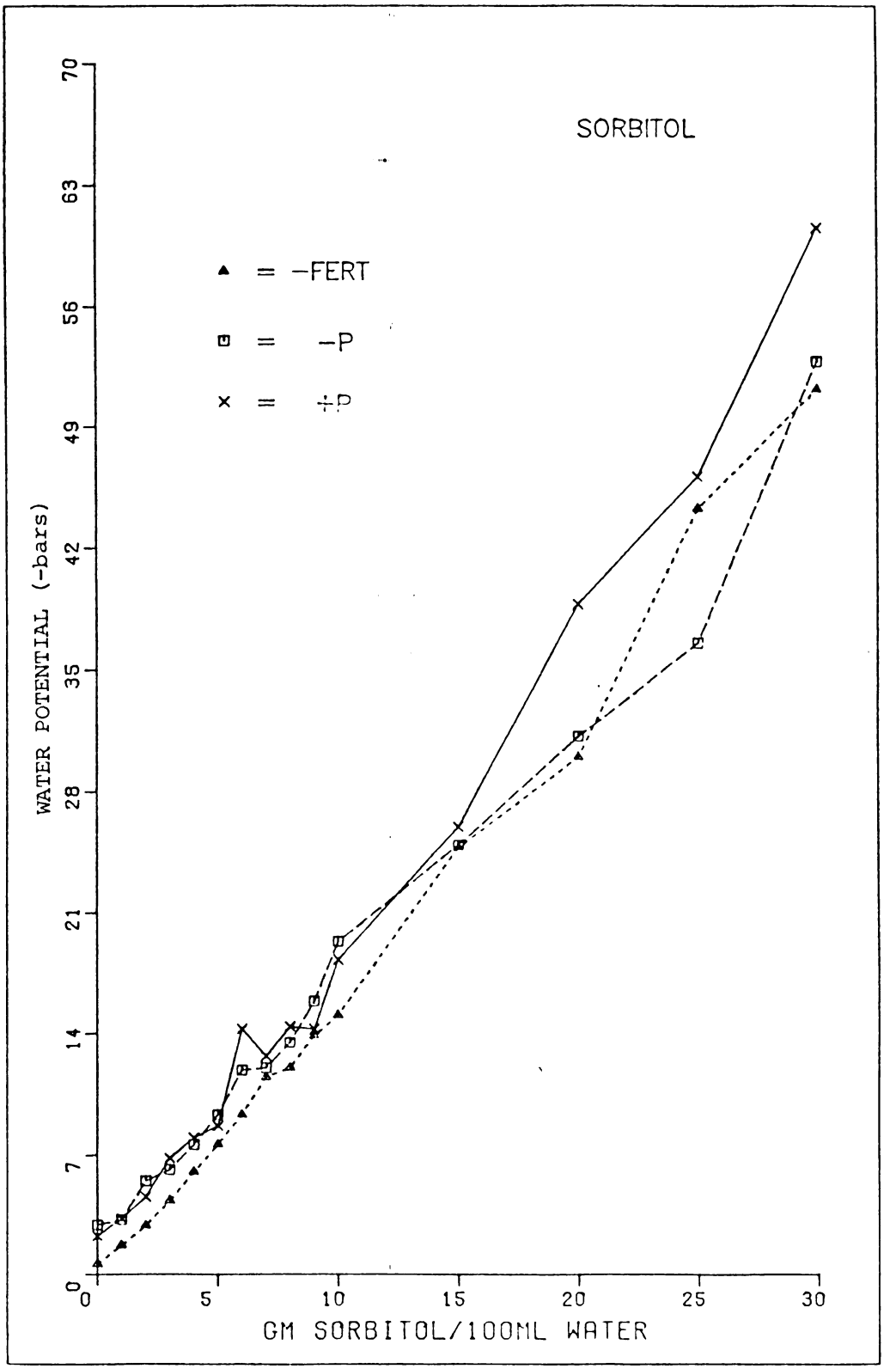

Figure 7. The effect of sorbitol on the water potential of a VAM growth medium. -FERT=water agar, -P=VAM growth medium without P, +P=VAM growth medium with P.

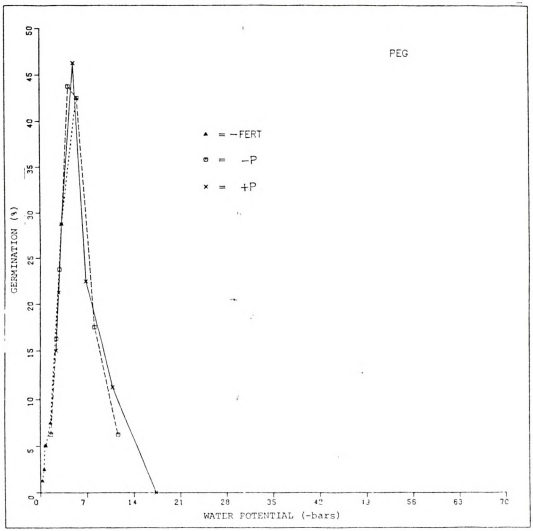

Figure 8. The effect of substrate water potential as adjusted by PEG on the germination of Glomus fasciculatus spores. -FERT=water agar, -P=VAM growth medium without P, +P=VAM growth medium with P. Each point is the mean of 4 replicate plates each containing 20 spores.

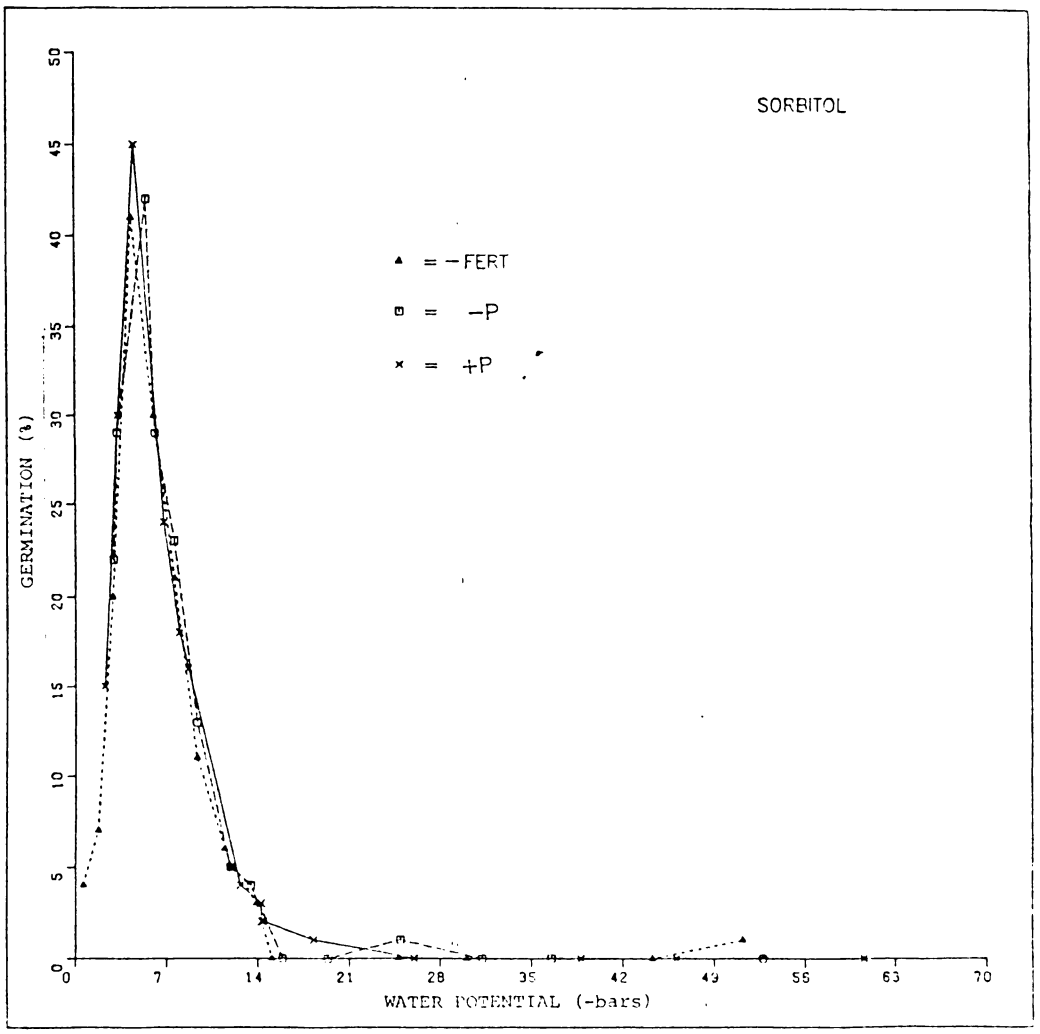

Figure 9. The effect of substrate water potential as adjusted by sorbitol on germination of Glomus fasciculatus spores. -FERT=water agar, -P= VAM growth medium without P, +P=VAM growth medium with P. Each point is the mean of 5 replicate plates each containing 20 spores.

Table 2. The influence of Bacto agar on water potential (-bars) and subsequent germination of Glomus fasciculatus spores.

Bacto Agar Added (%)	Media Used					
	A(-FERT)		B(-P)		C(+P)	
	% Germination[a]	-Bars	% Germination	-Bars	% Germination	-Bars
0.8	5.0 ± 1.8[b]	0.79	6.3 ± 2.1	1.41	7.5 ± 1.3	1.64
1.0	3.8 ± 2.1	0.59	6.3 ± 4.1	1.85	8.8 ± 2.7	1.89
1.5	3.8 ± 1.1	0.69	8.8 ± 1.1	1.93	8.8 ± 3.7	1.96
2.0	6.3 ± 2.1	0.97	6.3 ± 3.2	1.72	6.3 ± 2.1	1.68
2.5	3.8 ± 2.1	0.73	6.3 ± 2.7	1.69	5.0 ± 1.8	1.64
3.0	5.0 ± 1.8	0.68	8.8 ± 2.1	1.28	5.0 ± 1.8	1.39
3.5	6.3 ± 1.1	0.65	10.0 ± 3.1	1.88	6.3 ± 2.1	1.08
4.0	7.5 ± 1.3	1.20	10.0 ± 3.1	2.07	12.5 ± 3.8	2.23

[a]Values represent the mean of 4 plates each containing 30 spores.

