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Title: THE ROLE OF ETHYLENE IN ECTOMYCORRHIZA FORMATION AND FUSARIUM INFECTION OF DOUGLAS-FIR ROOTS

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Dr. Robert G. Linderman

The ectomycorrhizal fungi Cenococcum geophilum, Hebeloma crustuliniforme and Laccaria laccata produced ethylene in vitro in modified Melin-Norkrans liquid medium only if amended with 2.5 to 10 mM methionine; Pisolithus tinctorius failed to produce ethylene unless the cultures were renewed with fresh methionine-amended medium prior to ethylene assay. An additional 19 ectomycorrhizal fungi plus 5 isolates of Fusarium oxysporum f. sp. pini all produced ethylene in renewed and/or nonrenewed media. Although the rates varied, ethylene production by many ectomycorrhizal fungi equaled that of Fusarium.

Culture filtrates of H. crustuliniforme and L. laccata also evolved ethylene that was apparently of nonenzymatic origin.

Ethylene was produced by aseptically grown Douglas-fir seedlings inoculated with C. geophilum, H. crustuliniforme and L. laccata, and appearance of ethylene coincided with the formation of mycorrhizae;

production by P. tinctorius-inoculated seedlings was inconsistent. Lateral root formation of Douglas-fir was stimulated by inoculation with C. geophilum, H. crustuliniforme and L. laccata, but was inhibited by P. tinctorius. Fusarium-inoculated seedlings produced more ethylene sooner than seedlings inoculated with mycorrhizal fungi.

Two-month-old Douglas-fir seedlings were exposed to six ethylene concentrations, ranging from 0.006 (soil ambient control) up to 0.5 ppm, established by adding Ethrel, an ethylene-releasing compound, as a soil drench to the root zone. Exposure of roots to the different ethylene concentrations for 2 months either stimulated (0.01-0.05 ppm), had no effect on (0.05-0.15 ppm), or inhibited (> 0.15 ppm) lateral root formation. Root dry weight increased and shoot dry weight decreased as the ethylene concentration was increased.

Mycorrhiza formation by Hebeloma crustuliniforme was not increased by exposing inoculated 4-month-old seedlings to 0.1 ppm ethylene for 3 months whether or not the seedlings had been exposed to ethylene for two months prior to inoculation. By contrast, when 2-month-old seedlings pre-inoculated with Fusarium oxysporum f. sp. pini were exposed to 0.1 ppm ethylene for 2 months, disease was significantly increased.

These studies suggest that there is a fundamental difference

between pathogenic Fusarium and symbiotic mycorrhizal fungi in the levels of ethylene produced by the host-fungus interaction and in the function of ethylene in disease development versus mycorrhiza establishment and maintenance.

The Role of Ethylene in Ectomycorrhiza Formation and *Fusarium* Infection of Douglas-fir Roots

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in charge of major

Redacted for privacy

Head of Department of Botany and Plant Pathology

Redacted for privacy

Dean of Graduate School

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Typed by Opal Grossnicklaus for James Herbert Graham, Jr.

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CHAPTER 1

INTRODUCTION

Ethylene, a natural plant growth regulator, is produced by a wide variety of soilborne microorganisms including bacterial and fungal plant pathogens. Ethylene evolved from soil and diseased plant tissue has been implicated in a number of plant growth and development phenomena.

It is now well established that certain disease symptoms caused by *Fusarium* infection are due to ethylene. *Fusarium* species and *Fusarium*-infected plant tissue produce large quantities of ethylene. Ethylene production by ectomycorrhizal fungi and mycorrhizae has not been examined although other plant hormones, such as auxins and cytokinins, have been suggested as playing a key role in mycorrhiza formation. Culture filtrates of mycorrhizal fungi and synthetic auxins stimulate root initiation on pines. However, ethylene, which has root growth promoting properties similar to auxins, also stimulates root formation.

Ethylene has been reported both to increase and decrease the susceptibility of plants to fungal infection. Increased ethylene from non-susceptible reactions has been correlated with the synthesis of

host defense compounds, such as phytoalexins, and the development of disease resistance. In contrast, ethylene treatment has also been shown to decrease the disease resistance of normally non-susceptible plants.

There apparently are many functions for ethylene in host-fungus interactions which need further investigation. Accordingly, comparative studies on the role of ethylene in symbiotic ectomycorrhiza formation and pathogenic Fusarium infection of Douglas-fir (Pseudotsuga menziesii) were conducted. The first study determined the ability of ectomycorrhizal fungi and Fusarium oxysporum f. sp. pini to produce ethylene in vitro and as a result of inoculation of aseptically grown Douglas-fir. The study following examined the effects of ethylene on root growth of Douglas-fir and on the host response to mycorrhiza formation and Fusarium infection.

CHAPTER 2

LITERATURE REVIEW

I. Ethylene in Soil

A. Existence of Ethylene in Soil

Ethylene was first suspected as a component of the soil atmosphere in 1935 when Nielson-Jones observed that tomato plants became epinastic when exposed to gaseous and water extracts of high organic matter soils.

Actual measurements of ethylene in soil were not made until the late 1960's when K. A. Smith and Russell (1969) detected up to 20 ppm in waterlogged soils. In subsequent studies, K. A. Smith and coworkers (Smith and Restall 1971; Smith and Dowdell 1974) discovered that many factors influenced the levels of ethylene in soil. Soil conditions which promoted ethylene production included high temperature, low oxygen concentrations, and high moisture content. They also found that high concentrations of nitrate (2000 ppm) in soil greatly reduced the rate of ethylene production but did not suppress it completely.

A. M. Smith (1973) examined a large number of moist but drained Australian soils and reported that all produced ethylene in an open aerobic system at levels ranging from 0.1 ppm to be as high as

10 ppm in certain high organic soils. Later, A. M. Smith and Cook (1974) observed that ethylene concentrations were least where tillage was extensive and greatest in undisturbed sod pasture. They concluded that reduced aeration and/or high organic matter favors ethylene production in soils, whereas increased aeration resulting from repeated mechanical disturbance lowers ethylene production. They also found that nitrate additions of 20-200 ppm in soil delayed ethylene production until denitrification was complete.

Examining a wide range of soils, including grassland and cultivated soils, Goodglass and K. A. Smith (1978a) confirmed that ethylene production from soil was correlated closely with total organic matter content. In a second study (1978b), they found that evolution of ethylene from soils could be stimulated by amendments of cereal straw and hay.

In a recent study on factors affecting ethylene levels in soils, Cook and Smith (1977) demonstrated that the relationship between ethylene production and soil water potential was nearly identical for two soils differing extensively in physical and chemical properties. For the two soils, one from Australia and the other from Idaho, ethylene production was maximum at saturation, decreased as water potential was lowered to -1 bar, and was greatly reduced at -5 bars and below.

B. Sources of Ethylene in Soil

Although it is apparent that ethylene occurs in most soils, the principal sources of ethylene are still in doubt. Most evidence points to microorganisms as the major source of soil ethylene. Many microorganisms including fungi, yeasts, and bacteria produce ethylene *in vitro* under certain conditions.

1. Ethylene Production by Fungi

The initial report of ethylene production by fungi resulted from observations that the respiratory activity of citrus fruits increased when infected by the green mold fungus, *Penicillium digitatum* (Biale 1940; Miller *et al.* 1940; Biale and Shepherd 1941). Subsequently, Young *et al.* (1951) identified ethylene as the physiologically active emanation from *P. digitatum*.

More recently Ilag and Curtis (1968) showed that ethylene is a common metabolite of a wide range of fungal species. Of 228 fungi examined, 25% produced ethylene when mycelium was grown in corn steep liquor-glucose liquid medium and subsequently incubated in a sealed glass syringe for 24 hours. In their system *Aspergillus clavatus* was the most prolific ethylene producer.

Lynch (1972) incubated soil on glucose-methionine medium and isolated *Mucor hiemalis* and two soil yeasts which he considered to be the dominant ethylene-producing constituents of the soil microflora.

M. hiemalis produced large quantities of ethylene when grown on glucose-methionine agar but not on malt or Sabourand-dextrose medium. Lynch compared the production of Mucor with Aspergillus clavatus, the best producer identified by Ilag and Curtis (1968). A. clavatus also required glucose and methionine for ethylene production, but produced much less ethylene than M. hiemalis. Lynch concluded that M. hiemalis, which is a common primary colonizer of fresh organic residues, is an important source of ethylene in soil.

Dasilva et al. (1974) examined ethylene production by species of Aspergillus and Mucor isolated from soil. They reported that varying carbon sources and types of media did not stimulate ethylene production as much as the glucose-methionine medium used by Lynch (1972).

Glucose plus methionine, the precursors for ethylene production by Mucor, did not enhance production of ethylene by Penicillium cyclopium and P. crustosum compared to glucose alone (Considine et al. 1977). Tests of various other carbon sources for ethylene production by these two organisms suggested that the pathway for ethylene production differed. P. cyclopium produced higher levels of ethylene on media containing acetate or tricarboxylic acid cycle intermediates than on glucose. The pattern for P. crustosum was the opposite, i.e. glucose supported good ethylene production whereas tricarboxylic acid

cycle intermediates and glucose gave little or none. Phenolic acids, including vanillic, p-hydroxybenzoic, protocatechuic, and ferulic acid supported growth and ethylene production by both species. These results suggested that phenolic acid-utilizing organisms may contribute to the total ethylene production in soil, particularly in high organic matter soils.

Ethylene production by the green mold fungus of citrus, Penicillium digitatum, has been investigated extensively and the ethylene biosynthetic pathways have been partially elucidated. When the fungus is grown in static culture, ethylene is derived from α-ketoglutarate or glutamate in the tricarboxylic acid cycle but not from methionine (Chou and Yang 1973). However, Chalutz et al. (1977) recently reported that under shake conditions labelled ethylene was produced from ^{14}C-methionine. They suggested that in P. digitatum ethylene can be produced from two different types of precursors depending on the cultural conditions under which it is grown: shake or static.

In the same study, Chalutz et al. (1977) demonstrated that filtrates of methionine-amended shake cultures also evolved ethylene by both enzymic and nonenzymic reactions. The evolution of ethylene by the filtrate was only partially inhibited by boiling the filtrate or adding the protein-degrading enzyme, pronase, suggesting that much of the ethylene (50-60%) was produced nonenzymatically. Tracer experiments indicated that methionine was not the direct precursor of

ethylene in filtrates. Culture filtrates evolved labelled ethylene only when radioactive methionine was added to the incubating medium when it still contained fungal cells. These results suggested that the fungus took up methonine from the solution and released a metabolite of methionine into the medium which was converted to ethylene.

Lynch (1974) also observed the production of ethylene by filtrates of Mucor hiemalis. He found that treating the filtrate by boiling, addition of Fe^{2+} and Fe^{3+}, or illuminating the filtrate greatly stimulated ethylene evolution. He suggested that ethylene production occurred nonenzymatically via a flavin cofactor which catalyzed the breakdown of methionine into ethylene in the presence of light (Yang et al. 1967).

Thomas and Spencer (1978) investigated ethylene production by the yeast, Saccharomyces cerevisiae, under aerobic and anaerobic conditions. Ethylene was produced both aerobically and anaerobically by yeast cultures and cell-free filtrates when glucose was added to the medium. Ethylene production by anaerobically grown cultures and filtrates was low, but the levels increased rapidly when air was admitted. The authors hypothesized that a precursor of ethylene, synthesized by the yeast and excreted into the medium under anaerobic conditions, was converted to ethylene in the presence of oxygen.

Because ethylene has been implicated in the development of some fungal disease symptoms, several soilborne pathogenic fungi have

been examined for production in vitro. In an early paper on the cause of epinasty in Fusarium wilt of tomato, Dimond and Waggoner (1953) examined production of ethylene by Fusarium oxysporum f. sp. lycopersici. When the fungus was grown on maltose-nutrient broth agar, emanations from the culture caused a triple ethylene response in peas. Chalutz and DeVay (1969) reported that all of the isolates tested of Ceratocystis fimbriata, the cause of black rot in sweet potato, produced ethylene when grown on potato-dextrose agar, but the amounts produced varied greatly. Pegg and Cronshaw (1976) found that Verticillium albo-atrum, a wilt fungal pathogen of tomato, only produced significant amounts of ethylene when grown on glucose-methionine medium.

The only report of ethylene production by basidiomycetes in vitro is for the cultivated mushroom, Agaricus bisporus (Ward et al. 1978). Ethylene was evolved from cultures of mycelium growing on a medium containing malt or extracts of compost. On basal salts medium with only glucose as the carbon source, methionine was required to stimulate ethylene production.

2. Ethylene Production by Bacteria

K. A. Smith and Restall (1971), in their studies on ethylene production in anaerobic soil, proposed that facultative anaerobic bacteria were the most likely producers of ethylene. They gave several lines of evidence to support their hypothesis. Ethylene

production occurred only at reduced oxygen tensions but apparently not under strictly anaerobic conditions since buildup of ethylene preceded that of methane, a product of severely reduced conditions. After autoclaving or gamma irradiation of soil, ethylene production was lowered significantly. Ethylene production was also affected by temperature; below 10°C the evolution of ethylene was reduced, while at higher temperature production was increased. Addition of high concentrations of nitrate also depressed ethylene evolution, which suggested that nitrate may act as an alternative oxygen substrate for ethylene-producing bacteria and that a relationship between denitrification and ethylene evolution exists.

Subsequent studies by A. M. Smith and Cook (1974), and A. M. Smith (1976) supported the hypothesis that bacteria are the main producers of ethylene in soil. Their evidence suggested that spore-forming bacteria in anaerobic microsites were responsible. Ethylene was produced in soil when oxygen was depleted, when water potentials were near saturation, or when soils were heated to 60°C and 80°C, an indication that heat-tolerant spore-forming bacteria were active. This heat treatment also eliminated fungi, such as Mucor hiemalis, as possible sources of ethylene.

Smith and Cook (1974) also showed that amendment of soil with 20-200 ppm nitrate delayed ethylene production. They interpreted this effect as evidence for the participation of strict anaerobes in ethylene

production. Supplying nitrate to soil would raise the redox potential, even in the absence of oxygen, preventing the activity of strict anaerobes. At the same time nitrate would act as an efficient terminal electron acceptor for facultative denitrifying bacteria in the absence of oxygen. In this case, when denitrification was complete, strictly anaerobic conditions necessary for ethylene production would be re-established.

Although A. M. Smith and Cook (1974) reported that anaerobic spore-forming bacteria were responsible for ethylene formed in soil, they never attempted to isolate these organisms. In recent studies, Primrose and Dilworth (1976) isolated from waterlogged soils two aerobes and three facultative anaerobes which produced significant amounts of ethylene when grown aerobically on potato extract-yeast extract-manitol medium. The two aerobes were identified as Pseudomonas and 2 of the 3 facultative anaerobes were Escherichia coli. Methionine stimulated production by E. coli proportional to the amount added in the medium. Oxygen was also required since only a small amount of ethylene was formed under anaerobic conditions.

In further studies on ethylene-forming bacteria, Primrose (1976b) isolated a large number of bacteria from different soil and water sources capable of producing ethylene aerobically on methionine-amended medium. Sixty-five gram-negative ethylene producing isolates were identified of which 49 were facultative and the remaining

were strict aerobes. Of the fermentative isolates, 12 were classified as Aeromonas hydrophila, the remaining were enterics including E. coli. The 16 non-fermentative isolates were identified as Pseudomonas species. Of the gram-positive isolates, two were identified as Arthrobacter, the others as saccharolytic coryneforms close to Arthrobacter.

Primrose (1976a) also performed more detailed studies on the physiology of ethylene production by E. coli. He tested a variety of compounds in addition to methionine for their ability to stimulate production. He found that L-cysteine, L-homocysteine, and derivatives of methionine, all sulfur containing compounds, were active. Cell-free filtrates of E. coli also evolved ethylene, but only in the light, the amount depending on whether the tiltrates were derived from cultures grown in the light or dark. When cultures were grown in the dark, the derived filtrates produced more ethylene than filtrates from light-grown cultures. Filtrates also produced ethylene anaerobically if the filtrate came from dark-grown cultures. Addition of methionine to culture filtrates derived from non-methionine amended cultures did not result in ethylene production indicating that a metabolite of methionine was responsible for ethylene production as suggested by Chalutz et al. (1978) with Penicillium digitatum. The results with E. coli are also in accordance with Lynch's (1974) findings that illumination of dark grown cultures of Mucor hiemalis stimulated ethylene production.

Several of the isolates examined by Primrose in a later study (1976b) secreted yellow, water-soluble pigment with spectral properties of flavins into the medium. Upon illumination, cell-free filtrates from these cultures released ethylene at much greater rates than filtrates not secreting such pigments. Therefore Primrose, as did Lynch (1974) with Mucor hiemalis, suggested that production of ethylene from methionine and its derivatives was mediated by flavin compounds.

The role of flavins, specifically flavin mononucleotide (FMN), was first identified by Yang et al. (1967), who showed that methionine and related compounds were nonenzymatically oxidized to ethylene. The initial step in the reaction is a photochemical excitation of FMN, which attacks the substrate and induces the chemical conversion of methionine to ethylene.

Ethylene production by bacterial plant pathogens has also been examined in cases where disease symptoms are highly suggestive of ethylene effects. Pseudomonas solanacearum, the cause of bacterial wilt of tomato and tobacco and early ripening of banana, was examined for production in peptone-glucose broth (Freebairn and Buddenhagen 1964). Ethylene production was detected from several strains of the pathogen, with the tobacco-tomato strains producing higher levels than the banana strain. More recently, Bonn et al. (1975) examined ethylene production from virulent and avirulent forms of

P. solanacearum by passing a continuous air-stream over cultures grown on the inside surface of glass tubes. The highest rates of ethylene formation occurred at the end of the log phase of growth and at the beginning of the stationary phase. Ethylene production rates per cell were much lower for avirulent forms than the virulent forms. They suggested that ethylene production could be considered another physiological characteristic associated with change in virulence of this organism.

3. Other Sources of Ethylene in Soil

Ethylene is produced by plant roots growing in soil (Abeles 1973). Kays et al. (1974) demonstrated that when the axial growth of bean roots was impeded by a barrier, the rate of ethylene evolution increased dramatically up to 30 hours. When the barrier was removed, ethylene evolution decreased. Kays et al. indicated that ethylene evolved by roots in response to mechanical resistance may act as an endogenous growth regulator.

Ethylene may also be a product of soil organic matter decomposition. Incubated samples of Pinus radiata litter produced physiologically significant quantities of ethylene although the contributions of the litter microflora and the organic matter were not distinguished (Lill and McWha 1976). Rovira and Vendrell (1972) found that gamma-irradiated soil evolved ethylene for several weeks. They used a soil relatively low in organic matter and suggested that the amounts of

ethylene would be even greater in soils with high organic matter content.

Ethylene production in soil is also promoted by the addition of organic residues (Goodglass and Smith 1978b) and organic wastes in the form of slurries (Stevens and Cornforth 1974; Burford 1975). However, such effects on ethylene production may reflect the stimulation of microbial activity, or in the case of slurries, the sealing of soil pores that slows the gaseous diffusion of ethylene.

Chemical reactions may also contribute to soil ethylene production, particularly in the vicinity of microbes. Mucor hiemalis (Lynch 1974) and bacteria (Primrose 1976a, 1976b) produced ethylene from cell-free filtrates. Lynch (1974) suggested that metabolites released extracellularly in the soil could subsequently be chemically degraded into ethylene.

Air pollution is another important ethylene input into soil (Abeles 1973). Abeles (1971) reported that large quantities of ethylene are released in auto exhaust and industrial effluents, especially in large metropolitan areas.

C. Degradation of Ethylene in Soil

Soil bacteria are able to consume hydrocarbons including ethylene. Yet, the degradation of ethylene in soil has been only recently recognized

and studied in detail.

Abeles et al. (1971) discovered that when soil samples were placed in an atmosphere containing auto exhaust, ethylene and other hydrocarbons were removed. Ethylene consumption did not occur in the absence of oxygen or after soil sterilization by autoclaving, an indication that microorganisms were involved. K. A. Smith et al. (1973) examined steam-sterilized soils and confirmed the findings of Abeles et al. that microbial activity was responsible for ethylene consumption in soil.

Cornforth (1975) examined the role of soil micro-organisms in ethylene degradation in more detail. He demonstrated that ethylene decomposition occurred most rapidly in fresh soil, and that successive exposures of the soil to ethylene increased the rate of consumption suggesting that a population of ethylene consumers was increasing. Ethylene consumption required oxygen since ethylene persisted in soil under a nitrogen atmosphere. Based on the rate of ethylene production under nitrogen atmospheres and the rate of consumption under air, Cornforth judged that ethylene decomposition was about fifty times faster than production. When he examined field soils he found that the activity of ethylene consumers was significant, even in the upper horizons of poorly drained soil, but was much less in the lower soil horizons of either well or poorly drained soils. He concluded that ethylene is microbially decomposed in aerobic soils faster

than it is produced, thus questioning whether ethylene persists in soil for any length of time. He pointed out, however, that ethylene probably accumulates in soil under anaerobic conditions.

Yoshida and Suzuki (1975) also examined the factors affecting ethylene formation and degradation in continuously submerged rice soils. Ethylene was formed in rice soils in physiologically active amounts, and production was stimulated by addition of organic amendments. Rice roots, however, exhibited ethylene degradation activity which was attributed to rhizosphere microorganisms capable of degrading ethylene.

De Bont (1976) attempted to isolate the microorganisms responsible for ethylene degradation. He isolated 5 strains of gram-positive aerobic ethylene-oxidizing bacteria in the genus Mycobacterium. In further studies (de Bont and Albers 1976) one of the isolated strains was found to utilize ethylene as a sole carbon source.

D. Ethylene and Soil Fungistasis

Soil fungistasis is the term applied to the widespread inability of fungal propagules to germinate in soil under apparently favorable conditions unless exogenous nutrient is supplied (Dobbs and Hinson 1953).

Ethylene has been suggested as an inhibitor that can cause soil fungistasis. In studies by Smith (1973), ethylene was identified in

more than 50 soils of different origins and histories as the only substance present in all soils in biologically active amounts. Smith (1973), and Smith and Cook (1974) demonstrated that ethylene was a fungistatic factor by comparing germination and growth of fungal propagules placed on nonsterile soil and exposed to water-saturated airstreams containing 0.5, 0.7 and 1.0 ppm ethylene. Ethylene inhibited germination and growth of test fungi and these inhibitory effects were reversed when soils were exposed to an ethylene-free atmosphere. Hyphal growth of test fungi was also sensitive to ethylene. Based on their observations, Smith and Cook hypothesized that organic nutrients were the main stimulators of fungal propagule germination and ethylene the inhibitor.

Preliminary results indicated that not all fungi were equally sensitive in that some basidiomycetes and phycomycetes were more tolerant of ethylene (Smith and Cook 1974). Mycelial growth of Agaricus bisporus was even stimulated by low concentrations of ethylene. Smith and Cook (1974) hypothesized that tolerance of fungi to ethylene is a reflection of the ethylene concentrations in their soil habitat. Basidiomycetes, acting as litter decomposers in soils high in organic matter, and phycomycetes, in wet soil, are both active under soil conditions associated with high levels of ethylene production.

Smith and Cook (1974) made similar observations on the effect of ethylene on activity of other soil microorganisms. Actinomycetes, like

most fungi, were found to be sensitive to ethylene. However, ethylene concentrations had no measurable effect on bacterial activity as determined by O_2 consumption, CO_2 production, and bacterial counts.

Since the reports of Smith and Cook (Smith 1973; Smith and Cook 1974), the role of ethylene as a fungistatic factor has been reviewed by a number of researchers. Balis (1976) found that ethylene alone did not inhibit spore germination of Penicillium chrysogenum and P. italicum. However, propagules were not exposed in soil, eliminating potential indirect effects of ethylene on other microbes whose activity might inhibit germination. K. A. Smith (1978) observed that ethylene did not affect aerobic respiration of soils, and questioned its role as a microbial inhibitor. The most critical evidence refuting the role of ethylene in fungistasis was presented by Schippers (1978). Ethylene in concentrations from 1 to 10 ppm neither directly affected conidial germination of Botrytis cinerea nor indirectly affected it in the presence of nonsterile soil, even if the soil or the conidia were pretreated with ethylene or if the spores were depleted of nutrients by leaching.

II. Ethylene and Root Physiology

A. Growth and Developmental Effects

Plants respond to ethylene exposure in a multitude of ways as reviewed by Burg (1962), Pratt and Goeschl (1969), Abeles (1973).

Despite the voluminous literature, the effects of ethylene on root growth and development have remained unappreciated and until recently have been ignored by most researchers. The lack of a comprehensive knowledge of the role of ethylene in root growth is unfortunate, especially in light of the recent discovery that ethylene occurs in soil at physiologically active concentrations.

Generally there is a great consistency in the concentrations required for ethylene-mediated processes (Abeles 1973; Goeschl and Kays 1975). A concentration of 0.01 ppm is normally required to give a threshold response, 0.1 ppm for a half-maximal effect, and 1 ppm for a maximal response. The uniformity in the dose-response curves supports the view that the different effects of ethylene are all mediated by a single receptor. The fact that CO_2 acts as a competitive inhibitor of ethylene action furthermore suggests an affinity of a regulator for a receptor site.

Inhibition of root elongation has the same concentration dependency as other ethylene-mediated phenomena. The response is induced by a half-maximal concentration of 0.1 ppm and is reversed by 5-10% CO_2 (Chadwick and Burg 1967, 1970). The effect of ethylene is immediate and removal within 24 hours results in resumption of elongation.

A number of researchers have noted that concentrations of ethylene observed in waterlogged soils are considerably higher than

those required to inhibit root elongation (Smith and Russell 1969; Smith and Robertson 1971; Crossett and Campbell 1975). Smith and Robertson (1971) found that at the end of a 3-day exposure period, root elongation of barley was virtually inhibited at 10 ppm ethylene, reduced by 50% at 1 ppm, and slightly affected by 0.1 ppm. When ethylene was removed normal rates of root elongation resumed within 1-2 days.

Crossett and Campbell (1975) compared the growth and development of barley roots after short and long-term exposures to 1-10 ppm ethylene followed by a period of growth in an ethylene-free environment. Seminal root extension of barley was inhibited significantly at higher ethylene concentrations, while lateral root growth was stimulated. Transfer to an ethylene-free environment increased the rate of seminal root extension, the shorter the preceding ethylene exposure time the greater the recovery of root extension.

In addition to reducing elongation, ethylene also causes a characteristic coiling of roots (Curtis 1968; Cornforth and Stevens 1973). Cornforth and Stevens (1974) observed that ethylene treated roots of barley were shortened, swollen, and tightly curled. Root coiling appears to be due to an asymmetric growth inhibition. Chadwick and Burg (1967, 1970), have postulated that ethylene mediates root geotropism in this manner. They demonstrated that auxin-induced ethylene increased on the lower side of horizontal roots,

causing localized inhibition of root elongation and a bending of the root downward. Moderately high concentrations of CO_2 (10%) retarded this geotropic curvature. Since CO_2 is a competitive inhibitor of ethylene action, their data suggested that ethylene mediates root geotropism. Exogenously applied ethylene masked any gradient of ethylene in the root and caused diageotropic growth and root coiling to occur.

Ethylene can also cause swelling and hypertrophy of root cortex (Abeles 1973). The swelling of cortical cells occurs as a result of preferential lateral cell expansion at the expense of cell elongation (Chadwick and Burg, 1970). Hypertrophies in root cortical tissue appear to be due to the formation of cells outside the cork cambium. Harvey and Rose (1915) noted an abundance of cell proliferations on root systems of hybiscus exposed to illuminating gas. Sections of roots revealed that the proliferations were due to an increase in cell division of the phellogen layer. Ethylene-induced cork cells were unsuberized compared to those normally formed.

Similarly, ethylene promotes the proliferation of callus formation on damaged portions of roots. Chalutz and DeVay (1969) reported that ethylene stimulated callus formation at the cut surfaces of sweet potato roots. Orion and Minz (1969) noted that Ethrel, an ethylene releasing compound, increased the weight of root knot nematode galls on tomato roots, suggesting that ethylene enhanced the

proliferation of root tissue.

Ethylene stimulates the initiation of roots from leaves, stems, petioles, and pre-existing roots of both woody and herbaceous plants (Zimmerman and Hitchcock 1933). Zimmerman and Hitchcock (1933) observed that ethylene exposure of whole plants for 1-3 days followed by transfer to air was the most effective treatment to induce roots. In their system 10 ppm of ethylene was usually required for root induction, a relatively high concentration compared to other ethylene-induced phenomena. They also observed secondary root formation on the initial set of roots when follow up treatments of ethylene were applied.

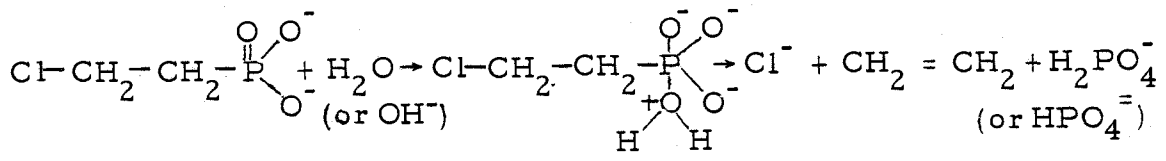
Crossett and Campbell (1975) also observed stimulation of root initiation when they exposed barley roots to various concentrations of ethylene, then removed them to an ethylene-free environment. They furthermore noted that the extension of lateral roots, initiated during ethylene treatment, was stimulated significantly.

B. Ethrel and Rooting of Cuttings

1. Mode of Action of Ethrel

Ethrel (Amchem, Ambler, Pa.), an ethylene releasing compound, has been used extensively in testing the effects of ethylene on rooting. The mode of action of the active ingredient in Ethrel, 2-chloroethyl phosphonic acid (ethephon), appears to be related to

its ability to release ethylene into plant tissue. The acid undergoes a chemical decomposition which is a base catalyzed elimination reaction (Yang 1969):



The rate of ethylene evolved increases with increasing pH. At pH 4.1 aqueous solutions of Ethrel yield small amounts of ethylene. The pH of the cell cytoplasm is greater than 4.1, thus the acid entering the plant is degraded (Amchem Technical Service Data Sheet H-96, 1969).

Ethylene is a gas that is five times more soluble in water than oxygen (Abeles 1973), and can move rapidly in the plant (Jackson and Campbell 1973). Ethrel is a liquid and is therefore not as mobile as ethylene. Kwong and Lagerstedt (1977), using labelled ethephon, demonstrated that it was absorbed by the roots of pea and bean and translocated to the most actively growing tissues. Radioactive ethylene was detected from all parts of the plant suggesting that ethephon was broken down along the pathway of translocation.

2. Rooting of Cuttings

According to Zimmerman and Hitchcock (1933) ethylene gas stimulated root formation of Salix cuttings. Kawase (1971) found that when cuttings of Salix alba were centrifuged in water, root formation was increased. Soaking cuttings also stimulated rooting, with

more roots formed at increasing depth, and the most formed by totally submerged cuttings. The result of submersion or centrifugation in water was an increase in ethylene in the cutting. Ethylene gas and Ethrel treatment of cuttings duplicated the stimulatory effects on rooting. Kawase suggested that all these treatments increased the ethylene concentration in Salix cuttings which in turn stimulated rooting.

In contrast, Mullins (1970) found that exposure of cuttings from dark grown mung bean seedlings to 1.0 ppm ethylene inhibited the formation of adventitious root primordia. Furthermore, Mullins re-examined the report of Zimmerman and Hitchcock (1933) that ethylene stimulated aerial root formation in seedlings of marigold. Marigold seedlings were enclosed in atmospheres of 1000 ppm ethylene for 48 hours then removed to the air. After 3-4 weeks numerous aerial roots grew from the hypocotyl portion of the stem. Examination of untreated hypocotyls revealed an abundance of preformed root initials. In etiolated mung bean cuttings no preformed root initials were observed and ethylene apparently inhibited the formation of new adventitious root primordia.

In a more recent study, Batten and Mullins (1978) examined the effect of Ethrel on rooting of dark grown mung bean cuttings. At very high concentrations (45 ppm of the active ingredient, ethephon), Ethrel had no significant effect on rooting of cuttings incubated in the dark.

Contrary to these findings, Krishnamoorthy (1970, 1972) reported that Ethrel, at a concentration of 10 ppm, increased the number of roots on etiolated mung bean hypocotyls incubated under continuous low light conditions. Roy et al. (1972) also showed that Ethrel promoted rooting of greenhouse grown mung bean and marigold cuttings when applied as a 0.5 ppm solution.

Swanson (1974) applied various concentrations of Ethrel to softwood cuttings of Prunus, Amorpha, Rhamnus, Forestiera, Juniperus, and Cotoneaster. Cuttings were treated with 3 minute soaks and then allowed to root in sand or peat-perlite for 60 days. Ethrel increased rooting success of all species examined, however, significant increases in root numbers were only observed for Forestiera.

In the above trials no attempt was made to control ethylene release from Ethrel by buffering the treatment solution. Because the release of ethylene from Ethrel is pH dependent, Mudge and Swanson (1978) tested the effect of Ethrel on rooting in buffered and nonbuffered solutions. Ethrel treatment, at the same concentrations used by Krishnamoorthy (1970, 1972) and Roy et al. (1972), increased tissue ethylene levels with increasing solution pH, but had no effect on rooting of etiolated mung bean cuttings incubated under 100% relative humidity conditions.

The results of Mudge and Swanson (1978) supported the findings of Batten and Mullins (1978), but conflicted with those of

Roy et al. (1972) and Krishnamoorthy (1970, 1972). Batten and Mullins used dark-grown seedlings and much higher concentrations of Ethrel than Krishnamoorthy and Roy et al., who used light-grown seedlings and low levels of Ethrel. Mudge and Swanson used light grown seedlings and low concentrations of Ethrel but conducted experiments under different humidity conditions. Taking these variables into consideration, Mudge and Swanson indicated that light effects and relative humidity influence rooting and could have accounted for the differences in rooting response to Ethrel.

Linkins et al. (1973), in another study on the role of ethylene in lateral root formation in bean stems, suggested that ethylene reduces the mechanical resistance of the cortex to emergence of root initials. Mudge and Swanson (1978) hypothesized that environmental conditions might affect the extent to which the cortex provides an effective mechanical barrier to root emergence. Cuttings in which cortical resistance is not limiting would not respond to ethylene treatment. In their experiment, increased cell turgor due to high relative humidity might have had an effect of overcoming resistance to root emergence, thus possibly explaining the lack of response to ethylene.

C. Auxin-Induced Ethylene Effects

The initial finding that auxin induced ethylene production was made in 1935 by Crocker et al. who observed that IAA and NAA

produced effects on plants similar to those caused by ethylene: epinasty, inhibition of growth, root induction, and cell hypertrophy. They hypothesized that growth effects attributed to auxins might be due indirectly to ethylene. Michner (1935, 1938) further investigated the phenomena of auxin-induced ethylene but concluded that no significant relationship existed. Unfortunately Michner's conclusions were accepted and the original discovery by Crocker and co-workers was ignored for about 30 years (Abeles 1973). Studies in the 1960's by Abeles and Rubinstein (1964) and Morgan and Hall (1962) confirmed that auxin treatment increased ethylene in plant tissue and that ethylene acts as an intermediate in a number of phenomena regulated by auxins.