[b]Standard Error of the Mean

-FERT = water agar
-P = without phosphorus
+P = with phosphorus

significant effect on germination of G. fasciculatus spores (Table 3). The amount of germination was consistant across various concentrations and times of collection of exudates. Similar results were obtained in a replicate experiment.

D. Influence of zearalenone on germination

The addition of zearalenone to ROC agar had no effect on water potential. However, it did inhibit spore germination (Table 4). Germination tended to decrease gradually with increased zearalenone concentration. This experiment was repeated and similar results were obtained.

Table 3. The influence of root exudates from Trifolium repens on germination of Glomus fasciculatus spores on agar.

Exudate Solution Added (ml)[a]		Exudates Collected Over Time % Germination		
		Week 2	Week 4	Week 6
Exd-P	0.0[b]	43	43	43
	0.0[c]	47	47	47
	0.1	40	43	50
	1.0	47	53	43
	2.0	63	43	43
	4.0	53	47	57
	8.0	43	53	50
Exd+P	0.0	43	43	43
	0.0	47	47	47
	0.1	43	40	47
	1.0	53	57	43
	2.0	17	47	37
	4.0	57	50	53
	8.0	43	43	50

aTaken directly from staining jars containing 50 ml sterile water in which 50 intact seedling root systems where allowed to exude for a 24hr period. Exudate solution was added to agar to bring it to 10 ml total volume.
bSterile water agar
cRoot organ culture agar
Exd-P=Exudates from P-deficient plants
Exd+P=Exudates from non-deficient plants

Table 4. The influence of zearalenone on water potential (-bars) and subsequent germination of Glomus fasciculatus sprores.

Zearalanone Added (ppm)	Media Used					
	A (-FERT)		B (-P)		C (+P)	
	% Germination[a]	-Bars	% Germination	-Bars	% Germination	-Bars
0(H_2O)	0.9 ± 0.8[b]	0.81	0.9 ± 0.8	2.53	2.7 ± 1.5	2.67
0(EtOH)	7.9 ± 3.0	0.56	2.7 ± 1.4	2.60	1.1 ± 0.9	2.53
1	3.1 ± 1.1	0.60	3.1 ± 1.8	2.40	8.8 ± 1.2	3.30
10	5.8 ± 1.7	0.64	1.7 ± 1.5	3.20	3.6 ± 2.0	3.60
100	2.0 ± 1.8	0.67	0.0 ± 0.0	2.40	0.0 ± 0.0	2.53
500	0.0 ± 0.0	0.51	0.0 ± 0.0	2.13	0.0 ± 0.0	2.53
1000	0.0 ± 0.0	0.53	0.0 ± 0.0	2.40	0.0 ± 0.0	2.13
5000	3.3 ± 3.0	0.80	0.0 ± 0.0	2.53	1.1 ± 1.0	2.40

[a] Values represent the mean of 5 plates each containing 20 spores.

[b] Standard Error of the Mean

-FERT = water agar
-P = without phosphorus
+P = with phosphorus

Discussion

A. Influence of storage solution over time on germination

The procedures involved in VAM spore isolation are very labor-intensive. It would be beneficial if large numbers of viable spores could be isolated at one time for storage. In this study, I was not able to demonstrate a storage solution which, over a period of several months, consistently maintained a high level of spore viability (Appendix 8). Both *G. margarita* and *G. fasciculatus* spores were still viable after 12 weeks in storage but there was a great deal of variation in their germination. In the case of *G. fasciculatus*, streptomycin/gentamicin was significantly better than the other storage solutions ($P<0.001$), while, conversely this same treatment had no significant effect on viability of *G. margarita*. The *G. mosseae* inoculum used was quite old and this factor alone may explain the poor germination observed at the outset and during the experiment.

It is well established that high spore germination can occur on agar media (Godfrey,1957; Mosse,1959b; Hepper and Smith,1976). I did not observe this in my studies so I decided to evaluate the effects of several additional physical and chemical factors on VAM spore germination.

Because of the inconsistent and poor results in this study all other experiments were conducted with freshly isolated spores of *G. fasciculatus*, because of its fairly

high germination rate and the extremely high spore populations (-50/gm) produced in the soil.

B. Influence of water potential on germination

Germination of G. fasciculatus was the highest at water potentials of -4.0 to -6.0 bars. Germination was severely decreased at water potentials above and below this narrow optimum range, which indicated that germination of G. fasciculatus is sensitive to excessive and deficent moisture levels common in the soil environment.

The inability of G. fasciculatus spores to germinate at high or low water potentials could possibly be important in the regulation of long term survival. In addition, this optimum water potential range could be utilized in studies involved with the eventual culture of VAM fungi. The use of Bacto agar, because it doesn't affect water potential, and PEG 8000, because it prevents agar from solidifying, are inappropriate if one wishes to take advantage of the water availability parameter. However, sorbitol did not have these disadvantages and moreover, is not a substrate of VAM fungi.

None of these observations on the influence of water availability on VAM germination are unexpected since similar germination curves have been reported for many fungi (Cook and Papendick,1970; Adebayo and Harris,1971).

C. Influence of root exudates on germination

In 1982 Graham observed that exudates from both citrus and sudangrass had a stimulatory effect on VAM spore germination. However, this was based on an experiment in which root exudates were collected under non-sterile conditions so in reality the root exudates were a mixture of both root exudates, microbes, and microbial metabolites. Because of this, it is impossible to determine the origin of the compounds that did in fact stimulate spore germination.

In my study, exudates, whether from phosphorus deficient or non-deficient plants, had no effect on the germination of VAM fungal spores. This subject has been recently discussed by Bowen (1984) who concluded that it is fairly well accepted that root exudates have little influence on stimulating VAM spore germination. This is not to say that exudates have no role in other phases of the VAM symbiosis, just that they play no part in germination. The possible role of root exudates in other phases of the VAM symbiosis will be addressed in Chapter 2.

D. Influence of zearalenone on germination

Work done by Wolf and Mirocha (1973) lead them to conclude that zearalenone was a sex-regulating hormone active in regulating the sexual stages of Fusarium roseum. It has hormonal activity in the sense that (1) it is produced by the organism it affects, (2) it is concentration -dependent since it is stimulatory at a low concentration

and inhibitory at a higher concentration, (3) its action is time dependent and (4) it is produced at the time of perithecial initiation. Zearalenone has also been shown to regulate sexual reproduction in a variety of other fungi (Mirocha et al.,1974).

In addition, Azcon et al. (1981) have shown the inhibitory effects of ethylene on germination and stimulatory effects of auxins, gibberellins and cytokinins on mycorrhizal hyphal growth.

With all this in mind, it is possible that zearalenone could have an effect on one or several stages of mycorrhizal development. However, the only effect observed in my studies was a significant inhibition of spore germination. Because of this inhibitory effect on germination and since there was no hyphal growth in the controls, no data could be collected to quantify any additional effects of the hormone.

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Chapter 2. VAM HYPHAL ELONGATION STUDIES

Literature Review

In 1904 Hiltner was the first to report that microbial populations were higher in the rhizosphere (soil closest to plant roots) as compared to soil outside of this zone. This phenomenon is referred to as "the rhizosphere effect". Many scientists speculated that organic substances in root exudates were wholly or partially responsible for this increase in the microbial populations around plant roots (Knudson,1920). Since that time there has been strong evidence to support the theory that root exudates have a large influence on soil microorganisms. (Lyon and Wilson,1921; Rovira,1956b,1965,1969a; Schroth and Hildebrand,1964; Balasubramanian and Rangaswami,1969; Hale et al.,1971).

A. The Influence of Exudation on VAM Fungi

Several studies have been conducted to determine the influence of plant root exudates on VAM colonization (Menge et al.,1978; Ratnayake et al.,1978; Bowen and Theodorou,1979; Jasper et al.,1979; Azcon and Ocampo,1981; Graham et al.,1981; Ferguson and Menge,1982; Graham,1982; Johnson et al., 1982a,1982b; McCool and Menge,1983).

Specificity

Very little is known about the recognition phenomena in VAM associations or the reasons for the lack of specificity between the fungi and plants involved. The lack of infection of Chenepodiaceae and Brassicaceae could be due to any number of reasons ranging from a physical barrier of the cell wall, to an absence of essential nutrients, to production of toxins by the plant (Bowen,1984). However, a lack of nutrients is not likely to be the major factor since VAM fungi can grow slightly in the rhizospheres of nonhost plants (Ocampo et al.,1980). In addition, Graham (1982) reported increased spore germination, germ tube growth and hyphal branching of Glomus epigaeum spores in vitro after exposure to either sudangrass or citrus root exudates. However, it should be noted that those root exudates were not collected from aseptically grown plants. Thus, few conclusions can be drawn about the influence of plant root exudates specifically. These findings are similar to those of Odunfa (1978) and Schroth and Snyder (1961) who reported a stimulation of conidial germination with Fusarium species after exposure to root exudates of either cowpea, sorghum or pinto bean.

In sharp contrast, there is well documented evidence of specificity of root exudates towards other soil microorganisms. The symbiotic association between a Rhizobium species and its legume host is a clear example of

specificity. Robinson (1967) has shown that in a field soil supporting a mixed pasture of subterranean clover and lucerne, clover stimulated R. trifolii but not R. meliloti, while lucerne stimulated both species in its rhizosphere. Robinson suggested that this selective stimulation may be due to a direct effect of the exudates from the legume roots, or it may be that clover root exudates stimulated a microflora which is antagonistic to R. meliloti. Investigations by Zentmyer (1961), confirming this specificity phenomenon, showed that Phytophthora cinnamomi zoospores were attracted to the zone immediately behind the root tips of avacado, a host and not mandarin orange roots, a nonhost.