It is now apparent that auxin-induced ethylene plays a role in root initiation, swelling of cortical cells (Zimmerman and Hitchcock 1933), inhibition of root elongation, and root tropistic responses (Chadwick and Burg 1967, 1970).

Chadwick and Burg (1967, 1970) presented evidence that auxin-regulated ethylene formation governs elongation of roots. They demonstrated that the kinetics of auxin-induced ethylene and auxin-induced inhibition of elongation were very similar. At low concentrations of IAA, the increase in ethylene production and decrease in elongation was short lived. Higher concentrations of IAA increased the duration of ethylene production and growth inhibition. Decreases in IAA

concentrations following initial application were correlated with a reduction in ethylene production and a return to normal growth rates.

Chadwick and Burg's observations generally supported the Cholodny-Went theory of geotropism which states that auxin levels increase on the lower side of roots. Chadwick and Burg hypothesized that auxin-induced ethylene production was responsible for root curvatures. Their support for this was that 5-10% CO_2, a competitive inhibitor of ethylene, retarded the geotropic response of horizontal pea roots. Applied ethylene masked the gradient of ethylene in the root and prevented the development of a normal geotropic curvature. The response to applied ethylene was also reversible as roots resumed normal geotropic growth when ethylene was removed.

The best evidence for the role of ethylene in root development is the work of Zobel (1973, 1974) who discovered a tomato mutant, diageotropica (dgt), deficient in auxin-induced ethylene biosynthetic capacity. The roots of dgt grew horizontally (plagiotropically) and failed to form lateral roots. Only Ethrel (10 ppm) and high concentrations of auxins (100 ppm) normalized the mutants root development. Normalization could also be achieved with ethylene exposures of 5 ppb, a concentration well below the threshold values for ethylene responses in most plants.

The response threshold of the dgt mutant to IAA treatments represented about a 10-fold decrease in normal sensitivity to auxins. The difference in IAA sensitivity was apparently due to a difference in the levels of auxin-induced ethylene produced, but not to increased destruction or conjugation of auxins. Treatments with auxin synergists, such as catechol, had little effect on the responses to IAA. It appeared, therefore, that the effect of the mutation was to cause a reduction in or elimination of auxin-induced ethylene synthesis (Zobel 1973).

Most of the research on effects of ethylene on plants has been carried out at concentrations in excess of 0.05 ppm. These concentrations are at least 10-fold greater than those necessary to normalize the tomato mutant. Zobel pointed out that the response of dgt to such extremely low concentrations of ethylene raises questions about the use of higher levels in previous research on ethylene-plant growth interactions.

III. Stress Ethylene and Plant Pathogenesis

A. Stress Ethylene

Ethylene production by plants increases rapidly following trauma by chemical toxins, temperature extremes, water stress, gamma irradiation, mechanical wounding, and disease (Abeles 1973). This

phenomena is commonly referred to as the wound response, and the product of the response is termed wound or stress ethylene. Stress ethylene is synthesized in living tissue by the same enzymatic system that controls normal ethylene production (Abeles and Abeles 1972). The primary function of stress ethylene seems to be the acceleration of abscission of damaged organs (Abeles 1973), but it may also play a role in disease resistance (Stahmann et al. 1966) and the growth of roots and shoots through soil (Kays et al. 1974; Goeschl et al. 1966).

Ethylene has been implicated in leaf abscission induced by environmental stress. Abscission of citrus fruits and leaves often follows a damaging freeze. Vines et al. (1968) reported that ethylene levels in grapefruit increased after freezing, and Young and Meredith (1971) noted a similar effect in freeze-injured citrus leaves. McMichael et al. (1972) found that the extent of leaf abscission in cotton was correlated with the increase in ethylene production during periods of drought stress. Jordan and coworkers (1972) furthermore demonstrated that increases in plant water stress caused leaves to become predisposed to the abscission-inducing effects of exogenously applied ethylene.

Waterlogging can rapidly affect the growth of plants, causing reduction in shoot extension, accelerated leaf and flower abscission, epinastic growth of leaves, and adventitious root production (Jackson

and Campbell 1979). Kramer (1951) and Jackson (1956) attributed the epinastic symptoms of tomato grown in waterlogged soils to ethylene. Kawase (1974) measured increased ethylene in roots of flooded sunflowers, which diffused through intercellular spaces into the shoots causing leaf epinasty, stem hypertrophy, and adventitious rooting. The increase of ethylene in flooded roots was thought to be caused by increased accumulation but not increased production.

Jackson and Campbell (1975, 1976) also showed that ethylene concentrations increased in roots of tomato during waterlogging and that ethylene moved from roots to stems. Low concentrations of oxygen in roots also promoted increases in ethylene in the shoot and epinasty of leaves. Bradford and Diley (1978) observed accelerated synthesis of ethylene in the shoots when roots were held under anaerobic conditions. Thus, oxygen deprivation may be the primary effect of soil flooding causing increased ethylene synthesis in the shoot.

Phytotoxins and hormones can stimulate ethylene production by affected tissue (Abeles 1973). Reports on the promotion of ethylene production by auxin in plant tissue are widespread (Abeles 1973). Phytotoxic compounds that increase ethylene production include organic salts, herbicides, and fungal exudates. Malformins, metabolic products of Aspergillus niger, are cyclopeptides capable of inducing ethylene production and causing abscission, epinasty and

root coiling (Curtis 1968). Fusaric acid, a fungal metabolite of several Fusarium species, also stimulates ethylene production by plant tissue (Wilson et al. 1978).

Because of the similarity in the response of plants to mechanical stresses and ethylene, it has been suggested that ethylene acts as an endogenous growth regulator. Goeschl et al. (1965) demonstrated that physical resistance to epicotyl elongation in peas caused a localized increase in ethylene evolution, a decrease in elongation, and an increase in radial expansion. The morphological effects of mechanical resistance were duplicated by ethylene applied at concentrations of 0.2 ppm or less. Based on their observations they proposed that ethylene plays a role as an endogenous growth regulator of seedling emergence through soil under conditions of nonwounding physical stress.

Similarly, Kays et al. (1975) found that when growth of bean roots was impeded by a physical barrier, the rate of ethylene evolution increased. When the barrier was removed, ethylene evolution decreased to near the rate of unimpeded roots. These results again suggested that ethylene synthesis may function as a growth regulating mechanism controlling growth of roots through soil.

B. Plant Pathogenesis

Increased ethylene production has been observed in plant

diseases caused by fungi, bacteria, and viruses (Archer and Hislop 1975). It appears that in some cases the host is the source of ethylene, while in others the pathogen. Because ethylene has a number of potential effects in the development of disease, it is difficult to distinguish its role in plant pathogenesis from its role as a product of plant stress or damage.

1. Production of Ethylene by Diseased Tissue

Since the initial report by Miller et al. (1940) demonstrating enhanced production by citrus fruits infected with Penicillium digitatum, diseased plants have been shown to evolve more ethylene than healthy plants in many cases. A number of disease symptoms, such as epinasty, abscission of leaves, chlorophyll breakdown, and adventitious root development, have been attributed to effects of ethylene.

Dimond and Waggoner (1953) were the first to conclude that petiolar epinasty in tomato plants infected with Fusarium oxysporum f. sp. lycopersici was due to ethylene. In a comparative study on defoliating and non-defoliating strains of Verticillium albo-atrum, Wiese and DeVay (1970) showed that infection of cotton with both strains increased ethylene production. The defoliating strain, however, induced production of more than twice as much ethylene as the non-defoliating strain. Exogenously applied ethylene reproduced the symptoms of the defoliating strain, causing epinasty and foliar abscission.

Early ripening of banana fruit infected with the bacterial pathogen,

Pseudomonas solancearum, suggested an ethylene effect. Freebairn and Buddenhagen (1964) detected high levels of ethylene in infected fruit, and found that strains of P. solanacearum produced large quantities of ethylene in vitro.

Levey and Marco (1976) demonstrated that epinasty of cucumber seedlings infected with Cucumber Mosaic Virus was due to ethylene. Exposure of healthy seedlings to ethylene reproduced epinastic symptoms, while exposure to hypobaric ventialtion to remove endogenous ethylene from diseased plants prevented symptom expression.

2. Source of Ethylene in Plant Pathogenesis

Increased ethylene produced by host-parasite interactions may be derived from either the infected host tissue, the pathogen, or both. While there is no definitive evidence for or against the contribution made by the pathogen, there are several studies suggesting that the host is largely responsible for ethylene production.

Hislop et al. (1971) examined the ethylene production from apple fruit infected with the brown rot fungus, Sclerotinia fructigena. Infected preclimacteric apples produced very large quantities of ethylene, but production by the fungus alone in vitro could not be demonstrated. Observations from infected plugs of apple tissue indicated that most ethylene produced came from the margin of the rot where hyphae penetrated healthy tissue. The rotted tissue, containing

large amounts of fungal mycelium, evolved little ethylene. Hislop et al. therefore concluded that the host produced ethylene in response to the infection by the fungus.

A similar case was reported by Lund (1971) for Erwinia carotovora infection of cauliflower floret tissue. Erwinia stimulated ethylene production in floret tissue, but the bacterium alone did not produce ethylene in vitro. Ethylene production by the host tissue could also be induced by bacterial culture filtrates which contained an enzyme, pectate lyase. Lund postulated that the bacterially produced enzyme increased the activity of host glucose oxidase, an enzyme required for the biosynthesis of ethylene from methionine in higher plants. While she recognized that it was possible that E. carotovora might produce ethylene in vitro under different cultural conditions than were tested, Lund felt that the ability of the bacterium to stimulate formation of ethylene was a more significant mechanism.

Virus diseases probably most convincingly demonstrate the role of the host in ethylene production. Work by Ross and Williamson (1951), Balázs et al. (1969), and Nagagaki et al. (1970) confirms that plants with local virus symptoms produce larger amounts of ethylene than those infected systemically, and that ethylene production coincides with the appearance of local necrotic lesions.

3. Ethylene and Disease Resistance

There has been much interest in the role of ethylene in the

increased or decreased susceptibility of plants to disease. It has been proposed that the rates of ethylene production from resistant and susceptible plants differ. Increased ethylene from the non-susceptible reaction has been correlated with the synthesis of defense compounds and the development of disease resistance. On the other hand, ethylene treatments have been shown to decrease the resistance of normally non-susceptible plants.

Stahmann et al. (1966) reported that non-pathogenic strains of Ceratocystis fimbriata induced more ethylene production by inoculated sweet potato than pathogenic strains. Furthermore, exposure of sweet potato tissue of a susceptible variety to ethylene induced resistance to infection by C. fimbriata and increased the activity of peroxidase and polyphenoloxidase. Based on these findings they suggested that increased ethylene synthesis in the resistant interaction may serve as a defense mechanism wherein ethylene stimulates host metabolism leading to a hypersensitive response.

The results of Stahmann et al. (1966) could not be confirmed by Chalutz and DeVay (1969). They challenged sweet potato roots with spores of Ceratocystis fimbriata before and after treatment with ethylene but found no differences in disease development.

Daly et al. (1970) found no correlation between disease resistance of wheat to infection by Puccinia graminis tritici and levels of peroxidase in the leaves. Ethylene treatment caused leaves of the

resistant variety of wheat to revert to a susceptible infection type even though ethylene induced additional peroxidase activity. Contrary to the observations of Stahmann et al. (1966) with sweet potato, Daly et al. demonstrated that infected leaves of the susceptible wheat produced more ethylene and higher levels of peroxidase than resistant wheat leaves. They concluded that total peroxidase activity is not causally related to resistance of wheat to rust.

Hislop and Stahmann (1971) reported that peroxidase and ethylene increased in response to inoculation with Erisyphe graminis f. sp. hordei in both susceptible and resistant barley leaves.

In addition to peroxidase, other enzymes and metabolic products increase following ethylene treatment. Chalutz and Stahmann (1972) showed that ethylene increased the production of pisatin, a phytoalexin involved in the natural resistance of peas. Similarly, Chalutz et al. (1969) reported that ethylene stimulated the production of an isocoumarin derivative, MMHD, in carrot disks and that continued synthesis of MMHD required the presence of ethylene. Jaworski et al. (1973) found that Ethrel not only induced MMHD synthesis in carrot disks, but also increased phenylalanine ammonia-lyase activity (PAL). Chalutz (1973) also demonstrated an increase in PAL activity in carrot disks after treatment with ethylene. Again the continuous presence of ethylene was required to maintain enzyme activity. The highest PAL activity occurred with 10 ppm ethylene treatment, and

CO_2 partially inhibited the ethylene induced activity.

PAL may play an important role in disease resistance in that it functions in the pathway from phenylalanine to a wide variety of plant phenols including phytoalexin compounds, such as the isocoumarins and pisatin. Although it is tempting to suggest that ethylene is the triggering agent for phytoalexin synthesis, Sequeira (1973) pointed out that phytoalexin production can be induced by a wide variety of chemical agents and that ethylene is not nearly as effective as some compounds. Thus, there seems little reason to single out ethylene as a specific inducer of phytoalexin production.

In some cases ethylene has been shown to increase disease susceptibility. Collins and Scheffer (1958) reported that ethylene enhanced infection of tomato plants inoculated with Fusarium oxysporum f. sp. lycopersici. Ethylene not only increased disease development in wilt-susceptible cuttings, but also caused normally resistant cuttings to show symptoms. Fusarium was isolated from all parts of ethylene-treated resistant cuttings, but was isolated from only the base of untreated cuttings. Daly et al. (1970) demonstrated that ethylene acted likewise in wheat infection by Puccinia graminis tritici. Ethylene caused leaves of the resistant variety of wheat to become completely susceptible to rust infection.

Cronshaw and Pegg (1976) identified a specific role for ethylene in disease enhancement. They discovered that pretreatment of

tomato leaves with 10 ppm ethylene, prior to treatment with culture filtrates of Verticillium albo-atrum containing toxins, increased wilting and necrosis of leaves. Fractions of the culture filtrate containing protein and pectolytic enzymes increased endogenous ethylene production when applied to leaves. They suggested that there is an interaction between ethylene and fungal toxins in early stages of pathogenesis whereby ethylene acts as a toxin synergist in Verticillium wilt of tomato.

Thus, it would seem that ethylene production in diseased tissue may trigger events leading to either disease resistance or disease enhancement, an indication that there exists a multiplicity of roles for ethylene in host-pathogen interactions.

IV. Ethylene as a Factor in the Development of Fusarium Disease

The earliest report on the involvement of ethylene in Fusarium disease was that of Dimond and Waggoner in 1953 who were the first to conclude that the disease symptoms in tomato plants infected with Fusarium oxysporum f. sp. lycopersici were due to ethylene. Although this early work was based entirely on bioassay and could not be regarded as quantitative, it clearly demonstrated that ethylene was produced by the diseased host and the fungus in culture and was responsible for the epinastic symptoms of Fusarium wilt. Disease

ethylene also induced the formation of root initials on the stem, but was not involved in the defoliation of the infected plant.

Since the studies of Dimond and Waggoner, other workers have examined the role of ethylene in Fusarium wilt of tomato in more detail. Collins and Scheffer (1958) confirmed the earlier findings and noted other effects of ethylene as well. Disease development was more rapid in an inoculated susceptible variety when treated with ethylene. Ethylene also broke the resistance of normally non-susceptible plants.

In a study by Gentile and Matta (1975) the rate of ethylene production from susceptible and resistant tomato plants infected with F. oxysporum f. sp. lycopersici was followed closely. In wilt-susceptible plants, infection increased ethylene production up to 10 days after inoculation which corresponded to the development of marked epinastic symptoms and basal leaf abscission. Changes in ethylene production were not observed in inoculated resistant plants or in plants infected with avirulent forms of F. oxysporum. Treatment of infected wilt-susceptible plants with ethylene increased the symptoms of disease.

Ethylene also plays a role in the development of Fusarium bulb rot of tulips. Tulip bulbs infected with Fusarium oxysporum f. sp. tulipae produced more ethylene than non-infected bulbs in cool storage (de Munk 1972). Ethylene was shown to be the cause of a number of

physiological disorders in tulips, including bud necrosis, flower-bud blasting, gummosis, and root and shoot inhibition.

A comparative study by Swart and Kamerbeek (1976) on the ethylene producing ability of Fusarium species and formae speciales revealed that all were capable of ethylene production to differing degrees. However, F. oxysporum f. sp. tulipae produced very high levels of ethylene, up to 5000 times more than other species examined. For this reason, Swart and Kamerbeek suggested that high ethylene production by the tulip Fusarium during the infection of bulbs might be a factor in the further development of disease.

Of further significance in the role of ethylene in the pathogenesis of tulips are the findings of Beijersbergen and Bergman (1973) who reported that de novo synthesis of the fungitoxic substance, tulipalin A, which may play a role in the resistance against infection by Fusarium, was inhibited by ethylene. They speculated that ethylene produced by Fusarium or by infections in bulb tissue may inhibit the build up of this compound near the site of infection.

V. Hormonal Factors Affecting Ectomycorrhizal Formation

Growth hormones produced extracellularly by ectomycorrhizal fungi may greatly influence growth and development of the host (Slankis 1973). Ectomycorrhizal fungi are known to produce auxins, cytokinins, gibberellins, B-vitamins (Slankis 1972), and auxin synergists

(Tomaszewski and Wojciechowska 1973). The ectomycorrhiza-like root morphology can be induced in roots by either exudates of ectomycorrhizal fungi or by applications of hormones (Slankis 1973). Furthermore, IAA and other compounds occur abundantly in mycorrhizal roots, but only traces in nonmycorrhizal roots (MacDougal and Dufrenoy 1944; Subba-Rao and Slankis 1959). These observations suggest that hormones supplied by the fungus are involved in the induction and establishment of the mycorrhizal relationship (Slankis 1973).