Plant Species

Schwab et al. (1984) investigated the differences in exudation pattern of three plant species which normally form VAM associations as compared to three plant species which normally do not form VAM associations. They observed that normally mycorrhizal plants generally leak larger quantities of compounds than nonmycorrhizal plant species. However, no specific reducing sugars or amino acids were present in the exudates from mycorrhizal versus nonmycorrhizal plant species. Previously, Hale et al. (1971) reviewed the subject of the influence of plant species on root exudation.

Plant Age

The stage of plant development can have a pronounced effect on root exudation (Rovira, 1959). For example, during early flower bud development there is mobilization of metabolites to the developing bud (Wedding et al.,1978), consequently fewer metabolites are available in the root for exudation. With this in mind, Johnson et al. (1982a) found that VAM formation was reduced during early stages of flower bud development but not during later stages of bud expansion. This was thought to result from decreased exudation of sugars and amino acids during early bud development. One of the first reports on root exudates as affected by plant age (Rovira,1956a) showed more amino acids and sugars exuded during the first 10 days of growth than during the second 10 days. In agreement with this, Balasubramanian and Rangaswami (1969) found that exudation from roots of sorghum, sunnhemp, ragi and tomato was greater during the first two weeks of growth than later.

Light

Johnson et al. (1982b) examined the effect of photoperiod on VAM formation. A longer daylength stimulated more plant growth and, subsequently, increased root exudation. The opposite was observed for short daylength, Johnson et al. (1982b) found that mycorrhizal formation was greatest under long-day photoperiods where exudation was greatest. In agreement with these findings, Ferguson and

Menge (1982) showed that high levels of root exudation in sudangrass were brought on by high light intensities and were associated with increased VAM infection and spore production. Previously, Rovira (1959) had shown that the light intensity under which plants were grown had a great deal of influence on the quantity and quality of compounds contained in root exudates. Both tomato and clover plants grown under high light intensity exuded higher quantities of certain amino acids than plants grown under 60% shade. He suggested that the effects of light on photosynthesis and translocation were responsible.

Ozone exposure is said to damage chloroplast membranes, thus reducing photosynthetic efficiency, and, in turn, carbohydrate availability. In addition, photosynthates are retained in injured leaves in order to supply energy for repairs (Koziol and Jordan,1978). Thus, there should be fewer carbohydrates available for translocation to the roots. McCool and Menge (1983) found that altered carbon partitioning does occur in tomato plants exposed to air pollutants and that certain tissues (roots) may suffer at the expense of other metabolic sinks. They suggested that reduced exudation may delay the establishment of a VAM symbiosis.

Bååth and Hayman (1983) found decreased VAM infection in tomato plants with Verticillium wilt. They suggested that, since Verticillium wilt decreases photosythetic

efficiency of the leaves (Selman and Pegg,1957), root exudation was decreased resulting in decreased VAM infection.

Phosphorus

Soil phosphorus (P) has received a great deal of study because it is probably the factor which most markedly affects VAM formation (Mosse,1972,1973a,1973b; Sanders,1975; Menge,1978). Jasper et al. (1979) found that application of phosphorus to soil depressed VAM formation by increasing plant phosphorus status, and not by direct effects of increased soil phosphorus. This conclusion is in agreement with a study by Sanders (1975). His experiments, involving foliar application of P to onions, showed that P supply regulates VAM infection through effects on plant P status. Menge et al. (1978), using a split-plate technique, observed this same phenomenon.

The mechanism by which P suppresses VAM infection is not clear. Ratnayake et al. (1978) found that reducing sugars and soluble amino acids were increased in P deficient roots and total root exudation increased as well. Leakiness of roots decreased rapidly as internal plant P increased. A threshold response for exudation was seen below 0.034% dry weight of root P in sudangrass and 0.068% root P in sour orange. At root P above these levels exudation of metabolites and ions was low, and declined slightly with increasing root P. Lipid P was inversely related to

leakiness, providing further evidence that membrane dysfunction is a result of low plant P. More recent studies have confirmed these results. Graham et al. (1981) showed that the rates of exudation are directly related to fundamental changes in root membrane permeability which is controlled by P.

Jasper et al. (1979) suggested that higher VAM infection in P-deficient clover was correlated with an increase in the soluble carbohydrate content of roots. However, Graham et al. (1981) found low levels of sugars in roots of P deficient sudangrass. They suggested that the dramatic increase in VAM infection at low root P status was due to an increase in membrane-mediated root exudation rather than to higher sugar and amino acid concentrations in the root. This is supported by a report from Bowen (1969) who grew Pinus radiata seedlings under phosphorus-deficient, nitrogen-deficient or complete nutrient solutions and then monitored the quantity and quality of amino acids in root exudates. He saw increased exudation by phosphorus or nitrogen deficient plants over the controls.

Until recently, most investigations had led scientists to conclude that P deficiency increases root exudation and that the quantity of exudates leaked into the rhizosphere increases VAM infection. However, Schwab et al. (1983b) found that there was no qualitative difference in the exudates from P deficient as compared to non-deficient

plants. These reults, however, do not eliminate the possibility that some unknown growth factor is a constituent of the root exudates of P-deficient plants (Bowen,1984). It is possible that this factor could be produced at the onset of P deficiency. This unknown factor, if it exists, has never been observed because experiments have not been designed to demonstrate it. For example, Ratnayake et al. (1978) collected exudates only once from 8-10 week old sudangrass or sour orange seedlings. Graham et al. (1981) collected exudates only once from 7-8 week old sudangrass seedlings. Schwab et al. (1983b,1984) collected exudates only once from 6 week old sudangrass and other seedlings. Since Rovira (1956a) has shown that root exudation decreases dramatically with age, a plant grown for 2 months without an external source of P is probably near death. If a VAM growth factor is produced at all, it should be looked for at the beginning of P deprivation and, since it is probably transient, its presence should be monitored over a time period of several weeks. To my knowledge this has never been attempted.

B. Axenic Culture of VAM Fungi

The fungal species that form vesicular-arbuscular mycorrhizae (VAM) in many plant roots are obligate symbionts, hence attempts to culture them have meet with varying degrees of success. Mosse (1962) was the first to establish VAM in monoaxenic cultures of clover seedlings in

an inorganic salt medium (Jensen,1942). However, root infection by the fungus (an unspecified Endogone sp.) could only be obtained on agar when a bacterium (Pseudomonas sp.) was present, or by including bacterial culture filtrates, EDTA or pectinase. Mosse and Phillips (1971) further modified the growth conditions by eliminating the bacterium and by manipulating the phosphorus amounts and sources. Ca-phytate in conjunction with plant macronutrients appeared to give the most intense infection and external hyphal growth.

The first report of establishment of VAM infections in root-organ cultures (Mosse and Hepper,1975) involved clover roots grown in a modified White's growth medium with Ca-phytate as the P source. VAM development was enhanced by using a pressure cooker for medium sterilization as opposed to an autoclave. Allen et al. (1979) noted that filter sterilization of Ca-phytate enhanced VAM establishment perhaps as a consequence of reduced mineralization of P. Allen et al. (1981) also found that plants grown in the Ca-phytate medium not only had higher infection levels but had lower internal P concentrations than plants grown with inorganic P. Thus, they suggested that in a high nutrient regime the use of a complex organic P source such as phytate may be beneficial to infection establishment.

Several recent studies have indicated that other complex P sources (Hepper,1981) or a continuous flow of a dilute nutrient solution (MacDonald,1981) can result in good

mycorrhizal establishment. Hepper (1981) showed that VAM infection could be initiated and maintained on agar slants, moistened filter paper or Fahraeus slides. The medium she used was adjusted to pH 6.8, contained micro- and macronutrients, but used bone meal as the P source. MacDonald (1981) established mycorrhizae on agar-coated slides maintained in hydroponic culture chambers. A defined dilute nutrient solution was allowed to drip over the mycorrhiza which improved aeration. Cultures could be maintained for as long as 3 to 4 months with periodic replacement of the nutrient solution.

In spite of these reported successes at limited growth in culture of VAM, a lack of reproducibility has left scientists without a standard method for generating either the endophyte in axenic culture or pure two-member cultures (the endophyte and an infected host plant).

Until VAM can be established reliably in axenic cultures it will be difficult to learn more about the biochemistry, physiology or genetics of VAM fungi. However, the VAM/root-organ culture system (Hepper and Mosse,1980) can still be a valuable tool in elucidating information about steps in the pre-infection phase of endophyte/host plant interactions.

The objective of this study was to determine the effects of root exudates, produced by plants experiencing phosphorus deprivation, on VAM hyphal elongation.

Materials and Methods

A. Exudate and Extract Studies

White clover (Trifolium repens L. cv `Ladino') seeds were surface sterilized with 70% ethyl alcohol for 30 seconds followed by 0.1% $HgCl_2$ in 1mM HCl for 5-7 minutes and washed with sterile distilled water (Appendix 6). The seeds were then placed on moist filter paper in a sterile petri dish and incubated at 23 C in the dark for 2 days to allow for germination. Fifty seedlings were then taken from each petri dish and placed on moist cheesecloth in sterilized square glass staining dishes (Figure 1) which contained 100ml of sterilized Hoagland's nutrient solution (Appendix 7), with phosphorus (+P) or without phosphorus (-P). The dishes were then enclosed in sterile clear plastic bags, tightly sealed and the seedlings grown under 16 hour daylength fluorescent lights (4.5 Klux).

The Hoagland's solution was replaced every 7 days. Each treatment (+P,-P) consisted of 6 replicate staining dishes which contained fifty seedlings. Parallel samples (5 replicate plants/treatment) were taken at weekly intervals for 6 weeks to measure growth of whole plants, root systems and root tissue phosphorus depletion rates (Appendix 13).