A. Production of Plant Growth Hormones by Ectomycorrhizal Fungi

1. Auxins

MacDougal and Dufrenoy (1944) reported the occurrence of abundant auxins in the mycorrhizae of Pinus radiata. Auxins were detected in the fungal hyphae and mantle, and were also present in the endodermis and pericycle of the root. The fact that auxins were not found in nonmycorrhizal roots was given as evidence that the fungus was the primary source of auxins in mycorrhizae.

In the late 1950's, Moser (1959) tested 23 species of ectomycorrhizal basidiomycetes for auxin production in vitro. As a nitrogen source and precursor for auxin production, he added large amounts of indolepropionic acid (IPA) and indolebutyric acid (IBA). Moser reported that some of the mycorrhizal fungi he tested were able to synthesize IAA in tryptophan deficient medium. When the medium was

amended with dl-alanine, l-asparagine, and indole, Suillus species were able to produce anthranilic acid and IAA. Later, Horak (1963, 1964) demonstrated that Boletus edulis formed tryptophan and IPA from indole and alanine. Ulrich (1960) also showed that Suillus vareigatus and S. granulatus synthesized IAA in a nutrient solution containing malt extract and glucose. However, other fungi she tested, including Boletus and Amanita species, required a supplement of tryptophan.

These studies revealed that the amounts and kinds of auxins produced vary greatly between species and even between strains. Ulrich (1960) found that Suillus variegatus produced IAA after 3 days in culture, whereas Amanita frostiana required up to 7 months to produce detectable amounts. She further noted that Suillus granulatus produced up to 15 times more auxin from tryptophan than S. variegatus. Moser (1959) examined 3 strains of Suillus grevellei and 12 strains of S. plorans, and found that strains of each species produced different amounts and kinds of auxins.

Apparently not all ectomycorrhizal fungi can produce auxin in vitro. Shemakhanova (1962) could not detect auxin-like substances in culture filtrates of Suillus granulatus, S. bovinus, S. luteus, Xerocomus subtomentosus, Amanita rubescens, and Cenococcum geophilum. Similar negative results were obtained by Wargo (1966) for cultures of Russula amigdaloides and Cenococcum geophilum.

2. Other Growth Substances

Miller (1967, 1971) demonstrated that mycorrhizal fungi produce and liberate cytokinins. Utilizing a cytokinin-requiring soybean callus bioassay, he detected cytokinins from cultures of Suillus cothurnatus, S. punctipes, Amanita rubescens, and Rhizopogon roseolus. Both zeatin and ribosylzeatin were crystallized from culture filtrates of R. roseolus. Not all fungi examined produced cytokinins; Cenococcum geophilum, Thelephora terrestris and several species in the genera Rhizopogon and Hebeloma failed to produce detectable amounts in the soybean callus bioassay.

Production of gibberellins and related compounds from culture filtrates, mycelium, and fruiting bodies of Boletus edulis var. pinicola was reported by Gogala (1971). She also detected one cytokinin thought to be zeatin.

Ectomycorrhizal fungi also synthesize and liberate vitamins. Shemakhanova (1962) found that nicotinic acid, biotin and pantohenic acid were produced by Suillus luteus, S. bovinus, Boletus edulis, B. scaber, and Paxillus involutus. Other fungi tested produced only biotin except Amanita pantherina which failed to produce biotin but did produce pyridoxine.

At present, no studies on ethylene production by ectomycorrhizal fungi have been reported.

B. Effect of Fungal Growth Hormones on Ectomycorrhizal Formation

Slankis (1972) observed that ectomycorrhiza formation depends on specific physiological and morphological conditions in the root. Indeed, there are no reports of intercellular infections occurring in either meristematic tissue of root apices, or in moribund or dead cells. For this reason, Marks and Foster (1972) demarcated a discrete zone, behind the growing apex and in advance of the moribund primary cortex, where mycorrhiza formation can occur. This region of the root, referred to as the mycorrhizal infection zone (MIZ), moves acropetally as the root grows. The extent of the MIZ, its rate of acropetal movement and the period for which it remains receptive are influenced by factors affecting root growth. Marks and Foster (1973) suggested that plant growth substances may have a profound effect on the morphogenesis of this zone, altering root growth and development.

Hatch's (1937) anatomical and morphological studies on pine defined certain structural characteristics of ectomycorrhizae including swelling of cortical cells, delayed senescence of cortex, lack of root hairs, and in pine, a characteristic dichotomy of short roots. Earlier work by Melin (1923, 1925) provided evidence in pure culture that the structural characteristics of pine mycorrhizae developed only in the presence of the symbiotic fungus. Later, Slankis (1948)

reported that excised roots of Pinus sylvestris grown aseptically in nutrient solution became dichotomously branched when inoculated with Suillus luteus and S. variegatus. Dichotomy was also induced by culture filtrates of these fungi.

Shemakanova (1962) reported that culture filtrates of Suillus luteus and S. bovinus induced dichotomy resembling ectomycorrhizae in both excised pine roots and roots of 10 week-old seedlings. Filtrate of S. bovinus, in particular, greatly stimulated lateral root initiation even on well developed, branched root systems. In addition to observations on pine roots, she also found that culture filtrates of these fungi promoted root formation in bean cuttings.

In studies investigating the effects of the growth promoting substances on pine roots, Slankis (1949, 1950, 1951) clearly demonstrated that short structures resembling simple, coralloid, and tuberculate mycorrhizae could be induced with synthetic auxins. These mycorrhiza-like roots were swollen due to radial expansion of cortical cells and were devoid of root hairs. To maintain these characteristic mycorrhiza-like structures a periodic supply of fungal exudates was required or the swollen apices began to elongate and the morphogenetic effect disappeared. Slankis (1951, 1958) concluded that auxin-like substances produced by the mycorrhizal fungus were responsible for the characteristic ectomycorrhizal root morphology.

Palmer (1954) likewise demonstrated that mycorrhiza-like root structures could be induced by synthetic auxins. IAA concentrations of 2 ppm induced dichotomy in about 5% of the short roots of one month-old Pinus virginiana. Concentrations of 10 ppm induced the maximum amount of dichotomy, whereas 20 ppm IAA induced prolific lateral root initiation. These results once again suggested that forking and proliferation of short roots are induced by fungus auxins or similar growth promoting substances.

Auxins have been reported to occur in great abundance in mycorrhizal roots but not in non-mycorrhizal roots. In mycorrhizae of Pinus radiata MacDougal and Dufrenoy (1944) detected auxins in the fungal hyphae and mantle as well as in the endodermis and pericycle of the root. In a later study, Subba-Rao and Slankis (1959) confirmed that mycorrhizal root tips of Pinus resinosa and P. strobus contained large amounts of unidentified indole compounds whereas uninfected root tips contained none.

In the creation of hyperauxiny in mycorrhiza formation some fungi produce so-called auxin synergists which inhibit the activity of auxin oxidases exuded by pine roots (Tomaszewski and Wojciechowska 1973). Tomaszewski and Wojciechowska (1973) showed that culture filtrates of Suillus variegatus contained up to 2×10^{-8} M equivalent of IAA. These filtrates induced mycorrhiza-like swellings that could be duplicated with IAA treatment, but only when very high concentrations

were supplied. They hypothesized that other substances present in fungal filtrates were acting as auxin protectants. When they analyzed ether extracts from culture filtrates of Suillus variegatus, S. bovinus, S. grevellei, and S. granulatus, they found polyphenol compounds, such as variegatic acid and boviquinones, which have well-established auxin-protecting properties (Haissig 1973).

Slankis (1976) summarized the role of auxins in ectomycorrhizal formation as follows: 1) auxins are produced by ectomycorrhizal fungi and mycorrhizal short roots of pine, 2) fungus exudates or synthetic auxins induce the formation of ectomycorrhiza-like short roots, 3) removal of auxins or fungus exudates results in reverse morphogenesis of short roots, 4) the ectomycorrhizal root morphology reflects a physiological state induced and maintained by fungus auxins. Slankis noted that cytokinins and auxin synergists may also regulate the establishment of mycorrhizae. He acknowledged that the possible interaction of different growth regulating substances in the formation of ectomycorrhiza greatly complicates the understanding of the hormonal mechanisms involved.

CHAPTER 3

ETHYLENE PRODUCTION BY ECTOMYCORRHIZAL FUNGI, *FUSARIUM OXYSPORUM* F. SP. *PINI*, AND BY ASEPTICALLY SYNTHESIZED ECTOMYCORRHIZAE AND *FUSARIUM*-INFECTED DOUGLAS-FIR ROOTS

J. H. Graham

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

and

R. G. Linderman

USDA SEA-AR Ornamental Plants Research Laboratory
Corvallis, Oregon 97330

ABSTRACT

The ectomycorrhizal fungi *Cenococcum geophilum*, *Hebeloma crustuliniforme* and *Laccaria laccata* produced ethylene *in vitro* in modified Melin-Norkrans liquid medium only if amended with 2.5 to 10 mM methionine; *Pisolithus tinctorius* failed to produce ethylene unless the cultures were renewed with fresh methionine-amended medium prior to ethylene assay. An additional 19 ectomycorrhizal fungi plus 5 isolates of *Fusarium oxysporum* f. sp. *pini* all produced ethylene in renewed and/or nonrenewed media. Although the rates varied, ethylene production by many ectomycorrhizal fungi equaled that of *Fusarium*.

Culture filtrates of *H. crustuliniforme* and *L. laccata* also

evolved ethylene that was apparently of nonenzymatic origin.

Ethylene was produced by aseptically grown Douglas-fir seedlings inoculated with C. geophilum, H. crustuliniforme, and L. laccata, and appearance of ethylene coincided with the formation of mycorrhizae; production by P. tinctorius-inoculated seedlings was inconsistent. Lateral root formation of Douglas-fir was stimulated by inoculation with C. geophilum, H. crustuliniforme and L. laccata, but was inhibited by P. tinctorius. Fusarium-inoculated seedlings produced more ethylene sooner than seedlings inoculated with mycorrhizal fungi. Thus, our data suggest that ethylene may function differently in the establishment of the mycorrhizal association compared to the development of Fusarium disease.

INTRODUCTION

Ethylene, a natural plant growth regulator, is produced by a wide variety of soilborne microorganisms including bacterial and fungal plant pathogens (Archer and Hislop 1975; Abeles 1973). Ethylene, evolved from soil and diseased plant tissues, has been implicated in a number of plant growth and development phenomena including inhibition of root elongation in waterlogged soil (Smith and Russell 1969), and development of disease symptoms such as petiolar epinasty (Dimond and Waggoner 1953), abscission (Williamson 1950), and early ripening of fruit (Freebairn and Buddenhagen 1964).

It is now well established that certain disease symptoms caused by Fusarium infection are due to ethylene (Gentile and Matta 1975). Bulbs infected with Fusarium oxysporum f. sp. tulipae evolve sufficiently high levels of ethylene to cause a number of physiological disorders in tulips (de Munk 1972). Swart and Kamerbeek (1976) reported that all Fusarium species and formae speciales they examined produced ethylene. F. oxysporum f. sp. tulipae produced the highest amounts, although isolates varied considerably.

Ethylene production by ectomycorrhizal fungi and mycorrhizae has not been examined, although a number of other plant hormones produced by ectomycorrhizal fungi, such as auxins and cytokinins, have been suggested as playing a key role in mycorrhiza formation (Slankis 1972, 1976). Apparently not all ectomycorrhizal fungi produce auxins (Shemakhanova 1962) or cytokinins (Miller 1971) in vitro, which raises the question of the universality of their role in ectomycorrhiza formation. In view of the similarity in effects of ethylene and auxins on root growth and development (Abeles 1973; Zobel 1973), and the widespread capability of fungi for ethylene production (Ilag and Curtis 1968), the role of ethylene in ectomycorrhiza formation needs to be characterized and contrasted with its function in disease development.

We therefore conducted comparative studies on ectomycorrhizal fungi and Fusarium oxysporum Schlecht. f. sp. pini (Hartig)

Synd. and Hans. to determine the ability of these fungi to produce ethylene *in vitro* and as a result of inoculation of aseptically grown Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco).

MATERIALS AND METHODS

Fungi

Cultures of ectomycorrhizal fungi were obtained from R. J. Molina, U. S. D. A. Forest Service, Corvallis, Oregon, and maintained on modified Melin-Norkrans (MMN) agar (Marx 1969). *Fusarium oxysporum* f. sp. *pini* was isolated from surface-sterilized and washed Douglas-fir seed, and infected and dying Douglas-fir seedlings. Isolates were tested for pathogenicity on Douglas-fir (Graham and Linderman, unpublished data) and maintained on V-8 juice (Toussoun and Nelson 1968) agar.

Experiment 1: Ethylene Production by Ectomycorrhizal Fungi and *Fusarium*

Ectomycorrhizal fungi, which had been grown in MMN liquid medium (Marx 1969) for 4-6 weeks, were prepared for experimental inoculations by aseptically filtering mycelium, and washing and resuspending the mycelium in 150-200 ml of sterile-distilled water (sdw). The suspension was then homogenized in a Waring blendor for 5-20 sec and 6-ml aliquots of the mycelial slurry, equivalent to 6-10 mg dry weight of mycelium, were pipetted into volume-calibrated 125 ml flasks containing 40 ml of MMN nutrient solution. *Fusarium*

oxysporum f. sp. pini, which had been grown on MMN agar for 7-10 days, was prepared by suspending 8 mm agar plugs containing macro- and microconidia and chlamydospores in 20 ml of sdw and shaking vigorously for 1 min. One-ml aliquots of the spore suspension (10^4-10^5 spores/ml) plus 5 ml of sdw were pipetted into the test flasks. Uninoculated control flasks received an equivalent amount of sdw to give the same total liquid volume. All treatments were incubated in the dark at 20°C for the requisite period of time before ethylene was assayed.

For the ethylene assay, flasks were flushed with sterile air in a laminar flow hood to remove any residual ethylene accumulated in the culture headspace, sealed with ethanol-sterilized serum stoppers, and incubated at 20°C in the dark. After 2 h a 1 cc gas sample was withdrawn and injected into a Perkin-Elmer 3920 gas chromatograph equipped with a flame-ionization detector and a 2.4 m poropak N (80-100 mesh) column operated isothermally at 80°C. Ethylene was identified by cochromatography with an ethylene-in-air standard. The quantity of ethylene produced in cultures was calculated as the total volume of ethylene in the headspace of treatment cultures minus the volume of ethylene produced by the control medium. Fungal biomass was determined as oven dry weight after 24 h at 70°C.

For treatments requiring renewal of the culture medium, mycelial cultures were transferred to tubes and centrifuged at 12,000 g for 15 min. The mycelial pellet was washed in sdw, recentrifuged

and transferred to a calibrated flask containing fresh medium and ethylene was assayed.

To identify the in vitro culture conditions required for ethylene production, four species of ectomycorrhizal fungi, Cenococcum geophilum Fr. (A-145), Hebeloma crustuliniforme (Bull. ex St. Am.) Quél (S-166), Laccaria laccata (Scop. ex Fr.) Berk. and Br. (S-167) and Pisolithus tinctorius (Pers.) Coker and Couch (S-210) were examined. To establish the role of methionine as a precursor for ethylene production, MMN liquid medium was amended with 0, 2.5, 5.0, 7.5 and 10.0 mM of DL-methionine. To determine the time of maximum ethylene production after inoculation, cultures were assayed over a period of 21 days. To eliminate effects of fungal metabolites accumulating in the culture medium during the incubation period, each culture was renewed with fresh medium prior to assaying for ethylene, and production from renewed and nonrenewed cultures was compared.

Having learned the culture conditions necessary to demonstrate ethylene production by the four test species, we then surveyed 19 additional ectomycorrhizal fungi and 5 isolates of Fusarium oxysporum f. sp. pini.

Ethylene evolution from cell-free culture filtrates was determined for two ectomycorrhizal fungi, H. crustuliniforme (S-166) and L. laccata (S-167). Ethylene production by fungus plus filtrate was assayed after 7 days incubation. Cultures were then filtered (0.45 μ

Millipore) to remove the mycelial fraction, and one-half of the cell-free cultures were assayed immediately. To identify whether or not the source of ethylene was enzymic, the remaining one-half of the cell-free cultures were aseptically transferred to test tubes, incubated at 60°C for 1 h, and cooled to 20°C. The heat-treated filtrates were then transferred to flasks and assayed immediately. For further assay, cell-free culture treatments were incubated at 20°C and ethylene evolution determined at 7 and 14 days.

Experiment 2: Ethylene Production by Ectomycorrhizae and Fusarium-Infected Douglas-fir

Aseptic Douglas-fir seedlings were grown in large test tubes (200X 32 mm), filled with 60 cc vermiculite and 5 cc finely ground peatmoss, mixed thoroughly, and moistened with 45 ml of MMN nutrient solution. The tubes were capped with 50 ml glass beakers and autoclaved at 121°C for 30 min.

Washed Douglas-fir seed was surface-sterilized in 30% H_2O_2 for 1 h and germinated on MMN agar. Germlings were transplanted into the culture tubes and the root portion of the tube was wrapped in foil to exclude light. To direct root growth along the side of the tube, cultures were incubated on a slant in a growth chamber under fluorescent-incandescent lights (250 $\mu E \cdot m^{-2} \cdot sec^{-1}$ at 400-700 nm) set on a 16 h photoperiod and 25/15°C day-night temperature regime. After one month, healthy seedlings, free of visible contamination, were

selected for inoculation.

To determine the production of ethylene by the fungus-host tree interaction, the same four ectomycorrhizal species examined in Experiment 1 and one isolate of F. oxysporum f. sp. pini (DF-4) were chosen for study. Aseptic cultures with and without Douglas-fir seedlings were inoculated with 10 ml blended mycelium of ectomycorrhizal fungi or 10 ml of Fusarium spore suspension prepared as described in Experiment 1. Uninoculated controls received an equal volume of sdw.

For the ethylene assay, culture tubes were sealed with ethanol-sterilized serum stoppers and incubated under growth chamber conditions. After 24 h a 1 cc gas sample was withdrawn and analyzed as described in Experiment 1. The quantity of ethylene produced in the treatments was calculated as the concentration of ethylene (ppm) in fungus-tree cultures minus the concentrations in the respective fungus culture alone and tree culture alone combined. Starting at time of inoculation, ethylene was assayed at 2 week intervals during a 14 week period.

Mycorrhizal and control tree cultures were harvested at 16 weeks and total number of short roots less than 2 cm in length, number of mycorrhiza-mantled short roots, and seedling dry weight (70°C/24 h) were determined. For confirmation of ectomycorrhiza formation, mycorrhizal tips were collected, fixed in 50% FAA,

embedded and sectioned in paraffin, and stained with safranin followed by fast green (Johansen 1940).

RESULTS

Experiment 1: Ethylene Production by Ectomycorrhizal Fungi and Fusarium

Cenococcum geophilum, H. crustuliniforme and L. laccata produced ethylene in MMN liquid medium only if amended with DL-methionine (Fig. 1). The levels of ethylene produced by L. laccata and H. crustuliniforme were similar and reached a maximum at 7.5 mM methionine; production by C. geophilum was 100 times lower with a maximum at 2.5 mM; P. tinctorius failed to produce ethylene at any methionine concentration tested. For further treatments, MMN medium was amended with a 5 mM concentration of DL-methionine.

Ethylene production levels peaked within 7 days after inoculation and by 21 days decreased to about 50% of the maximum level (Fig. 2). P. tinctorius only produced ethylene at the time of inoculation, suggesting that fungal metabolites accumulating in the medium over time might be inhibitory to ethylene production by this fungus.

When cultures were renewed with a fresh medium prior to the ethylene assay, P. tinctorius was stimulated to produce ethylene at 7, 14 and 21 days after inoculation (Fig. 3). Culture renewal also significantly increased ethylene production by C. geophilum, but decreased production by H. crustuliniforme and L. laccata.

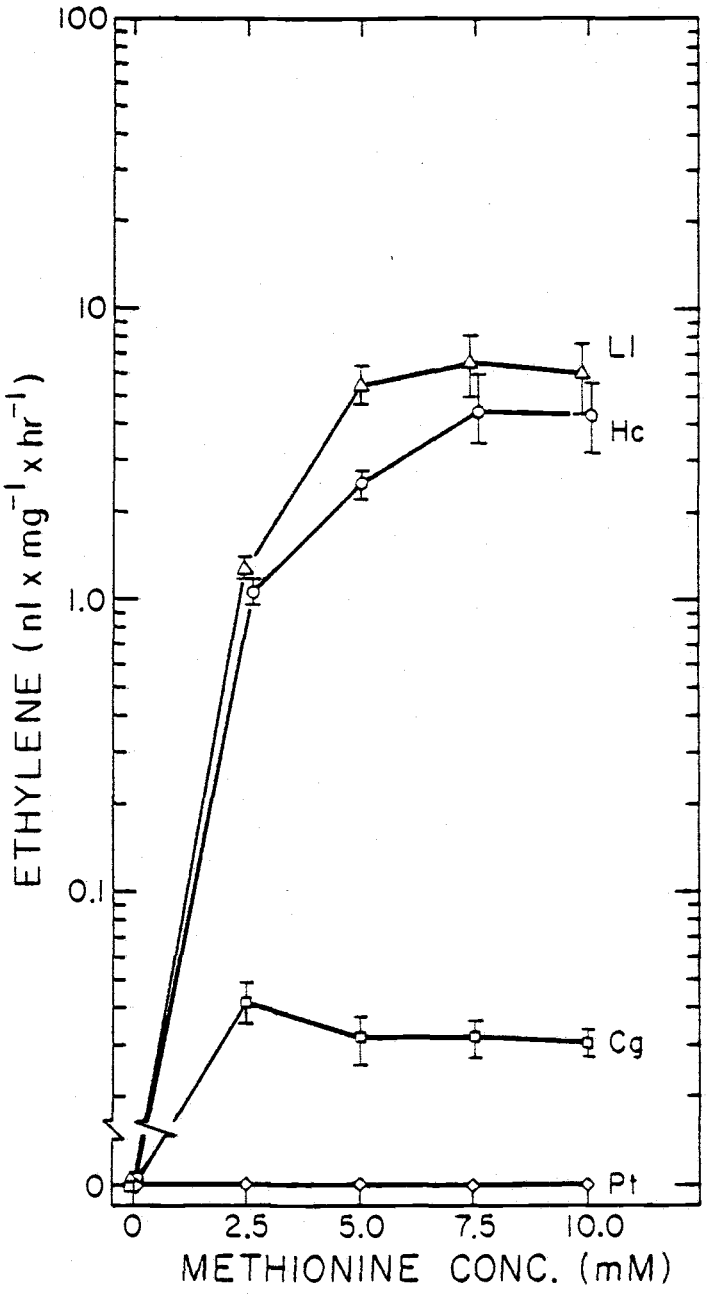


Figure 1. Effect of methionine on ethylene production by ectomycorrhizal fungi grown in MMN liquid medium for 21 days. Cenococcum geophilum (Cg), Hebeloma crustuliniforme (Hc), Laccaria laccata (Ll), Pisolithus tinctorius (Pt). Error bars represent the standard deviation of the mean of 5 replications.

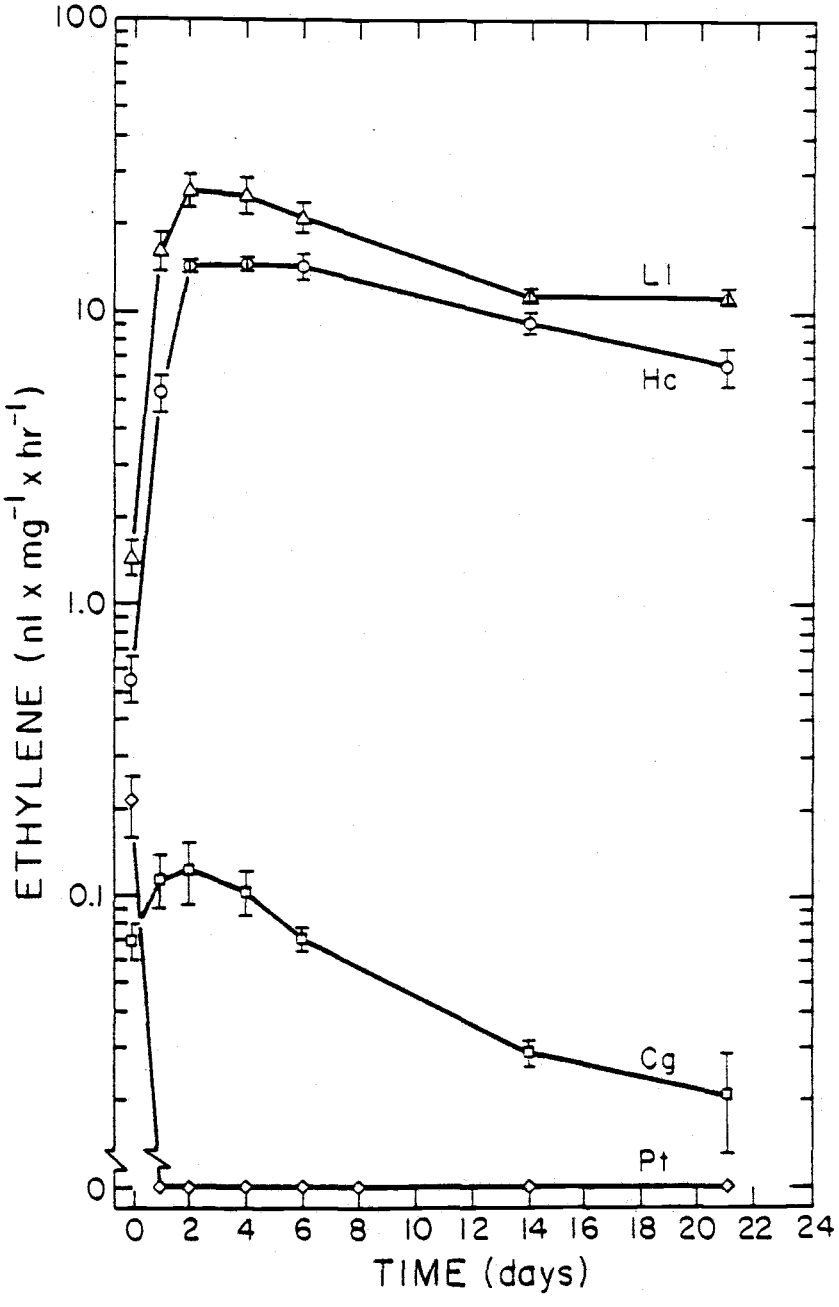


Figure 2. Production of ethylene by ectomycorrhizal fungi during a 21 day period. Fungi were grown in 5 mM methionine-amended MMN liquid medium. Cenococcum geophilum (Cg), Hebeloma crustuliniforme (Hc), Laccaria laccata (L1), Pisolithus tinctorius (Pt). Error bars represent the standard deviation of the mean of 5 replications.

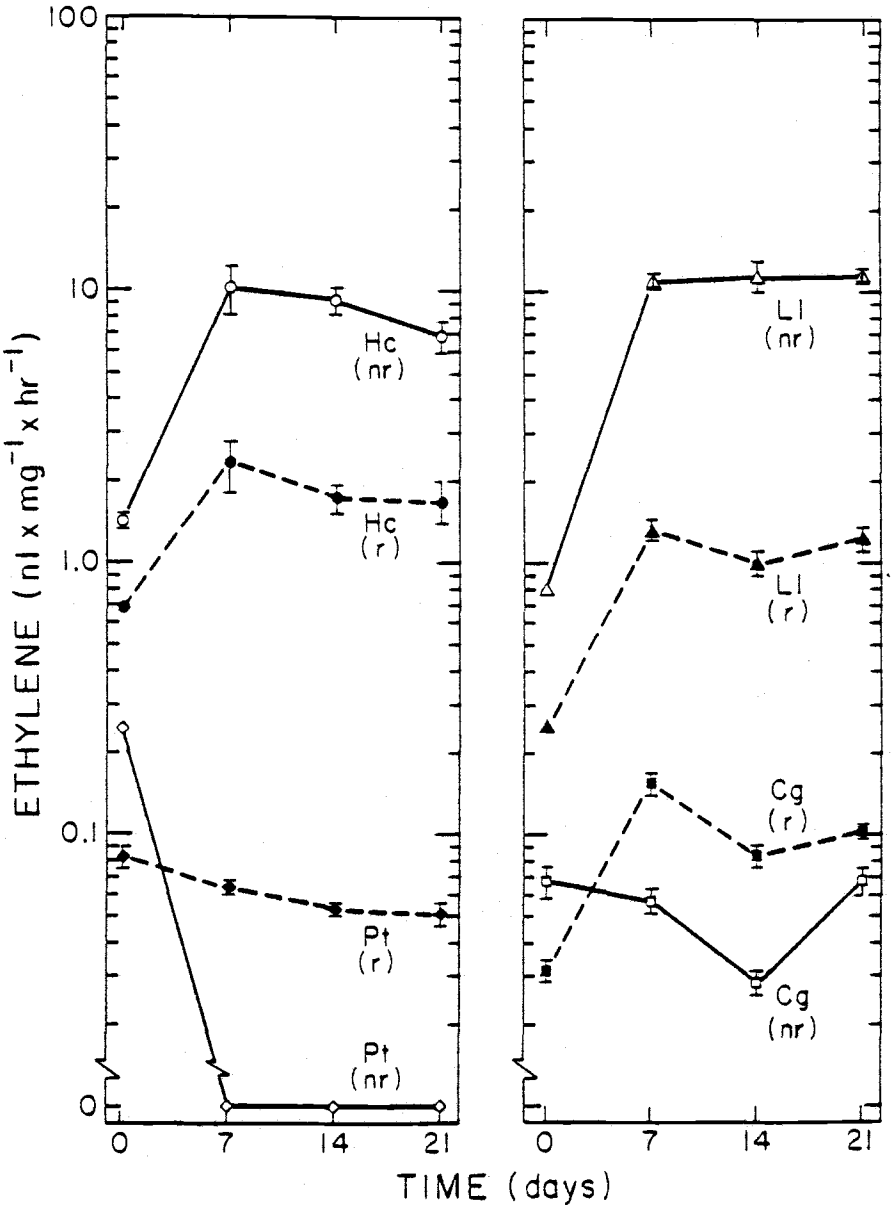


Figure 3. Effect of renewal of the culture medium on ethylene production by ectomycorrhizal fungi during a 21 day period. Fungi were grown in 5 mM methionine-amended MMN liquid medium. For renewed treatments the culture media was replaced with fresh medium prior to ethylene assay. Nonrenewed (nr), renewed (r), Cenococcum geophilum (Cg), Hebeloma crustuliniforme (Hc), Laccaria laccata (Ll), Pisolithus tinctorius (Pt). Error bars represent the standard deviation of the mean of 5 replications.

All of the additional 19 ectomycorrhizal fungi tested in renewed and nonrenewed cultures produced ethylene. Amounts of ethylene produced by three isolates each of H. crustuliniforme and L. laccata varied only slightly. All isolates of P. tinctorius required culture renewal to produce ethylene, whereas isolates of C. geophilum differed in their requirement for culture renewal (Fig. 4). Suillus species also varied in their cultural requirements for ethylene production but Rhizopogon species did not (Fig. 5). The levels of ethylene production by F. oxysporum f. sp. pini isolates were similar and comparable to the most productive mycorrhizal fungi (Fig. 6). A number of mycorrhizal species (Rhizopogon versisporus, R. abietis, Amanita pantherina and Cortinarius elegantior) behaved like C. geophilum isolate A-145 in that culture renewal increased ethylene production.

In many cases culture renewal decreased ethylene production suggesting that the filtrate contributed to production. Ethylene evolved by cell-free culture filtrates of H. crustuliniforme and L. laccata represented a substantial proportion (> 50%) of the total ethylene evolved by the fungus cultures (Table 1). Heating the filtrate at 60°C to nullify extracellular enzyme activity did not lower ethylene production.

Experiment 2: Ethylene Production by Ectomycorrhizae and Fusarium-Infected Douglas-fir

Aseptic Douglas-fir cultures inoculated with ectomycorrhizal fungi or F. oxysporum f. sp. pini produced significantly more ethylene

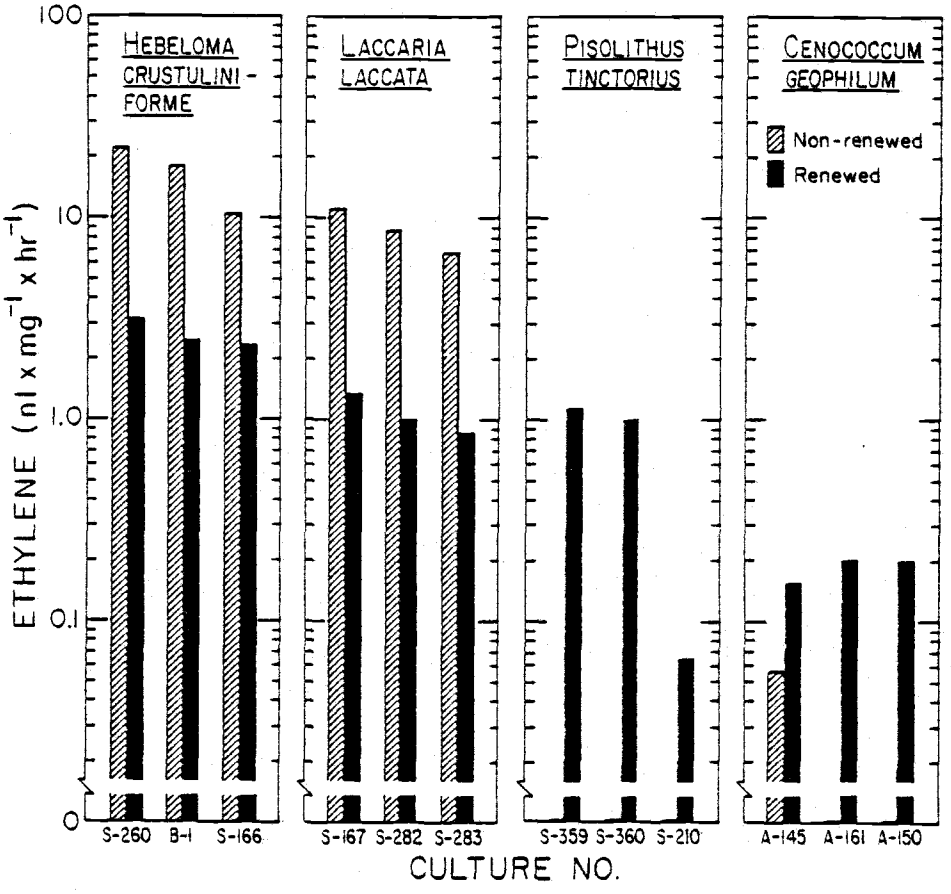


Figure 4. Ethylene production by selected isolates of Cenococcum geophilum, Hebeloma crustuliniforme, Laccaria laccata, and Pisolithus tinctorius. Fungi were grown in 5 mM methionine-amended MMN liquid medium for 7 days, and ethylene assayed in renewed and nonrenewed cultures. Each ethylene level is the mean of 5 replications.