During initial trials of this procedure Gentamicine (0.5mg/100ml nutrient solution) or streptomycin (1.0mg/100ml nutrient solution) was added to the Hoagland's nutrient solution with or without phosphorus. Streptomycin or

gentamicine was also added each time the nutrient solution was replaced. Each initial treatment consisted of 2 replicate staining dishes each of which contained fifty seedlings. Five plants were harvested from each treatment at weekly intervals to monitor growth of the whole plant, the root system and root phosphorus content (Appendix 13). At 2, 4 and 6 weeks exudates were collected from the roots of these clover seedlings. Briefly, at the end of each 2 week period, all plants were taken out of the nutrient solution, rinsed with sterile distilled water several times and then placed in sterile distilled water for a 24 hour period. The relative conductivities of the exudates from each treatmemt were separately measured immediately after collection. The pooled exudates within a treatment were filter sterilized (0.45um mesh), rotory evaporated at 40 C to 1/10 original volume, filter sterilized again and stored at 4 C. This was repeated at 4 and 6 weeks. Contamination checks were done once a week by plating out spent nutrient solution on water agar and PDA at the time of nutrient solution replacement. No contamination was observed. After the final exudate collection at 6 weeks, all root systems from each treatment were weighed (fw) and homogenized in 100ml glass distilled water. The mixture was then passed through a Nalgene filter (0.45um mesh) to remove root tissue and to sterilize the aqueous extracts.

Root exudates kor extracts from phosphorus deficient

and non-deficient plants at 2, 4 and 6 weeks were added to root-organ culture (ROC) agar (Hepper and Mosse,1980; Appendix 5) at a range of concentrations from 0.0 to 8.0ml exudate or extract solution per 10ml total volume of media. Sorbitol was added to attain the optimum water potential of -5 bars for germination of G. fasciculatus spores, as determined in a previous experiment. At the same time T. repens roots, propagated from root tips (Appendix 11), were grown on phosphorus deficient ROC agar. Uniform 1 cm long root segments were placed singly on plates for exudate or extract treatment. On plates with root-organs, G. fasciculatus spores (30/plate) were placed in close proximity to the root segment. G. fasciculatus spores (30/plate) also were placed on plates amended with root exudate or extract, without roots. All plates were incubated at 25-27 C in the dark. Germination and hyphal elongation were monitored at 5 day intervals. Germination is defined as a germ tube that is at least twice the diameter of the spore. Hyphal elongation levels are the mean hyphal length of only those spores that germinated. Colonization of root-organs was checked by clearing and then staining (Appendix 12) the root organs at the end of the experiment (40 days after inoculation). This experiment was performed twice.

B. Root-Organ Culture

White clover (*Trifolium repens* cv `Ladino') seeds were surface-sterilized with 70% ethyl alcohol for 30 seconds, placed in 0.1% $HgCl_2$ in 1mM HCl for 5-7 minutes, and then washed with sterile distilled water (Appendix 6). The seeds were placed on moist filter paper in a sterile petri dish and incubated at 23 C in the dark for 2 days to allow for germination. Root tips of uniform length were transferred to ROC agar with phosphorus (+P) or without phosphorus (-P). Root-organs were transfered to fresh media every 10 days. At weekly intervals root tissue was harvested to monitor tissue P levels (Appendix 13).

Statistical analysis by analysis of variance (ANOVA) or standard error of the mean (SEM) was conducted where appropriate and stated in the results.

Results

A. Exudate and Extract Studies

Influence of Antibiotics

Trifolium repens cv `Ladino' seedlings grown with the addition of either gentamicin or streptomycin to the nutrient solution were severely effected. Even though both antibiotics were added at very low concentrations after only three weeks of growth those plants which received streptomycin began to senesce (Table 5). Plants which received Gentamicin began to senesce after 4 weeks. By the end of the experiment (5 weeks) only the +P and -P controls were still alive. This phytotoxicity problem plus the fact that there were no contamination problems, makes it apparent that the addition of streptomycin, gentamicin or any other antimicrobial agent is an unnecessary precaution. Figure 10 shows the effect of phosphorus treatment on growth of the control plants. The non-deficient plants grew faster than the P-deprived plants. Note the rapid growth of the P-deprived root systems while growth of P-nondeficient plant root systems leveled off, however, root growth lagged for the first 2 to 3 weeks possibly because the plants were not P-deficient at that time. Figure 11 shows the rate of depletion of phosphorus in root tissue over time. Note that there was no change in the P concentration in plants which were provided a P source while the P concentrations of the P-deprived plants rapidly declined and eventually leveled

Table 5. The influence of antibiotics on growth of Trifolium repens.

TIME In Weeks	Media Used					
	-P			+P		
	A	B	C	A	B	C
1 Whole Plant DW(gms)[a]	0.5 ± 0.0[b]	0.5 ± 0.1	0.4 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.0
Root System DW(gms)	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Root %P (DW)	0.63 ± 0.11	1.09 ± 0.26	1.50 ± 0.22	0.53 ± 0.07	0.85 ± 0.19	0.51 ± 0.20
2 Whole Plant DW	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	0.9 ± 0.1	1.1 ± 0.2	0.6 ± 0.2
Root System DW	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0
Root %P (DW)	0.58 ± 0.13	0.47 ± 0.07	1.10 ± 0.50	0.57 ± 0.18	0.51 ± 0.17	0.28 ± 0.07
3 Whole Plant DW	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	1.3 ± 0.2	2.0 ± 0.0	-
Root System DW	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	-
Root %P (DW)	0.45 ± 0.14	0.92 ± 0.21	0.36 ± 0.09	0.59 ± 0.13	0.89 ± 0.00	-
4 Whole Plant DW	2.0 ± 0.3	1.4 ± 0.1	1.1 ± 0.2	2.3 ± 0.2	-	-
Root System DW	0.6 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	-	-
Root %P (DW)	0.14 ± 0.00	0.57 ± 0.12	0.86 ± 0.25	0.52 ± 0.15	-	-
5 Whole Plant DW	2.9 ± 0.4	-	-	3.3 ± 0.7	-	-
Root System	0.7 ± 0.1	-	-	0.3 ± 0.1	-	-
Root %P (DW)	0.17 ± 0.02	-	-	0.42 ± 0.09	-	-

[a] Values represent the mean of 5 dishes (50 plants/dish)

[b] Standard Error of the Mean

A = Hoagland's
B = Hoagland's + gentamicin
C = Hoagland's + streptomycin
- = No data collected

-FERT = water agar
-P = without phosphorus
+P = with phosphorus

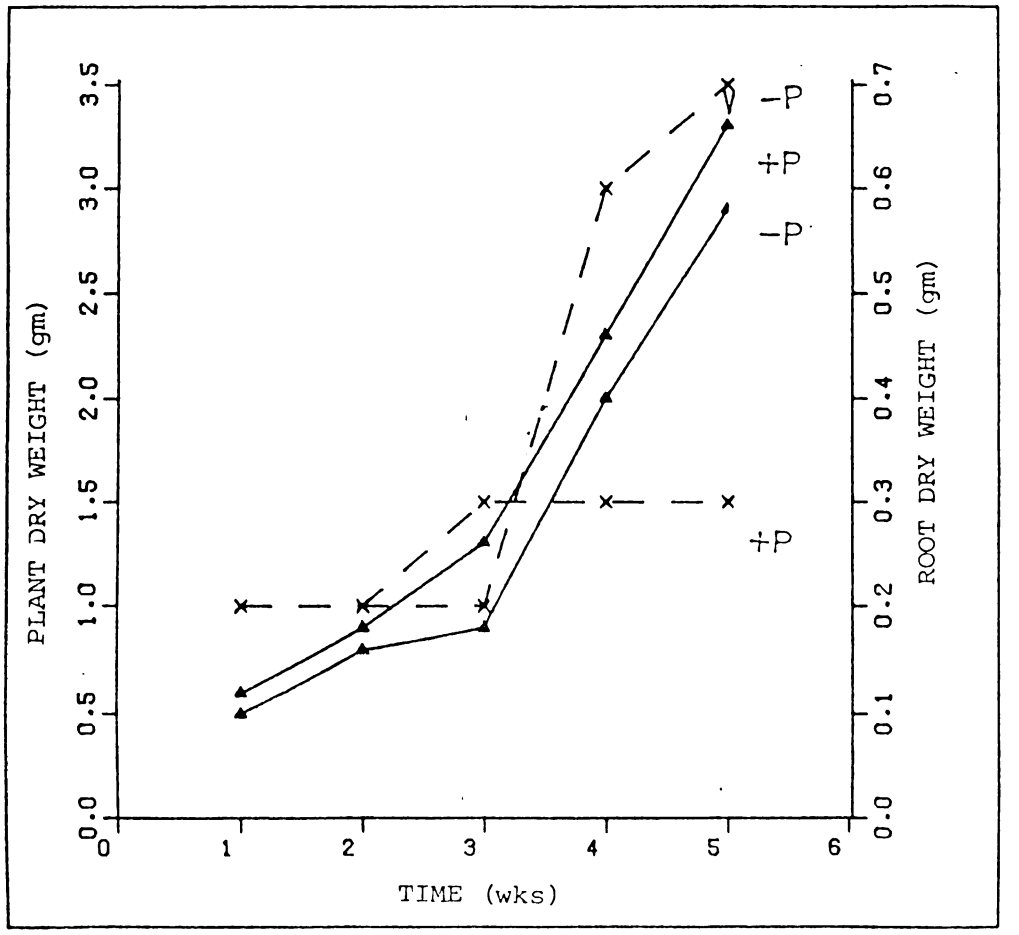

Figure 10. The effect of phosphorus nutrition on Trifolium repens whole plant and root growth in root-organ culture medium. x---x = dry weight (gm), ▲——▲ = whole plant dry weight(gm), +P = with phosphorus, -P = without phosphorus. Each point is the mean of 5 replicate whole plants or root systems.

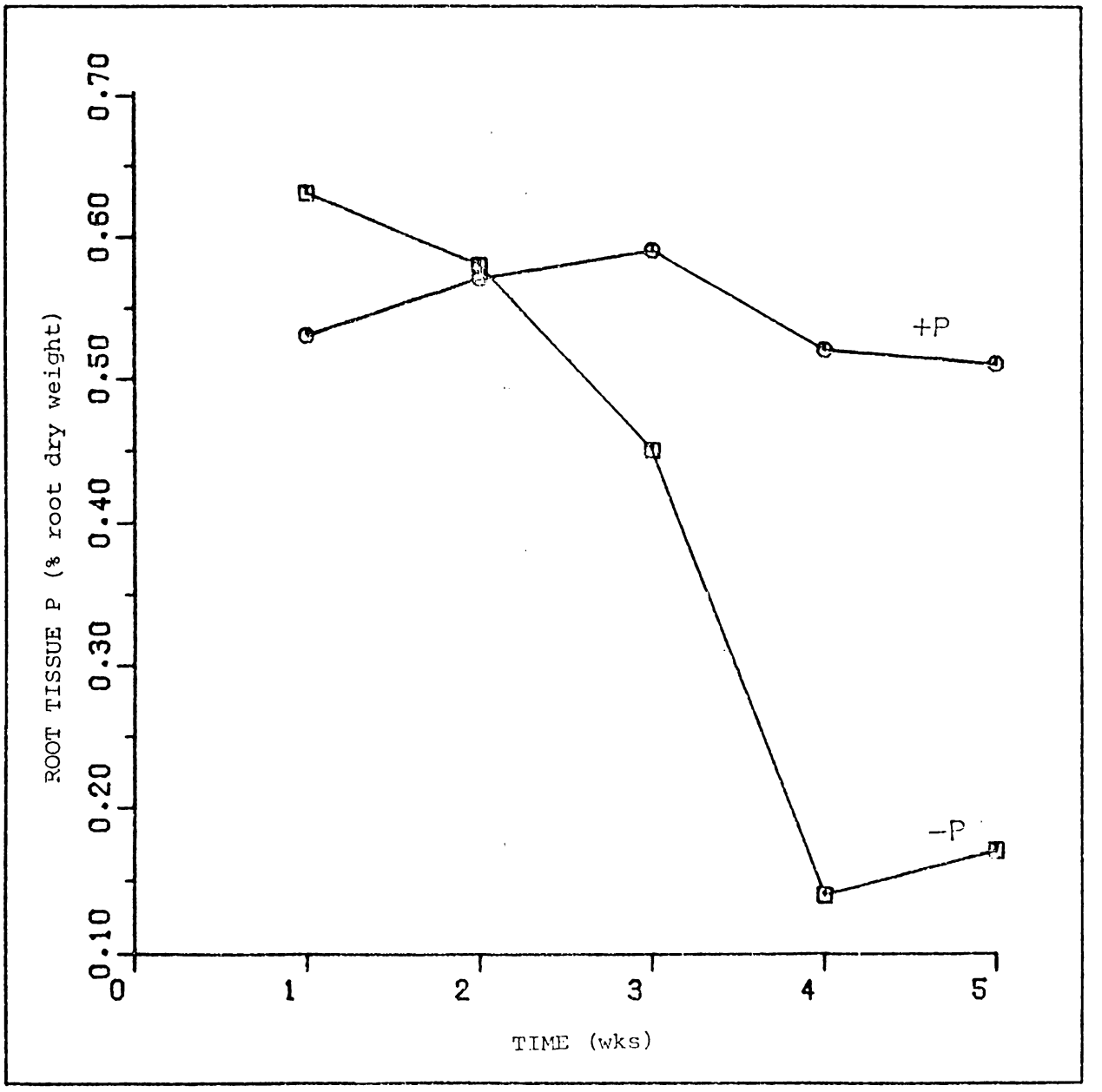

Figure 11. Depletion of phosphorus from Trifolium repens root tissue of plants grown on root-organ culture medium. +P = with phosphorus, -P = without phosphorus. Each point is the mean of 5 root systems selected randomly from dishes each containing 50 plants.

off at 4 weeks to a threshold level (Epstein,1972).

Parallel Sample Data

Figure 12 shows the growth of +P and -P root systems and the depletion of phosphorus from roots over time. The plants grown in a nutrient solution without phosphorus were quickly depleted of root P down to the critical level (0.13%,Epstein,1972) within 4 weeks while plants that received P maintained a somewhat constant root P concentration. These -P plants also developed more extensive root systems as compared with the +P treatment plants.

Conductivity Measurements

The relative conductivity of the root exudates collected from plants grown with and without phosphorus decreased slightly over time (Figure 13). However, only exudates collected at 6 weeks from plants grown with phosphorus had a significantly lower relative conductivity. Similar results were obtained in a replicate experiment.

Hyphal Elongation

Exudates from plants grown under P deprivation stimulated greater hyphal elongation than exudates from plants grown with a P source (Figure 14 and 15). Exudates collected at 2 weeks were more stimulatory to hyphal elongation than exudates collected at 4 or 6 weeks. The control treatments were not stimulatory to hyphal elongation.

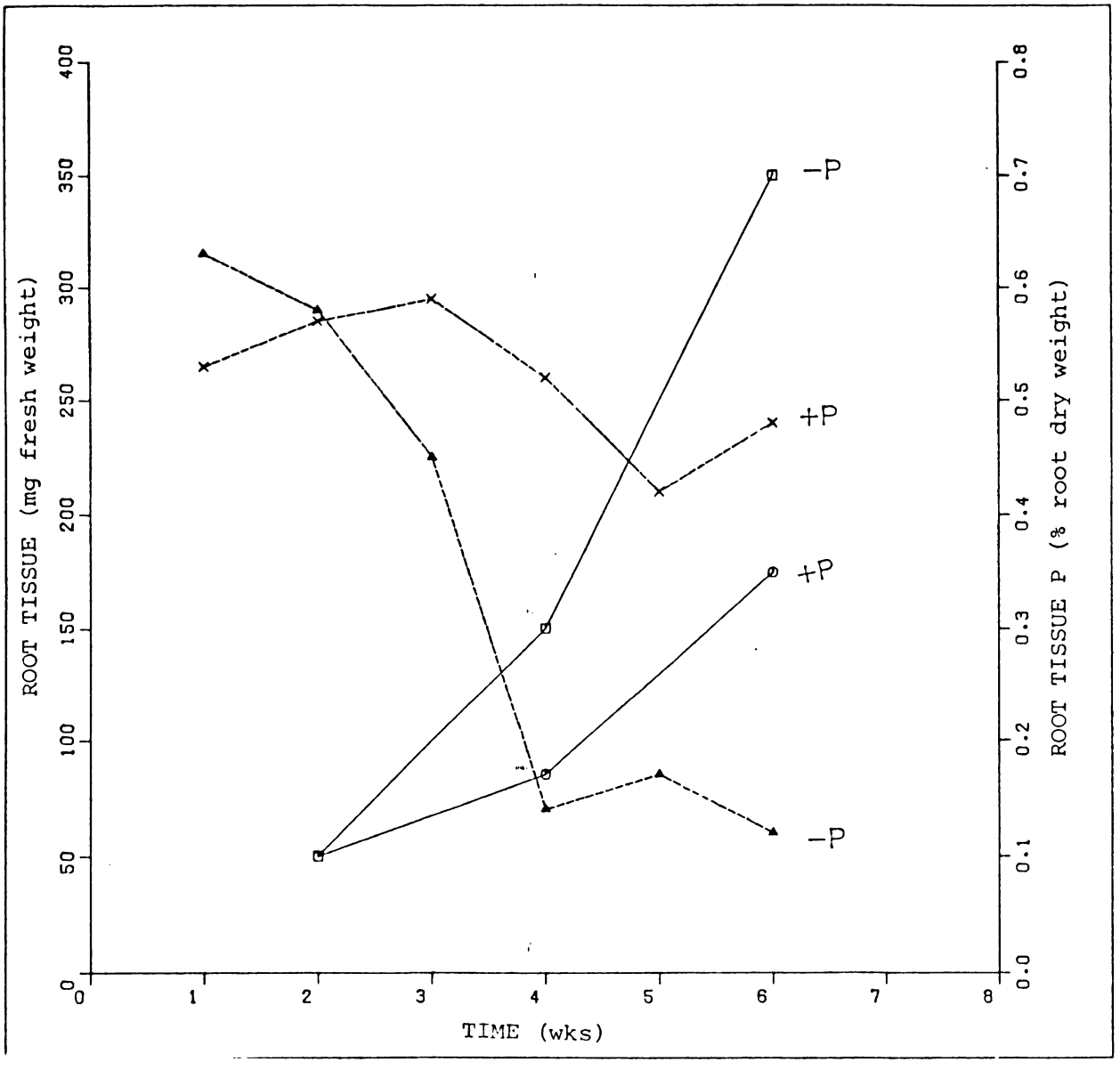

Figure 12. The effect of phosphorus nutrition of Trifolium repens root growth and root phosphorus depletion. o---o = total dry weight(mg) of roots at the time of exudate collection, x——x = root tissue phosphorus on a % dry weight basis, +P = with added phosphorus, -P = without added phosphorus. Root tissue phosphorus is expressed on the mean phosphorus level(% dw of root tissue) of 5 root systems selected randomly from dishes each containing 50 plants.