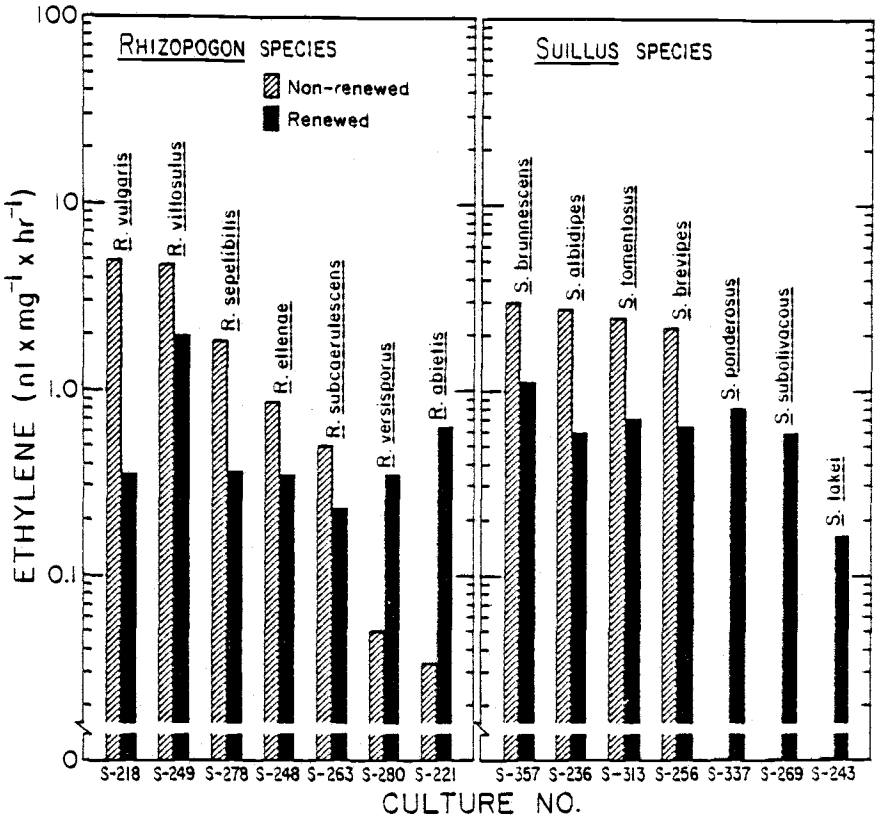


Figure 5. Ethylene production by selected species of Rhizopogon and Suillus. Fungi were grown in 5 mM methionine-amended MMN liquid medium for 7 days, and ethylene assayed in renewed and nonrenewed cultures. Each ethylene level is the mean of 5 replications.

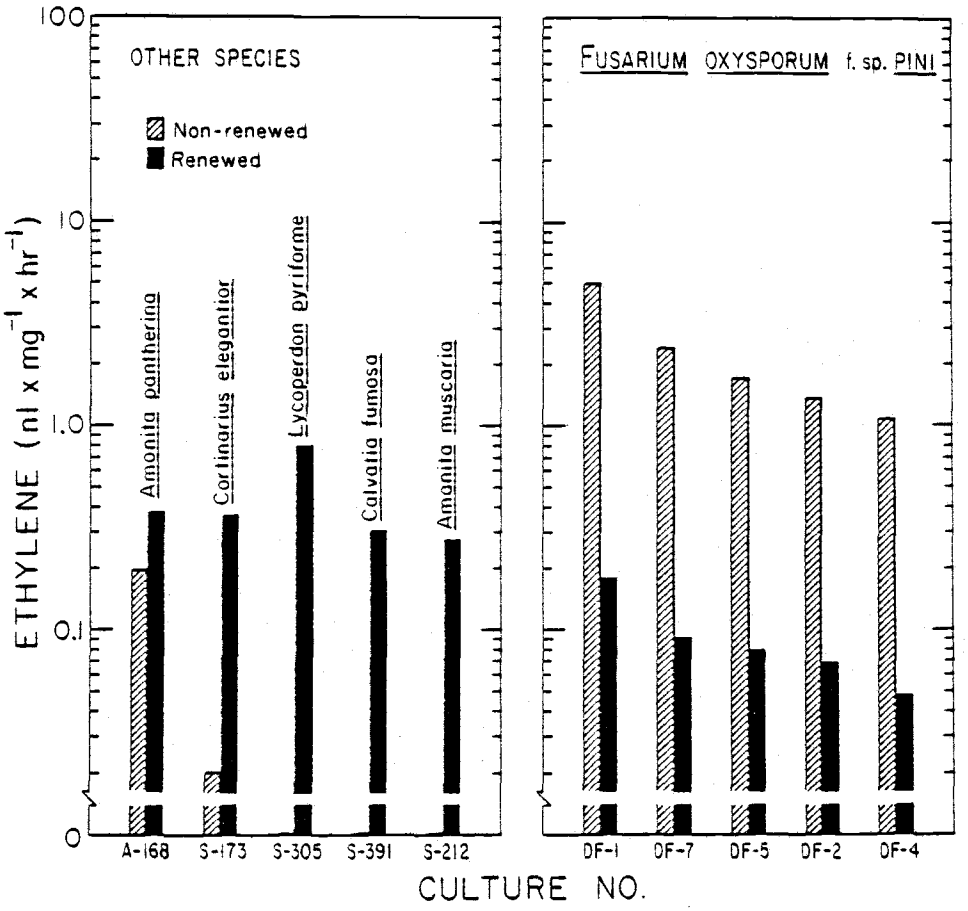


Figure 6. Ethylene production by selected ectomycorrhizal fungi and isolates of Fusarium oxysporum f. sp. pini. Fungi were grown in 5 mM methionine-amended MMN liquid medium for 7 days, and ethylene assayed in renewed and nonrenewed cultures. Each ethylene level is the mean of 5 replications.

Table 1. Comparison of ethylene evolution from cultures and cell-free culture filtrates of Hebeloma crustuliniforme (S-166) and Laccaria laccata (S-167).

Treatment	7 day[a]		14 day[a]		21 day[a]	
	S-166	S-167	S-166	S-167	S-166	S-167
Fungus and filtrate	43.4[b]	49.9	--	--	--	--
Filtrate	28.9	37.5	5.4	8.8	0	0
Heated Filtrate	29.4	36.9	5.0	9.1	0	0
% Contribution from Filtrate[c]	66.4	75.2	--	--	--	--

[a]Days after time of inoculation.

[b]Ethylene evolved in nl/h. Values are the mean of 5 replications.

[c]Percent contribution of filtrate to total ethylene evolved from fungus plus filtrate.

than the total of fungus alone and seedling alone cultures (Fig. 7). Time of ethylene production by seedlings inoculated with C. geophilum, H. crustuliniforme and L. laccata coincided with formation of mycorrhizal short roots. Production by P. tinctorius-inoculated seedlings was inconsistent. Fusarium-inoculated seedlings produced more ethylene 1 week after inoculation than mycorrhizal fungus-inoculated seedlings did at any time during the 14 week assay period. Ethylene production continued to increase up to 4 weeks after inoculation and was correlated with the colonization of the root cortex and development of stunting, chlorosis, and wilting of the top. After 4 weeks, ethylene production decreased as a result of seedling mortality.

Seedlings inoculated with the four different mycorrhizal fungi showed no differences in dry weight or percent mycorrhizal formation, but C. geophilum, H. crustuliniforme and L. laccata stimulated lateral root formation, while P. tinctorius inhibited compared to uninoculated controls (Table 2). Transverse sections of Pisolithus-mantled roots revealed the absence of a Hartig net, while the other three fungi formed a Hartig net between 1 to 3 layers of cortical cells.

DISCUSSION

Ectomycorrhizal fungi and F. oxysporum f. sp. pini required methionine for ethylene production in MMN liquid medium containing mineral salts and glucose. Glucose and methionine are the

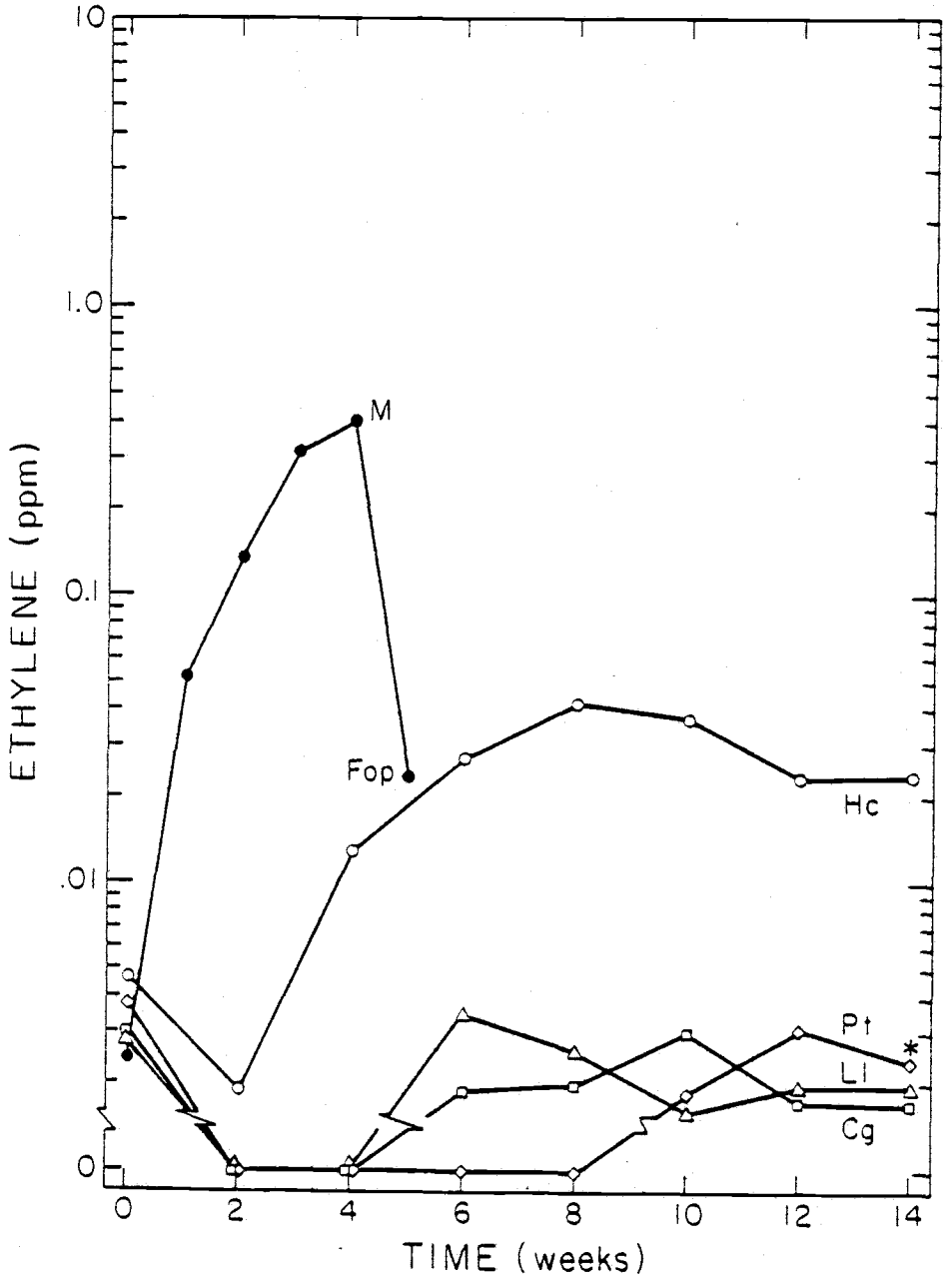


Figure 7. Ethylene production by aseptically synthesized ectomycorrhizae and Fusarium-infected roots of Douglas-fir. Cenococcum geophilum (Cg), Hebeloma crustuliniforme (Hc), Laccaria laccata (Ll), Pisolithus tinctorius (Pt), Fusarium oxysporum f. sp. pini (Fop). M indicates time of tree mortality in Fusarium-inoculated cultures. All ethylene levels greater than zero are significantly different from the controls at the .01 level except (*).

Table 2. Effect of ectomycorrhizal fungi on growth and development of Douglas-fir in aseptic culture.

Fungal Treatment	Seedling Dry Weight (g)	% Mycorrhizal Short Roots	Short Root Count
Uninoculated control	.33 a*	--	210 a*
Cenococcum geophilum	.31 a	47 a*	293 b
Hebeloma crustuliniforme	.33 a	55 a	358 b
Laccaria laccata	.32 a	41 a	287 b
Pisolithus tinctorius	.30 a	65 a	121 c

*Comparison of means of 7 replications by Duncan's multiple range test; column values followed by the same letter are not significantly different at the .01 level.

precursors for ethylene production by a number of fungi and bacteria (Lynch and Harper 1974; Chalutz et al. 1977; Primrose 1976a).

Several ectomycorrhizal fungi also required renewal of the culture medium to induce or enhance ethylene production, suggesting that fungal metabolites accumulating in the medium during incubation may be inhibitory. Problems in assessing microbial ethylene production in liquid cultures over extended incubation periods have been noted by other workers (Lynch and Harper 1974; Bonn et al. 1975). In our experiments the effect of fungal metabolites was minimized by assaying for ethylene production in fresh culture medium.

In many cases, culture renewal decreased ethylene production, an indication that the culture filtrate was a source of ethylene. Cell-free culture filtrates of H. crustuliniforme and L. laccata evolved substantial quantities of ethylene and contributed to over half of the ethylene produced by fungus plus filtrate. Ethylene from filtrates appeared to be of nonenzymatic origin because ethylene evolution was unaffected by a heat-treatment that would nullify enzymatic activity.

Nonenzymatic release of ethylene from cell-free filtrates has been reported for both fungi and bacteria. Lynch (1974) suggested that Mucor hiemalis released flavin compounds into the culture medium which act as a cofactor in the photochemical conversion of methionine to ethylene (Yang et al. 1967). Primrose (1976b) identified several

isolates of bacteria that exuded pigments with spectral properties of flavins into the culture medium. Cell-free filtrates from these cultures released ethylene more rapidly than filtrates from bacterial isolates not exuding pigment. Chalutz et al. (1977) reported that filtrates of Penicillium digitatum evolved ethylene by both enzymatic and nonenzymatic reactions. Boiling the filtrate or treating with protein-degrading enzyme reduced ethylene formation by over 50%. Tracer experiments indicated that the fungus took up methionine and released a metabolite which was then converted to ethylene. Chalutz et al. suggested that the ethylene-evolving systems in cell-free filtrates of fungi and bacteria may be similar but require further investigation.

Ethylene was also produced by aseptically synthesized ectomycorrhizae of Douglas-fir. Inoculation with C. geophilum, H. crustuliniforme and L. laccata stimulated lateral root development on Douglas-fir, but P. tinctorius inhibited formation. The absence of a Hartig net in P. tinctorius-mantled short roots indicated that a functional mycorrhizal association was not formed (Marks and Foster 1972). Thus, the lack of a clear ethylene response may relate to the failure of P. tinctorius to form a true mycorrhizal association.

Our studies have shown that ethylene is produced by ectomycorrhizal fungi in culture and in the mycorrhizal association. Methionine, the precursor for ethylene production by ectomycorrhizal fungi and their culture filtrates, is in root exudate of ectomycorrhizal hosts

such as Pinus radiata (Bowen, cited in Rovira 1965) and Eucalyptus calophylla (Malajczuk and McComb 1977). Thus, ethylene formation by mycorrhizal fungi in the rhizosphere or in mycorrhizal association may depend on a supply of methionine from plant roots.

Culture filtrates of mycorrhizal fungus and synthetic auxins are known to stimulate root initiation (Slankis 1972). However, ethylene has root growth promoting properties similar to auxins (Abeles 1973; Zobel 1973). We found that when a functional mycorrhizal association was formed, low levels of ethylene were produced and lateral root formation increased. These observations suggest that there is a relationship between ethylene, lateral root formation, and establishment of ectomycorrhizae.

Compared to mycorrhiza formation, the role of ethylene in Fusarium disease development has been well characterized (Gentile and Matta 1975; de Munk 1972). Douglas-fir seedlings infected with Fusarium produced much more ethylene than mycorrhizal seedlings. High levels of ethylene associated with Fusarium disease may function in lowering the disease resistance of the host as shown with Fusarium wilt of tomato (Collins and Scheffer 1958).

The disparity in the levels of ethylene associated with mycorrhiza formation compared to Fusarium infection suggests a differential role for ethylene in symbiotic and pathogenic fungus-host interactions. Further comparative studies will examine the effect of

ethylene on ectomycorrhizae and Fusarium disease of Douglas-fir in more detail.

ACKNOWLEDGMENT

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CHAPTER 4

EFFECT OF ETHYLENE ON ROOT GROWTH, ECTOMYCORRHIZA FORMATION, AND *FUSARIUM* INFECTION OF DOUGLAS-FIR

J. H. Graham

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

and

R. G. Linderman

USDA SEA-AR Ornamental Plants Research Laboratory
Corvallis, Oregon 97330

ABSTRACT

Two-month-old Douglas-fir seedlings were exposed to six ethylene concentrations, ranging from 0.006 (soil ambient control) up to 0.5 ppm, established by adding Ethrel, an ethylene-releasing compound, as a soil drench to the root zone. Exposure of roots to the different ethylene concentrations for 2 months either stimulated (0.01-0.05 ppm), had no effect on (0.05-0.15 ppm), or inhibited (> 0.15 ppm) lateral root formation. Root dry weight increased and shoot dry weight decreased as the ethylene concentration was increased.