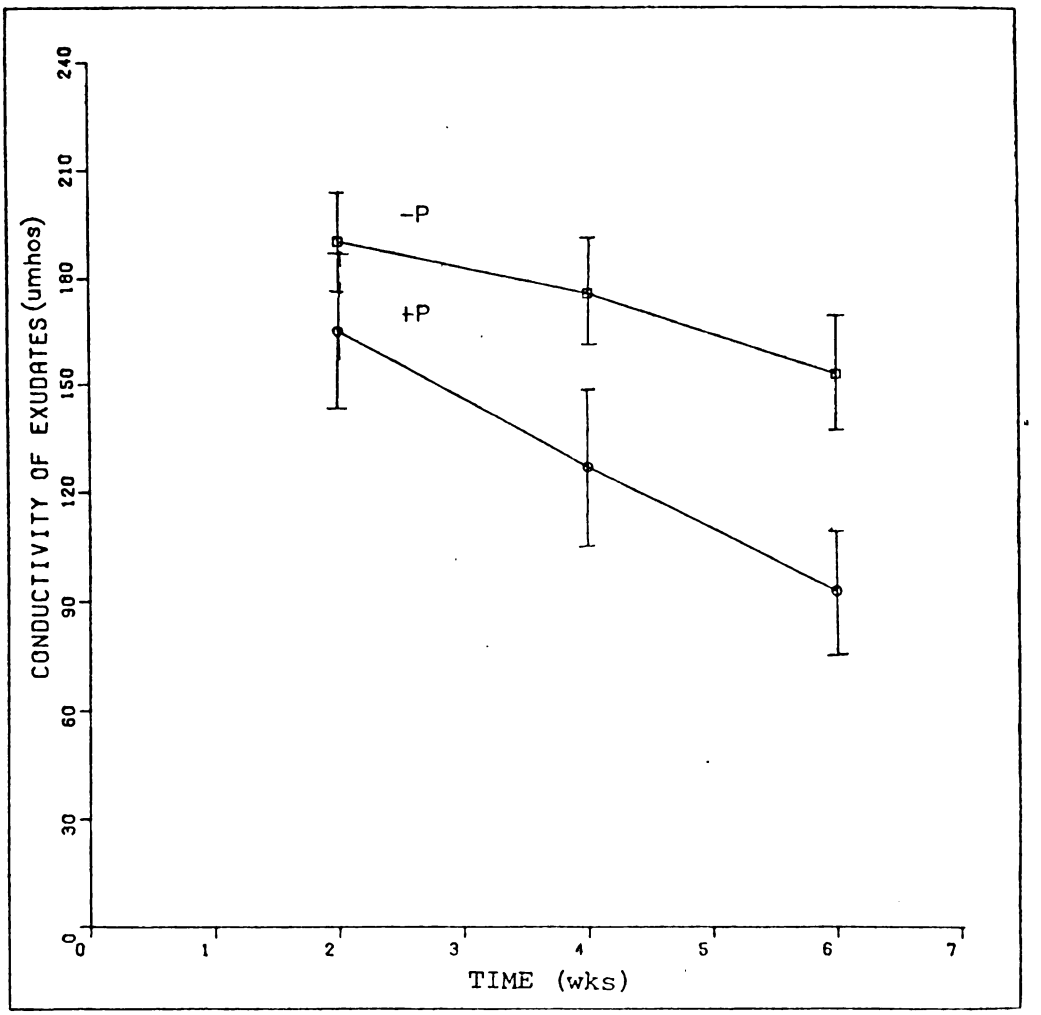

Figure 13. The relative conductivity of exudates from Triflium repens plants grown with or without phosphorus over a 6 week period. -P = without phosphorus, +P = with phosphorus. Each point is the mean of 5 dishes in which exudates were collected over a 24 hour incubation.

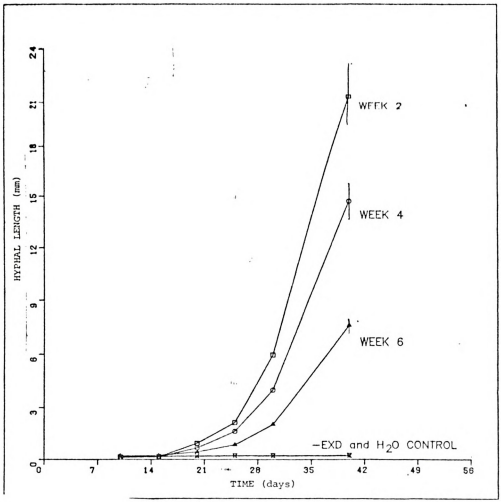

Figure 14. The influence of exudates collected from 2,4, and 6 week-old Trifolium repens seedlings deprived of phosphorus on hyphal elongation of Glomus fasciculatus spores. Each point is the mean of all spores that germinated.

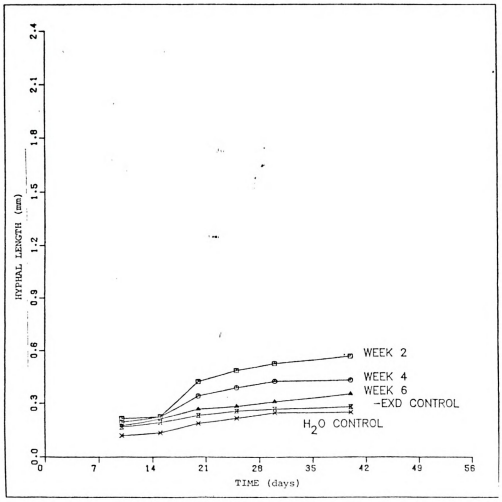

Figure 15. The influence of exudates collected from 2,4, and 6 week-old Trifolium repens seedlings supplied with phosphorus, on hyphal elongation of Glomus fasciculatus spores. Each point is the mean of all spores that germinated.

All exudate solutions more concentrated than 1.0ml exudate solution/10ml total volume of media had no stimulatory effects on hyphal elongation. Only the 0.1 and 1.0ml exudate amounts from P deprived plants stimulated hyphal elongation while the controls had no effect. The extracts from plants grown without phosphorus at 1.0ml extract solution per 10ml total volume of media treatment also stimulated hyphal elongation (Table 5).

Root-organs placed on the agar with VAM spores had no effect on hyphal elongation and none were colonized by VAM fungi. Similar results were obtained in a replicate experiment.

B. Root-Organ Culture

Phosphorus was depleted from the root-organs in a very short time period (Figure 16). The critical level for phosphorus (0.13%,Epstein,1972) was reached by root-organs grown in ROC agar without phosphorus after approximately 3 weeks. However, the root-organs grown on ROC agar with phosphorus lost some phosphorus but then the concentration leveled off to roughly 0.50% root P/gm root tissue (on a dry weight basis).

Table 6. The influence of exudates and extracts of Trifolium repens on VAM hyphal elongation over time.

Treatment[a] and Age of Seedlings[b]	Mean Hyphal Length (mm)[c]
EXD-P	
Week 2	21.42 ± 1.93
Week 4	14.72 ± 1.03
Week 6	7.62 ± 0.37
EXD+P	
Week 2	0.57 ± 0.26
Week 4	0.43 ± 0.30
Week 6	0.35 ± 0.40
EXT-P	18.73 ± 1.75
EXT+P	0.54 ± 0.37
Controls	
H_2O	0.25 ± 0.10
-EXD or -EXT	0.28 ± 0.20

[a] Exudates were taken from staining jars containing 50 ml sterile water in which 50 intact seedling root systems were allowed to incubate for a 24 hour period.

[b] The age of seedlings when exudates or extracts were collected.

[c] Values represent the mean hyphal length of germinated spores after 40 days.

EXD-P = Exudates from P-deprived plants(0.1ml)
EXD+P = Exudates from nondeficient plants(0.1ml)
EXT-P = Extracts from P-deprived plants(0.1ml)
EXT+P = Extracts from nondeficient plants(0.1ml)
H_2O = Sterile water control
-EXD or -EXT = No exudate or extract control

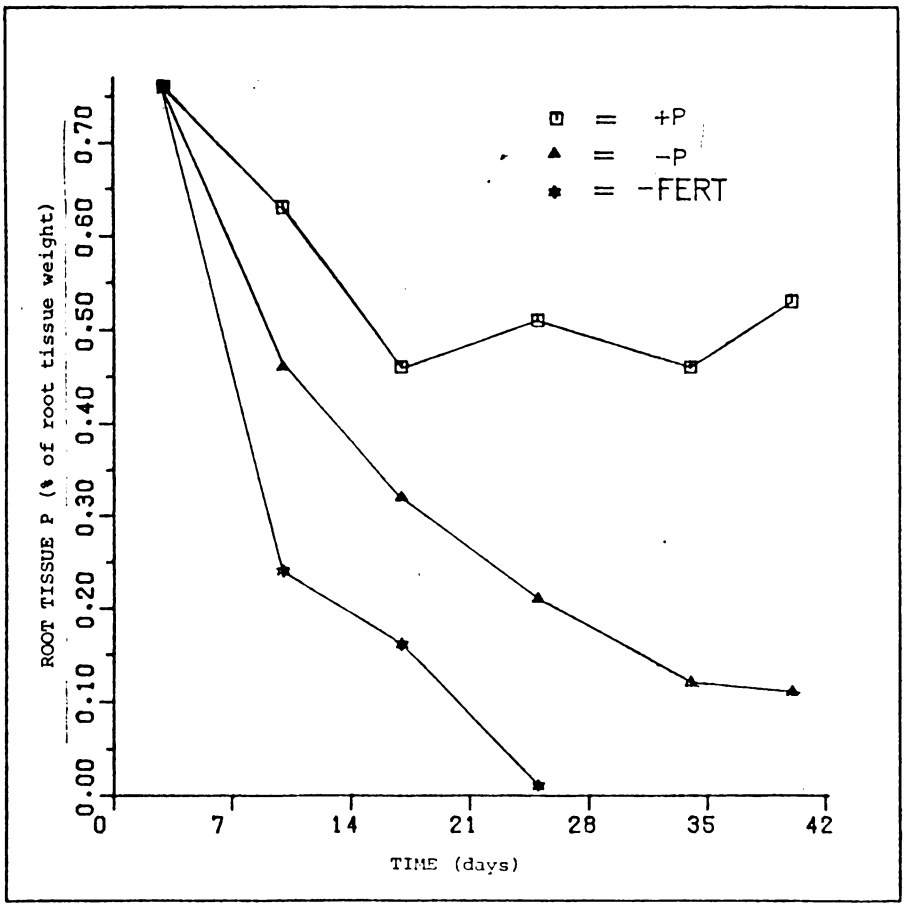

Figure 16. The rate of depletion of phosphorus from root-organs grown on root-organ culture medium. +P = with phosphorus, -P = without phosphorus, -Fert = water agar. Each point is the mean of 5 root phosphorus levels.

Discussion

A. Exudate and Extract Studies

Bowen (1984) divided VAM formation into three phases: (1) the preinfection phase, which includes spore germination and hyphal growth, (2) the infection process and (3) spread of infection in the host root system and/or in the adjacent rhizosphere soil. My investigation was involved with only the preinfection phase of VAM formation.

My study reveals a stimulatory influence of exudates or extracts from P-deficient clover seedlings as compared to P supplimented seedlings on VAM fungal hyphal growth on agar. My data also suggests that the quanlity of exudates from the root systems deprived of phosphorus may be responsible for this difference. Previously, however, Ratnayake et al. (1978), Menge et al. (1978) and Graham et al. (1981) suggested that the quantity of exudates rather than a specific compound, was responsible for the increased VAM infection. They also provided very strong evidence to support the theory that the P level of plant roots is correlated with increased root membrane permeability and subsequent increased root exudation. This increased root exudation was suggested as the cause for increased VAM infection.

All of the previous studies concerning the influence of exudates or extracts on VAM were conducted with exudates collected at only one time, whereas I collected exudates

from the same plants over a 6 week period. I did this for a number of reasons. First, both Rovira (1969) and Hale et al. (1971) reported that plant age has a significant influence on exudation. Second, Schwab et al. (1983a) showed that the P nutritional status of plants has very little influence on the quantity of root exudation and has no clear influence on the quality of root exudates. Hence, I thought that if there was an unknown factor being produced by root systems deprived of phosphorus which stimulates VAM formation, it would probably be exuded in the greatest quantities at very early stages in seedling growth and then taper off over time. Production of this VAM formation factor at the earliest stage of seedling growth would be the most advantages to the plant since potential VAM benefits to the plant would be initiated quickly. This is also the time when VAM hyphal growth and infection usually increases rapidly. The data in Table 6 support my hypothesis that an unknown VAM formation factor may be present and transient. Root exudates from 2-week-old P deprived (-P) seedlings stimulated more hyphal growth than did root exudates from 4 or 6 Week old P deprived (-P) seedlings. Figure 12 and 13 offer supporting evidence for the existence of a VAM formation factor. At 2 weeks, the time of the first exudate collection, the tissue P levels in the root systems of plants grown with or without P were not significantly different, thus, their membrane permeabilities were probably

similar. In addition, at 2 weeks, the size of the root systems and the relative conductivities of exudates between treatments were also similar (Figure 13). However, there was a drastic difference in the effect those exudates from P deprived plants had on hyphal elongation as compared to exudates from nondeficient plants. Similarly, at 4 and 6 weeks, root systems of plants grown both with and without phosphorus grew larger but the relative conductivity measurements changed very little. More importantly, even with a four-fold increase in the amount of root system from which exudates were collected, the influence on hyphal elongation decreased. Root extracts from 6-week-old, P-deprived seedlings also had a significant but smaller stimulatory effect on VAM hyphal elongation. This would be expected if an infection factor first accumulated in the root system before being exuded. The effect of the quantity of exudated has not been ruled out, however, because the concentrations of exudate constituents were not determined. In future experiments this should be done.

Plant growth curves and P depletion curves similar to those in Figure 12 and 13 have been shown for other root systems (Epstein,1972). P-deficient plants generally produce larger root system having greater surface area to explore a larger volume of soil *in situ* than other plants (Brady,1974). In my study, it was not necessary for plants receiving P to make more roots since sufficient P was

available. However, I would have expected the root systems to continue growing but at a slower pace. Possibly other environmental factors such as low light intensities were indirectly limiting root system growth.

Bowen (1969) showed that the nutritional status of a plant has an effect on the quantity and quality of root exudates. In my research (Table 6) similar results were observed. Plants that were deprived of P presumeably had leaky membranes and hence they exuded more than plants that received sufficient phosphorus. Exudation also is affected by plant age (Rovira,1969) and my results further support this phenomenon (Table 6). Whether an unknown stimulatory factor is produced and exuded at the onset of P deprivation or only during the early stages of seedling growth could be determined by testing the effects of exudates that were collected, after P deprivation, from plants initially supplied with P.

The level of phosphorus in root-organs grown with added phosphorus in my study is probably high enough to inhibit VAM infection (Bowen,1984). However, the level of phosphorus in root-organs grown without phosphorus added was extremely low. This tissue phosphorus level would probably not be inhibitory to VAM fungal colonization (Bowen,1984). These phosphorus-deprived root-organs were still growing, unlike the root-organs grown in ROC agar containing no nutrients. Hence, these phosphorus deprived root-organs

would probably be acceptable for use in initial attempts to establish VAM infected root-organs in vitro.

Most of the reports on the extent of VAM hyphal growth in vitro have been qualitative rather than quantitative. Allen et al. (1979), for example, reported only that Glomus fasciculatus grew in culture for up to 4 months. Other scientists have devised their own growth rating systems which makes it difficult to compare and evaluate published data (Hepper and Jakobsen,1983; Mosse and Phillips,1971; Mosse and Hepper,1975; Schwab et al.,1983b). In my study, when exudates from P-deprived plants were used, I observed hyphal elongation of up to 34.17 mm in length compared to less than 0.5 mm for the control; a 70-fold difference. Menge et al. (1978) observed up to 39 mm growth of G. fasciculatus hyphae with their split-root technique in soil over a 3 month period. Siqueira et al. (1982) observed up to 55 mm of growth of Gigaspora margarita hyphae on agar after 30 days. However, this VAM species produces very large spores compared to G. fasciculatus, hence, stored spore reserves may be responsible for this hyphal growth.

In summary, because of the similarity of exudate conductivity levels, the P depletion curves, the root system growth levels and the great differences in hyphal elongation between treatments, my data suggest that it is the quality of exudates from plants experiencing P deprivation which is important in stimulating VAM hyphal growth. In addition,

the 70-fold increase in growth of hyphae in exudates from P-deprived plants is one of, if not the largest, stimulation that has ever been reported.

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APPENDICES

Appendix 1. Spore Isolation Procedure

1. Sieve soil through 400 um and 40 um screens with 40 um screen on top and 400 um screen on the bottom. Spores will be trapped on the bottom screen.

2. Decant spores, soil and organic debris into centrifuge tube, add water to balance tubes, and spin for 3 minutes at 2500 rpm in a tipping-bucket centrifuge.

3. Decant water and light organic debris, spores are in pellet.

4. Add 3 ml 60% Ficoll to centrifuge tube and mix to suspend spores.

5. Carefully add 5 ml 60% Ficoll letting it run slowly down the side of the tube.

6. Repeat Step 5 with 5 ml 50% Ficoll, then 45%, 35% and finally 15% Ficoll last. Add them all slowly to prevent disturbance of the layers of the gradient.

7. Add water to balance tubes and then centrifuge 10 minutes at 2500 rpm.

8. Spores are now in supernatant. Pour off supernatant into 400 um cup sieve and rinse spores with water until all Ficoll is washed off.

9. Transfer spores to petri dish or beaker for storage. Spores must be cleaned up, surface sterilized and stored in Strep/Gen solution at 4 C.

Appendix 2. Procedure for VAM Spore Surface Sterilization

1. Collect VAM spores that have been picked clean of organic debris, in a 38 um cup sieve, then wash them into a sterile 250 ml flask with 30 ml of filter -sterilized solution containing 2% (w/v) Chloramine-T, 0.2% streptomycin sulfate and a trace of sodium lauryl sulfate (SLS) (MacDonald,1981).

2. Apply vacuum to flask for 30 minutes with a change into 30 ml of fresh sterilized solution after 15 minutes using a sterilized 38 um cup sieve. Swirl occasionally to insure full contact of Chloramine-T with spores.

3. Collect spores again in another sterilized 38 um cup sieve. Rinse spores with sterile glass distilled water several times to wash out all Chloramine-T.

4. Transfer spores into a sterile vessel by washing them off the cup sieve screen with 30 ml filter sterilized Strep/Gen solution (25 mg streptomycin sulfate and 16.7 mg Gentamicin Sulfate/100 ml water.

5. Store spores at 4 C for use within 5 days.

Appendix 3. Materials for VAM Storage Experiment

Strep/Gen Solution

Streptomycin Sulfate 25.0 mg
Gentamicin Sulfate 16.7 mg

Add to 100 ml glass distilled water and filter sterilize with 0.45 um Nalgene filter unit.

Ringer's Solution

NaCl 6.0 gm
CaCl 0.1 gm
KCl 0.1 gm

Add to 1 liter glass distilled water.

Adjust to pH 7.4 with 0.1N NaOH and then autoclave for 15 minutes.

Appendix 4. VAM Growth Medium

Macronutrients (per liter of media):
(modified to contain no micronutrients)

KNO3	303.0	mg
Ca(NO3)2-4H2O	2.04	gm
MgSO4-7H2O	368.0	mg
KH2PO4	44.0	mg*
Iron Tartrate	46.0	mg
Difco Bacto Agar	8.0	gm

Adjust to pH 6.8 with 0.1 N NaOH and autoclave for 15 minutes.

*Add only for medium with phosphorus (W/P).