Mycorrhiza formation by *Hebeloma crustuliniforme* was not increased by exposing inoculated 4-month-old seedlings to 0.1 ppm ethylene for 3 months whether or not the seedlings had been exposed

to ethylene for two months prior to inoculation. By contrast, when 2-month-old seedlings pre-inoculated with Fusarium oxysporum f. sp. pini were exposed to 0.1 ppm ethylene for 2 months, disease was significantly increased. These studies suggest that ethylene may have a differential role in the host response to a root pathogen compared to a mycorrhizal fungus.

INTRODUCTION

Plant roots respond to ethylene exposure in many ways (Abeles 1973). Ethylene reportedly plays a role in lateral root initiation, swelling of cortical cells (Zimmerman and Hitchcock 1933), inhibition of root elongation, and geotropic responses (Chadwick and Burg 1970).

Studies by Zobel (1973) on the ethylene-requiring tomato mutant, diagetropica, suggest that auxin-induced ethylene may be responsible for the control of root growth and development. Roots of diageotropica grow horizontally and fail to form lateral roots unless treated with as little as 5 ppb ethylene or high concentrations of auxin (100 ppm). Ethrel, an ethylene-releasing compound, at concentrations of 10 ppm, also normalizes root tropism and lateral root formation. Thus, ethylene may act as an intermediate in root growth phenomena regulated by auxins (Abeles 1973).

Slankis (1972) observed that morphological changes resembling ectomycorrhizae, such as swelling of cortical cells and

lateral root initiation, occur when roots of pine are treated with mycorrhizal fungus exudates or synthetic auxins. He suggested that fungus auxins are responsible for the induction and maintenance of the ectomycorrhizal root morphology. In view of the similarities in the effect of ethylene and auxins on root growth and development, the role of the ethylene in the morphological response of roots to mycorrhizal formation should be characterized.

Ethylene has also been reported to both increase and decrease the susceptibility of plants to fungal infection (Archer and Hislop 1975). Increased ethylene from non-susceptible host-fungus interactions has been correlated with the synthesis of defense compounds, such as phytoalexins (Chalutz et al. 1969; Jaworski et al. 1973), and the development of disease resistance (Stahmann et al. 1966). In contrast, ethylene treatment has been shown to increase the disease susceptibility of normally resistant plants (Collins and Scheffer 1958; Daly et al. 1970).

The apparent multiple roles for ethylene in host-fungus interactions need further investigation. Accordingly, we determined the effects of ethylene on root growth of Douglas-fir, Pseudotsuga menziesii (Mirb.) Franco, and conducted comparative studies on the influence of ethylene on host response to mycorrhiza formation and Fusarium infection.

MATERIALS AND METHODS

Experiment 1: Effect of Ethylene on Root Growth

The effect of exogenously applied ethylene on root growth of containerized Douglas-fir was examined. Twenty-four blocks, each with fifty 65 cm^3 Leach Fir cells (Leach Cone-Tainer Nursery, Aurora, Oregon), were filled with a steam-pasteurized (70°C/30 min) 1:1:1 (v:v) peat-vermiculite-sand mix (pH 6.0). Douglas-fir seed was prepared for sowing by washing in running tap water for 24 h and then surface-sterilizing in 30% H_2O_2 for 30 min. Three seeds per cell were sown and germlings thinned to one per cell. Randomized blocks of seedlings were grown for the duration of the experiment in a growth chamber under fluorescent-incandescent lights (350 $\mu E \cdot m^{-2} \cdot sec^{-1}$ at 400-700 nm) set on a 16 h photoperiod and 25/15°C day-night temperature regime. Starting at 4 weeks, seedlings were fertilized weekly with 6 ml of nutrient solution of the following composition: NH_4NO_3, 286 ppm; KH_2PO_4, 264 ppm; $MgSO_4 \cdot 7H_2O$, 247 ppm; $CaCl_2 \cdot 2H_2O$, 147 ppm; Fe sequestrene 330, 30 ppm; and Hoagland's micronutrients.

For ethylene exposure, 4 blocks of fifty 2-month-old seedlings were assigned to each of six ethylene treatments. Ethylene concentrations were established in the root zone of seedlings by adding Ethrel (Amchem, Ambler, Pa.), an ethylene-releasing compound, as a soil drench. Ethrel, diluted with 1/4-strength nutrient solution,

was applied in 6 ml aliquots at concentrations of 0.15 to 12.5 ppm to achieve the ethylene levels desired. Ethylene release from Ethrel was determined by inserting a syringe needle into the middle of the soil core, withdrawing a 1 cc gas sample, and injecting the sample into a Perkin-Elmer 3920 gas chromatograph equipped with a flame-ionization detector and a 2.4 m poropak N (80-100 mesh) column operated isothermally at 80°C. Ethylene was identified by co-chromatography with an ethylene-in-air standard. The rate of ethylene release into the soil medium was determined by sampling every 1-4 h for 3 days. In peat-vermiculite-sand, ethylene release from Ethrel occurred over a 48 h period after which time Ethrel was again added and the cycle of release repeated (Fig. 1B). Ethylene levels in each treatment were monitored at weekly intervals for 2 months by sampling 5 randomly selected seedlings 6 h after Ethrel addition. Seedlings were supplementally watered by subirrigation on alternating days with Ethrel treatment.

After 2 months, 80 seedlings per treatment (20 seedlings per block) were randomly selected for harvest. Symptoms of ethylene exposure were noted, lateral roots less 2 cm long counted, and oven dry weight (70°C/48 h) of roots and shoots determined. For anatomical evaluation of ethylene effects on root growth, root tissue was fixed in 50% FAA, embedded and sectioned in paraffin, and stained with safranin followed by fast green (Johansen 1940).

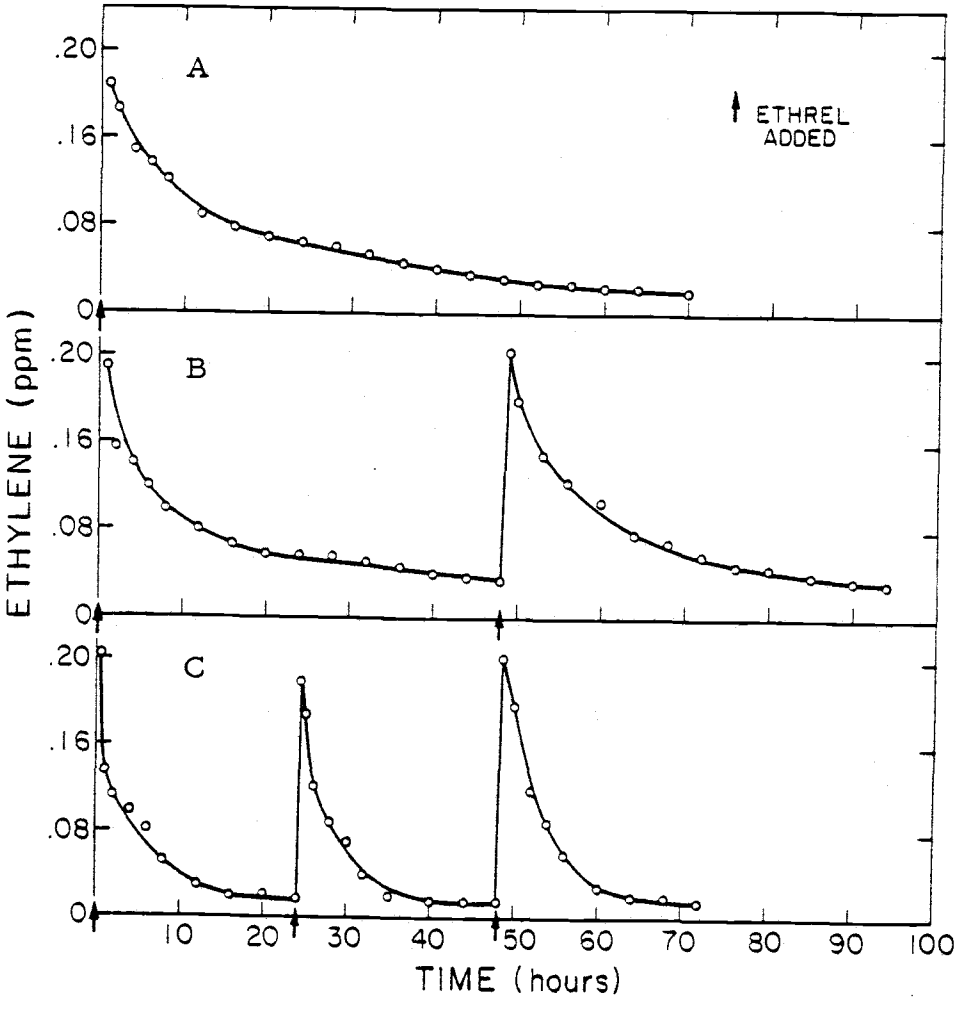

Figure 1. Ethylene release from Ethrel in three artificial soil mixes. (A) Peat-vermiculite (1:1), (B) Peat-vermiculite-sand (1:1:1), (C) Vermiculite. Each point is the mean of 5 observations.

Experiment 2: Effect of Ethylene on Ectomycorrhiza Formation

To determine the effect of ethylene on ectomycorrhiza formation of Douglas-fir, seedlings from Experiment 1 were used. Thirty-two 4-month-old seedlings were selected from each of the control (no ethylene) treatment blocks and from each block of the ethylene treatment which had no significant effect on lateral root formation (0.1 ppm). Seedling treatments (4 blocks of 16 seedlings each) were as follows: (1) ethylene pretreatment, ethylene treatment; (2) no ethylene pretreatment, ethylene treatment; (3) ethylene pretreatment, no ethylene treatment; (4) no ethylene pretreatment, no ethylene treatment. Ten-liters of vegetative peat-vermiculite inoculum of Hebeloma crustuliniforme (Bull. ex St. Am.) Quél (S-166) were prepared as described by Marx and Bryan (1975). Flasks containing 1-liter of peat-vermiculite medium were inoculated with 25 ml of blended mycelium from 4-week-old cultures grown in modified Melin-Norkrans liquid medium (Marx 1969). After 16 weeks mycelium had permeated the medium and the inoculum was harvested and mixed with 50 liters of steam-sterilized (121°C/30 min) vermiculite (pH 6.0). Seedlings from Experiment 1 with attached soil mix were transferred into 150 ml Rootrainers (Spencer-Lemaire Industries, Edmonton, Alberta) and 100 ml of vermiculite inoculum was packed around the root-soil core. Planted seedlings were leached with an excess of water to remove non-assimilated nutrients from the inoculum (Marx and Bryan

1975) and returned to growth chamber conditions as described in Experiment 1.

For ethylene treatment, Ethrel, diluted with 1/8-strength nutrient solution, was added in 18 ml aliquots to establish an ethylene exposure level comparable to that of pretreated seedlings (0.1 ppm). Ethylene release in vermiculite medium occurred over a 24 h period, so Ethrel was applied daily (Fig. 1C). Ethylene levels were monitored for 3 months as described in Experiment 1.

After 3 months, 20 seedlings per treatment (5 per block) were randomly selected for harvest and evaluated as follows: (1) number of lateral roots less than 2 cm long, (2) number of mycorrhiza-mantled short roots, and (3) seedling root and shoot oven dry weight (70°C/48 h).

Experiment 3: Effect of Ethylene on Fusarium Infection

Fusarium oxysporum Schlecht f. sp. pini (Hartig) Synd. and Hans. (DF-4) was isolated from a diseased Douglas-fir container seedling and maintained on V-8 juice (Toussoun and Nelson 1968) agar. Vermiculite inoculum of this isolate was prepared by mixing 750 ml of vermiculite with 350 ml of V-8 juice solution in 1 liter flasks, autoclaving (121°C/30 min), and inoculating the medium with V-8 juice agar disks of Fusarium. After 6 weeks, the inoculum was harvested and non-assimilated nutrients removed by leaching with tap

water. Eight blocks with twenty-five 65 cm^3 Leach Fir cells each were filled with a soil mixture of 1 part inoculum and 9 parts 1:1 (v:v) peat-vermiculite (pH 5.0) and sown with Douglas-fir seed prepared as described in Experiment 1. Seedlings were grown for the duration of the experiment in the greenhouse under high pressure sodium-vapor lamps (minimum 200 $\mu E \cdot m^{-2} \cdot sec^{-1}$ at 400-700 nm) set on a 16 h photoperiod and 25/15°C day-night temperature regime. Starting at 4 weeks, seedlings were fertilized weekly with 6 ml of nutrient solution for 1 month.

Ethrel, diluted in 1/3 strength nutrient solution, was added in 6 ml aliquots to establish an ethylene level in the root zone of 2-month-old seedlings comparable to that of Experiment 2 (0.1 ppm). Ethylene release in peat-vermiculite occurred over a 72-h period, so Ethrel was added every 3 days (Fig. 1A). Ethylene levels were monitored for 2 months as described in Experiment 1.

After 2 months, 40 seedlings from each treatment (10 per block) were randomly selected for evaluation of Fusarium infection, and another 40 seedlings for lateral root counts and dry weight analysis. To determine the percentage of roots infected with Fusarium, the entire root system was cut into 0.5 cm sections, spread on a 1 cm line grid and the sections intersecting grid lines were surface-sterilized in 30% H_2O_2 for 2 min, washed in sterile-distilled water for 1 min, and 10 sections were plated on Komada's medium (Komada

1976). Root infection was calculated as the percentage of Fusarium-positive root pieces per plate.

RESULTS

Experiment 1: Effect of Ethylene on Root Growth

The average ethylene concentrations (ppm) established in the root zone of Douglas-fir over the 2 month period were 0.006 ± .001 (ambient control), 0.02 ± .01, 0.04 ± .02, 0.10 ± .06, 0.34 ± .23 and 0.54 ± .35. At the two lower ethylene concentrations (approx. 0.01-0.05 ppm range) lateral root formation was significantly stimulated when compared to non-exposed controls (Table 1). The intermediate ethylene level (approx. 0.05-0.15 ppm range) had no effect, while the two higher levels (> 0.15 ppm) significantly inhibited lateral root formation. With increasing level of ethylene concentration, root dry weight increased and shoot dry weight decreased, thereby increasing the root/shoot ratio. At the two higher levels of exposure, total dry weight decreased.

Several ethylene-induced symptoms were evident on treated seedlings (Table 1). Hypertrophy of tissue at root lenticels (Fig. 2A) and at root branch points (Fig. 2B) was the most sensitive response to ethylene; loss of a normal geotropic response resulting in root twisting was evident at intermediate levels of exposure; at the two highest concentrations an increase in diameter of secondary roots and a stunting of the entire root system was observed. Sections

Table 1. Effect of ethylene on root growth of Douglas-fir.

Ethylene Level (ppm)[x]	Short Root count	Seedling Dry Weight (mg)			R/S	% Seedlings w/symptom[y]			
		Root	Shoot	Total		1	2	3	4
0.006 ± .001 (control)	357 a[z]	201 a	556 a	753 ab	.37 a	0	0	0	0
0.02 ± .01	443 b	221 b	544 ab	761 a	.41 b	20	13	0	0
0.04 ± .02	400 c	218 b	537 b	754 ab	.41 b	31	21	1	0
0.10 ± .06	377 c	241 c	496 c	734 bc	.50 c	84	76	10	1
0.34 ± .23	290 d	242 c	484 c	724 c	.51 c	100	93	18	5
0.54 ± .35	270 d	241 c	434 d	677 d	.57 d	100	100	20	9

[x]Column values are the mean of weekly ethylene readings taken over a 2 month period.

[y]Symptoms of ethylene exposure: (1) hypertrophy of root lenticels and at root branch points, (2) root twisting, (3) increase in diameter of secondary roots, (4) stunting of root system.

[z]Table values except (x) are the mean of 4 replications of 20 observations. Comparison of column means by Duncan's multiple range test; means followed by the same letter are not significantly different at the .01 level.

through hypertrophied root tissue revealed an increase in cell formation causing a rupture of the epidermis (Fig. 2C, 2D). Increased diameter of roots was apparently due to the radial expansion of cortical cells (Fig. 2E, 2F).

Experiment 2: Effect of Ethylene on Ectomycorrhiza Formation

Ethylene exposure (0.1 ± .06 ppm level) for 3 months in all treatment combinations did not significantly increase the percentage of short roots that were mycorrhizal compared to non-exposed controls (Table 2). As in Experiment 1, this level of ethylene had no effect on lateral root formation, but did significantly increase root dry weight and thus the root/shoot ratio, irrespective of previous treatment.

Cross-sections of mycorrhizal short roots revealed that ethylene exposure caused no significant changes in short root morphology or in extent of mantle and Hartig net development.

Experiment 3: Effect of Ethylene on Fusarium Infection

Ethylene exposure (0.1 ± .07 ppm level) for 2 months significantly increased the percentage of Douglas-fir roots infected with F. oxysporum f. sp. pini (Table 3). The treatment combination of Fusarium plus ethylene also reduced seedling dry weight and lateral root formation compared to the Fusarium treatment alone.