Appendix 5. Root-Organ Culture Agar

(Hepper and Mosse,1980)

To 1 liter of glass distilled water add:

KCl	65.0	mg
KNO3	80.0	mg
Ca(NO3)2-4H2O	300.0	mg
MgSO4-7H2O	720.0	mg
NaH2PO4-2H2O	10.7	mg*
Fe Tartrate	4.6	mg
MnCl2-4H2O	4.9	mg
KI	0.75	mg
H3BO4	1.5	mg
ZnSO4-H2O	1.9	mg
CuSO4-5H2O	1.0	ug
Na2MoO4-2H2O	0.17	ug
Glycine	3.0	mg
Thiamine-HCl	0.1	mg
Nicotinic Acid	0.5	mg
Pyridoxine	0.1	mg
Sucrose	20.0	gm
Bacto agar	8.0	gm

Adjust medium to pH 4.9 and autoclave for 15 minutes.

*Omit from medium without phosphorus (-P).

Appendix 6. Procedure for Seed Surface-Sterilization

1. Place seeds in flask with 25 ml of 70% ethyl alcohol for 30 seconds, swirl then pour off liquid.

2. Add 25 ml 0.1% $HgCl_2$ in 1 mM HCl to flask. Attach vaccum and swirl frequently for 5-7 minutes. Then drain off liquid.

3. Wash seed exhaustively (7X) with 25 ml aliquots of glass distilled water.

Appendix 7. Hoagland's Nutrient Solution

To 1 liter of glass distilled water add:

Major elements

KNO_3	606.6	mg
$Ca(NO_3)_2 \cdot 4H_2O$	656.4	mg
$MgSO_4 \cdot H_2O$	240.8	mg
$NH_4H_2PO_4$	110.0	mg*

Minor elements

H_3BO_3	2.86	mg
$MnCl_2 \cdot 4H_2O$	1.81	mg
$ZnSO_4 \cdot 7H_2O$	0.22	mg
$CuSO_4 \cdot 5H_2O$	0.08	mg
H_2MoO_4	0.02	mg
Fe Tartrate	5.0	mg

Adjust to pH 6.8 with 0.1N NaOH and autoclave 15 minutes.

*Omit in medium without phosphorus (W/OP)

Appendix 8. Additional Data for Methods of Storage

Table 7. Percent germination for three VAM species stored in three treatment solutions

Treatment Solution	% Germination(± S.E.M.)[a] after indicated Weeks in Storage							
	0	1	2	4	6	8	10	12
Glomus mosseae								
A	6.5± 4.1[b]	1.6± 1.4	1.4± 1.2	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0
B	10.0± 8.7	4.9± 2.6	2.1± 1.1	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0
C	7.8± 3.9	2.8± 1.4	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.3± 0.0	0.0± 0.0	0.0± 0.0
Glomus fasciculatus								
A	13.4± 2.3	6.0± 1.3	4.1± 1.5	1.4± 1.1	0.9± 0.5	1.2± 0.6	1.1± 0.6	0.7± 0.4
B	17.5± 1.4	9.4± 1.5	7.6± 2.4	6.2± 1.5	8.1± 1.3	5.5± 1.8	3.5± 1.1	4.8± 0.8
C	19.1± 2.1	12.1± 3.0	8.1± 1.9	5.9± 0.6	7.3± 2.6	8.5± 2.3	4.2± 1.4	3.2± 1.2
Gigaspora margarita								
A	21.3± 2.9	34.2±14.7	18.6± 5.6	25.0±12.5	36.1±12.0	24.2±13.7	16.7±14.4	13.6± 7.7
B	30.5± 8.9	45.6± 8.1	20.7± 7.1	13.2± 8.8	17.7± 8.9	11.9± 7.7	8.5± 4.5	6.3± 5.4
C	27.2± 2.4	22.8±11.2	16.5± 6.0	14.9± 7.4	10.4± 3.8	3.6± 3.1	4.2± 3.6	2.4± 2.1

[a] S.E.M. = Standard Error of the Mean

[b] Values represent the mean of 4 plates containing spores.

A = Streptomycin/Gentomicin

B = Ringer's Solution

C = Sterile Water

Appendix 9. Additional PEG-Water Potential Data

Table 8. The influence of PEG on water potential (-bars) and on subsequent germination of *Glomus fasciculatus* spores.

PEG Added (g)	Media Used					
	A(-FERT)		B(-P)		C(+P)	
	% Germination[a]	-Bars	% Germination	-Bars	% Germination	-Bars
0	1.3 ± 1.1[b]	0.27	6.3 ± 2.1	1.60	15.0 ± 1.8	2.40
1	2.5 ± 1.3	0.53	16.3 ± 2.7	2.40	21.3 ± 1.1	2.80
5	5.0 ± 1.8	0.67	23.8 ± 2.1	2.87	28.8 ± 2.1	3.20
10	5.0 ± 1.8	0.84	43.8 ± 2.1	4.27	46.3 ± 2.7	5.00
15	7.5 ± 1.3	1.53	42.5 ± 2.8	5.55	22.5 ± 2.8	6.80
20	28.8 ± 3.7	3.20	17.5 ± 2.8	8.07	11.3 ± 2.7	10.80
30	42.5 ± 2.8	5.47	6.3 ± 2.1	11.60	0.0 ± 0.0	17.33

[a] Values represent the mean of 4 plantes, each containing 20 spores

[b] Satandard Error of the Mean

-FERT = water agar

-P = without phosphorus

+P = with phosphorus

Appendix 10. Additional Sorbitol-Water Potential Data

Table 9. The influence of sorbitol on water potential (-bars) and subsequent germination of Glomus fasciculatus spores.

Sorbitol Added (g)	Media Used					
	A(-FERT)		B(-P)		C(+P)	
	% Germination[a]	-Bars	% Germination	-Bars	% Germination	-Bars
0	4.0 ± 1.7[b]	0.60	22.0 ± 2.3	2.93	15.0 ± 1.4	2.27
1	6.8 ± 1.2	1.73	29.0 ± 2.6	3.20	30.0 ± 3.2	3.27
2	20.0 ± 1.6	2.87	42.0 ± 2.3	5.47	45.0 ± 3.2	4.53
3	41.0 ± 2.2	4.33	29.0 ± 3.0	6.13	25.0 ± 3.2	6.80
4	30.0 ± 3.2	6.00	23.0 ± 3.0	7.60	18.0 ± 2.3	8.00
5	21.0 ± 2.2	7.60	13.0 ± 2.3	9.33	16.0 ± 1.7	8.67
6	11.0 ± 3.0	9.33	5.0 ± 1.4	11.87	3.0 ± 1.1	14.27
7	6.0 ± 1.7	11.47	5.0 ± 1.4	12.00	4.0 ± 1.7	12.67
8	5.0 ± 2.0	12.00	4.0 ± 1.7	13.47	2.0 ± 1.1	14.40
9	3.0 ± 1.8	13.87	0.0 ± 0.0	15.87	2.0 ± 1.1	14.27
10	0.0 ± 0.0	15.07	0.0 ± 0.0	19.33	1.0 ± 0.9	18.27
15	0.0 ± 0.0	24.80	1.0 ± 0.9	24.93	0.0 ± 0.0	26.00
20	0.0 ± 0.0	30.00	0.0 ± 0.0	31.20	0.0 ± 0.0	38.80
25	0.0 ± 0.0	44.27	0.0 ± 0.0	36.53	0.0 ± 0.0	46.13
30	1.0 ± 0.9	51.20	0.0 ± 0.0	52.80	0.0 ± 0.0	60.53

[a] Values represent the mean of 4 plantes, each containing 20 spores

[b] Satandard Error of the Mean

-FERT = water agar

-P = without phosphorus

+P = with phosphorus

Appendix 11. Root-Organ Culture Method

1. Surface sterilize white clover (Trifolium repens) seeds (See Appendix 6).

2. Transfer seeds to moist filter paper in a sterile petri dish and incubate at 23 C in the dark for 2 days to allow for germination.

3. Using sterile technique remove root tips of uniform length and transfer them to root-organ culture (ROC) agar.

4. Transfer root pieces to fresh ROC agar every 10 days.

Appendix 12. Procedure for Root Staining

1. Cover roots in large test tubes with 10% KOH.
2. Heat tubes at 85 C in water bath for 50 minutes.
3. Wash roots to remove all KOH.
4. Acidify roots by soaking for 1 hour in 0.1 M HCl.
5. Stain overnight in 0.1% acid fuchsin in lactophenol. Lactophenol is made by mixing by weight: one part phenol, one part lactic acid, one part glycerol and one part glass distilled water.
6. Destain roots in clear lactophenol twice, with roots remaining in each solution overnight.

Appendix 13. Procedure for Tissue Phosphorus Assay

1. Weigh <1.0 to 50 mg dry tissue into 10 ml Kjeldahl flasks.
2. Add 0.5 ml 10 N sulfuric acid to each flask. The amount of sulfuric acid is critical. To make 10 N sulfuric acid combine 270 ml concentrate and 730 ml water.
3. Heat at #3 on microkjeldahl apparatus.
4. When white fuming starts, reduce to #2.
5. After 30 minutes cool tubes and add 0.5 ml 30% hydrogen peroxide.
6. Shake gently than repeat steps 3 and 4 for 30 minutes.
7. Cool tubes again and add 5.0 ml glass distilled water.
8. Heat tubes in boiling water bath (100 C) for 10 minutes.
9. Cool tubes and add 0.2 ml of 5% ammonium molybdate.
10. Add 0.2 ml Fiske-Sabbarow* reagent and vortex.
11. Heat in boiling water bath (100 C) for 7.5 minutes.
12. Read absorbance at 620 nm.

*Fiske-Sabbarow Reagent:

1.0 gm 1-amino-2-napthol sulfonic acid
1.0 gm sodium sulfite (Na_2SO_3)
58.4 gm sodium meta-bisulfite ($Na_2S_2O_5$)

Mix and grind to a dry powder. Dissolve 0.77 gm in 5.0 ml warm glass distilled water 15 minutes before use.