DISCUSSION

According to Abeles (1973) most ethylene-mediated responses

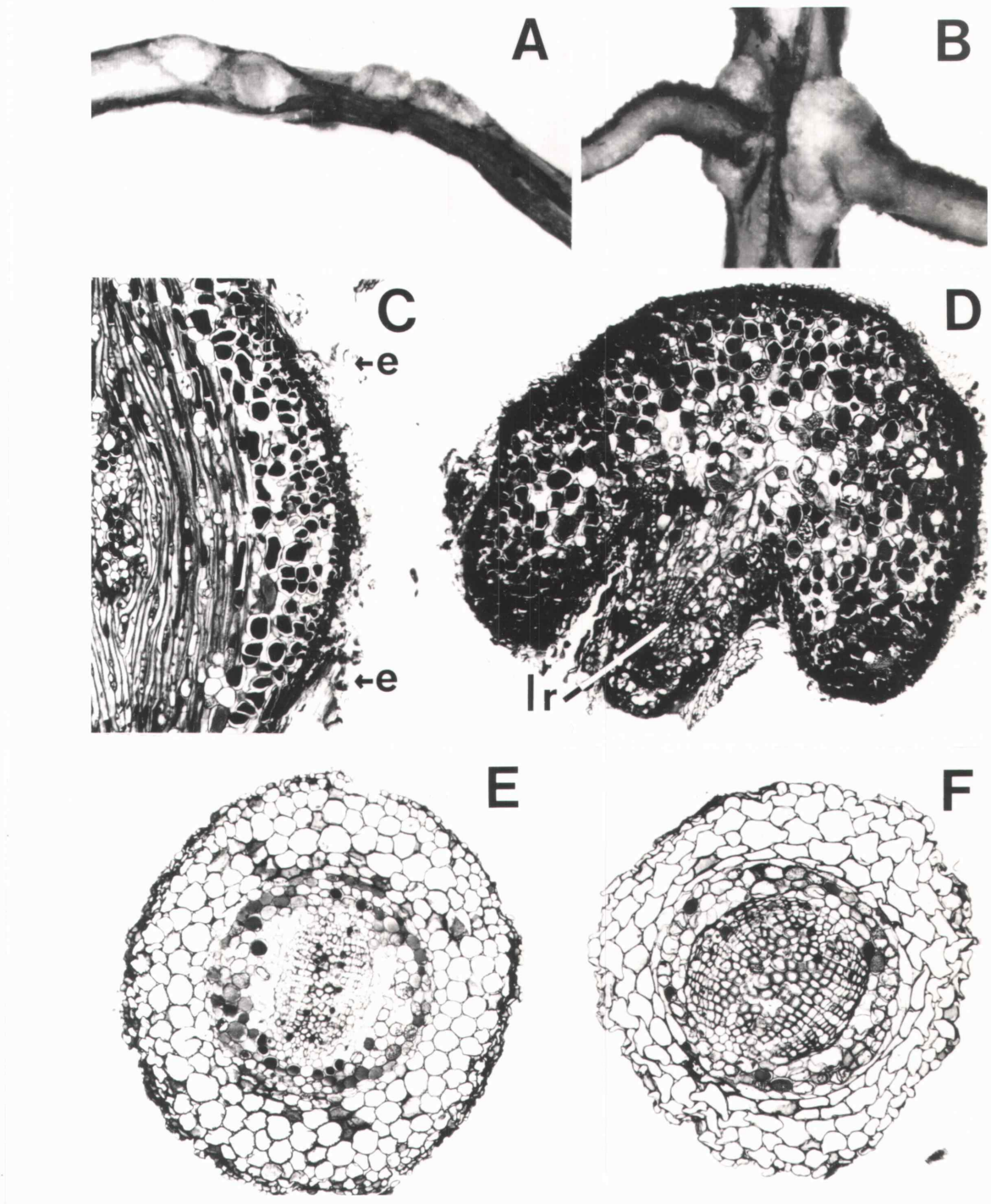

Figure 2. Ethylene effects on root growth of Douglas-fir. (A-B) Root hypertrophies at lenticels (A) and at root branches (B). (C) Longitudinal section of hypertrophied root lenticel showing rupture of epidermis (e) (X 96). (D) Transverse section of hypertrophied tissue at a lateral root (lr) branch point showing proliferation of undifferentiated cells (X 96). (E-F) Transverse section of ethylene-treated root (E) (X 96) showing radial expansion of cortical cell layers compared to control (F) (X 192).

Table 2. Effect of ethylene on ectomycorrhiza formation by Hebeloma crustuliniforme on Douglas-fir.

Ethylene[a] Pretreatment	Ethylene[b] Treatment	Short Root count	Mycorrhizal Short Roots	% Mycorrhizal Short Roots	Dry Weight (g) Root	Shoot	Total	R/S
-	-	1741[c]	758	47	1.27	1.69	2.96	.77
+	+	1630	664	40	1.41*	1.70	3.12	.83*
+	-	1804	709	39	1.32	1.75	3.07	.76
-	+	1562	667	46	1.42*	1.73	3.15	.84*

[a]Ethylene pretreatment at 0.1 ± .06 ppm level for 2 months

[b]Ethylene treatment at 0.1 ± .07 ppm level for 3 months

[c]Table values are the mean of 4 replications of 5 observations; means followed by (*) are significantly different from the non-ethylene treated control at the .01 level.

Table 3. Effect of ethylene on infection of Douglas-fir by Fusarium oxysporum f. sp. pini.

Treatment	% Root Infection	Short Root count	Dry weight (mg)			R/S
			Root	Shoot	Total	
Control	9.3[b]	112	90.7	179	267	.50
Ethylene Treated[a]	17.8*	36*	59.0*	158*	218*	.38*

[a]Ethylene treatment at 0.1 ± .07 ppm level for 2 months

[b]Table values are the mean of 4 replications of 10 observations; means for ethylene treatment followed by (*) are significantly different from the non-ethylene treated control at the .01 level.

require a concentration of 0.01 ppm to give a threshold response, 0.1 ppm for a half-maximal response, and 1 ppm for a maximal response; normally, after a maximal response to ethylene is observed, higher concentrations have no additional effect. In our study, stimulation of lateral root formation by ethylene did not follow this typical dose-response curve. Depending on concentration, ethylene either increased, had no effect, or decreased lateral root formation. Thus ethylene can both stimulate and inhibit root formation. This observation is supported by the report of Crossett and Campbell (1975) that long-term ethylene exposure inhibited extension of barley seminal roots, and stimulated extension primary roots, as well as significantly increased the number and length of secondary laterals. Zobel (1973) also observed a stimulation of lateral root development for the ethylene-requiring tomato mutant, diageotropica, after whole plant exposures to ethylene concentrations as low as 5 ppb, and noted that this concentration was 10 fold less than those used in previous studies. Our results with Douglas-fir support the role of low levels of ethylene as a stimulator of lateral root development.

Other root responses to ethylene exposure exhibited a typical dose-response relationship. Hypertrophy of root tissue and geotropic responses developed at the lowest ethylene concentrations, while increase in diameter and stunting of roots occurred at higher levels. The concentration dependencies for geotropic responses, root

elongation, and increase in root diameter of Douglas-fir are generally in agreement with those found for legumes and cotton (Goeschl and Kays 1975). A minimum concentration of 0.01 ppm required for hypertrophy of root tissue was also reported for hypertrophy of apple stem lenticels (Wallace 1927 as reported by Abeles, 1973).

The results showing a shift in dry weight from shoot to roots at concentrations below 0.15 ppm are contrary to the findings of Crossett and Campbell (1975), and Cornforth and Stevens (1974) who reported a decrease in root dry weight with long term exposure of barley to ethylene. However, in both cases ethylene concentrations in excess of 0.1 ppm were inhibitory.

Our studies suggest that very low levels of ethylene in the rhizosphere can influence the partitioning of dry matter into roots and stimulate lateral root formation. Ectomycorrhizal fungi may contribute to the production of low levels of ethylene in the rhizosphere (Graham and Linderman, unpublished results), and therefore indirectly control mycorrhiza formation by triggering the development of receptive short-roots.

Ethylene, however, did not appear to directly affect mycorrhiza formation, but did increase the development of Fusarium root disease of Douglas-fir. Similarly, Collins and Scheffer (1958) reported that ethylene enhanced infection of tomato by Fusarium oxysporum f. sp. lycopersici. Ethylene not only increased disease development in

wilt-susceptible tomato cuttings, but also caused normally resistant cuttings to show symptoms. Fusarium was isolated only from the base of untreated resistant cuttings, but was found in all parts of ethylene-treated plants. Furthermore, it has been shown that Fusarium oxysporum f. sp. tulipae produces large amounts of ethylene in vitro (Swart andKamerbeek 1976), and when inoculated into tulip bulbs (de Munk 1972). Beijersbergen and Bergman (1973) suggested that ethylene resulting from Fusarium infection inhibits the synthesis of a fungitoxic substance (tulipalin A) in bulb tissue, thereby increasing the susceptibility of bulbs to further infection.

Ethylene is produced in large quantities from Fusarium-infected Douglas-fir roots compared to very low levels from ectomycorrhizae (Graham and Linderman, unpublished results). While high levels of ethylene produced by Fusarium or infected host tissue may decrease host resistance, low levels appear not to influence the host response to mycorrhizal fungus colonization. Our experiments indicate that ethylene at concentrations up to 1.0 ppm has no effect on growth of Fusarium or mycorrhizal fungi in Melin-Norkrans liquid medium (Graham and Linderman, unpublished results). These results support the hypothesis that ethylene influences the host response to infection rather than the fungus activity.

Thus, there appears to be a fundamental difference between pathogenic Fusarium and symbiotic mycorrhizal fungi in the levels

of ethylene produced by the host-fungus interaction and in the function of ethylene in disease development versus mycorrhiza establishment and maintenance.

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CHAPTER 5

DISCUSSION AND CONCLUSIONS

With the discovery that plant pathogenic fungi produce ethylene, a natural plant growth regulator, it became apparent that infection of the plant must involve hormone-induced changes in host physiology. The relationship between ethylene and the development of symptoms in Fusarium disease is especially suggestive. The production of ethylene by Fusarium species and Fusarium disease has been demonstrated for vascular wilt (Dimond and Waggoner 1953; Gentile and Matta 1975), bulb rot (de Munk 1972; Swart and Kamerbeek 1976), and is now reported for Fusarium root rot of Douglas-fir.

Apparently ethylene is also a product of ectomycorrhizal fungi, their culture filtrates, and mycorrhizae. Methionine, the precursor for ethylene production by mycorrhizal fungi and culture filtrates is in root exudate of ectomycorrhizal tree hosts (Bowen, cited in Rovira 1965; Malajczuk and McComb 1977). Thus, ethylene formation by mycorrhizal fungi in the rhizosphere or in mycorrhizal association may depend on a supply of methionine from plant roots.

In aseptic culture, culture filtrates of mycorrhizal fungi and synthetic auxins stimulate root initiation on pines (Slankis 1972). Exposure of Douglas-fir roots to very low levels of ethylene (0.05 ppm) also stimulates the formation of lateral roots. Furthermore,

in the formation of a functional mycorrhizal relationship, low levels of ethylene are produced and lateral root development is increased. These findings support the role of ethylene produced by ectomycorrhizal fungi and mycorrhizae in the initiation of lateral roots. Ethylene production in the rhizosphere may therefore indirectly control mycorrhiza formation by triggering the development of receptive short roots.

Ethylene, however, does not appear to directly effect mycorrhiza formation of Douglas-fir but does increase the development of Fusarium root disease. Ethylene has been shown in other cases to decrease the disease resistance of normally non-susceptible plants (Collins and Scheffer 1958; Daley et al. 1970). Ethylene, produced in large quantities by Fusarium root disease, may function in lowering host resistance to root infection. By contrast, low levels of ethylene associated with mycorrhizal formation may not affect the host response to mycorrhizal colonization.

These studies suggest that there is a fundamental difference between pathogenic Fusarium and symbiotic mycorrhizal fungi in the levels of ethylene produced by the host-fungus interaction and in the function of ethylene in disease development versus mycorrhiza establishment and maintenance.

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APPENDIX

Purpose of Study

The following study was conducted to develop a reliable technique for inoculating Douglas-fir with ectomycorrhizal fungi. The goal was to obtain uniform levels of ectomycorrhiza formation as a baseline for testing ethylene effects. A modification of the post-emergence inoculation technique developed in this study was utilized in an experiment presented in Chapter 4.

INOCULATION OF CONTAINERIZED DOUGLAS-FIR WITH THE ECTOMYCORRHIZAL FUNGUS, CENOCOCCUM GEOPHILUM

J. H. Graham

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

and

R. G. Linderman

USDA SEA-AR Ornamental Plants Research Laboratory
Corvallis, Oregon 97330

Abstract. Containerized Douglas-fir (Pseudotsuga menziesii) was inoculated with vegetative peat-vermiculite inoculum of three isolates of Cenococcum geophilum (A-145, A-161, A-166) at time of seeding (pre-emergence), and after 1, 2 and 3 months (post-emergence). For all isolates, post-emergence inoculation resulted in more mycorrhizae than pre-emergence inoculation. Percent mycorrhization resulting from pre-emergence inoculation appeared to be correlated with the mycelial growth rate of C. geophilum isolates in vitro: A-145 (48%) > A-161 (24%) > A-166 (6%). Pre-emergence inoculation success with C. geophilum may be in part a function of the growth rate of the fungus in vitro as well as the availability of lateral short roots for mycorrhizal fungus colonization.

Additional key words. Fungal isolate variation, vegetative peat-vermiculite inoculum, pre- and post-emergence inoculation.

INTRODUCTION

The concept of inoculating seedlings with specific ectomycorrhizal fungi to improve their growth and survival after outplanting has been applied to containerized seedling production (Marx and Barnett 1974). Vegetative peat-vermiculite inoculum of Pisolithus tinctorius (Pers.) Coker and Couch incorporated into the soil mix was used successfully to inoculate containerized loblolly pine (Pinus taeda L.) in the Southeast (Marx and Barnett 1974; Ruehle and Marx 1977). Trappe (1977) discussed the merits of P. tinctorius for vegetative mycelial inoculation and at the same time noted that practical mycelial inoculation with other fungi may be limited by their poor growth in culture. However, he emphasized the need to examine other fungal species and ecotypes for their adaptability to nursery and containerized inoculation, as well as performance after outplanting.

Cenococcum geophilum Fr. (syn. C. graniforme (Sow.) Ferd. et Winge) is distributed throughout the northern hemisphere and forms mycorrhizae with all known ectomycorrhizal hosts within its range (Trappe 1962). In a coastal Oregon Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) stand, sclerotia of C. geophilum comprised 14% of the standing crop of fungal biomass (Fogel and Hunt 1979). Thus, C. geophilum is an extremely adaptable and important ectomycorrhizal species in the Pacific Northwest. However,

Marx and others (1978) reported that C. geophilum was less effective than P. tinctorius in forming ectomycorrhizae and stimulating growth of loblolly pine in fumigated and nonfumigated nursery soil. They suggested that C. geophilum was unsuitable for artificial inoculation in their nursery situation because of its inherently slow growth rate.

This study was designed to determine if mycorrhization success with C. geophilum is related to the growth rate of the fungus in vitro and limited by the availability of lateral short roots for mycorrhizal fungus colonization during the period of maximum inoculum potential.

MATERIALS AND METHODS

Cenococcum geophilum Isolates.-Three isolates of C. geophilum, obtained from R. J. Molina, U.S.D.A. Forest Service, Corvallis, Oregon, were as follows: (1) A-145, collected 1974 under Pseudotsuga menziesii at Blue River, Oregon; (2) A-161, collected 1975 under Tsuga heterophylla (Raf.) Sarg.-Abies amabilis (Dougl.) Forbes at Tombstone Pass, Oregon; (3) A-166, collected 1975 under P. menziesii at Marys Peak, Oregon.

Mycelial growth rates of the isolates were determined in liquid culture. Inoculum of each isolate, which had been grown for 4 weeks in modified Melin-Norkrans (MMN) liquid medium (Marx, 1969), was prepared by aseptically filtering the mycelium, and washing and resuspending the mycelium in sterile-distilled water. The suspension

was then homogenized in a Waring blender for 20 sec and 5 ml aliquots of the mycelial slurry, equivalent to about 5 mg dry weight of mycelium, were pipetted into 125 ml flasks containing 40 mls of MMN liquid medium. Test flasks were incubated in the dark at 20°C, the mycelium was filtered off, and oven dry weights (70°C/24 hr) were determined after 0, 7, 14, 21 and 28 days growth.

Inoculum Preparation. -Vegetative peat-vermiculite inoculum of each isolate of C. geophilum was prepared as described by Marx and Bryan (1975). Each flask containing 1-liter of peat-vermiculite mix was inoculated with 25 ml of blended mycelium from 4-week-old cultures grown in MMN liquid medium. After 20 weeks mycelium had permeated the medium and the inoculum was harvested and stored in plastic bags at 5°C for use the following day.

Seedling Preparation. -Spencer-Lemaire (Edmonton, Canada) folding book containers (4-150 ml cavities per book) were filled with steam-pasteurized (70°C/30 min) 1:1:1 (v:v) peat-vermiculite-sand mix (pH 6.0). Douglas-fir seed was prepared for sowing by washing in running tap water for 24 hr and then surface-sterilizing in 30% H_2O_2 for 30 min. Four seeds per cavity were sown and germlings thinned to one per cavity. Randomized blocks (4 books per block) of seedlings were grown for the duration of the experiment in the greenhouse under high-pressure sodium-vapor lamps (minimum 200 $\mu E \cdot m^{-2} \cdot sec^{-1}$ at

400-700 nm) set on a 16 hr photoperiod and 25/15°C day-night temperature regime. Seedlings were fertilized weekly with nutrient solution of the following composition: NH_4NO_3, 286 ppm; KH_2PO_4, 264 ppm; $MgSO_4 \cdot 7H_2O$, 247 ppm; $CaCl_2 \cdot 2H_2O$, 147 ppm; Fe sequestrene 330, 30 ppm; and Hoagland's micronutrients. The seedling preparation procedure was repeated 1 and 2 months later such that after 4 months there were nine blocks of 1-, 2- and 3-month-old seedlings available for inoculation.

Pre-emergence Inoculation. - Three blocks, each with four folding book containers, were inoculated with vegetative inoculum of each isolate. Three book containers per block were filled with a soil mixture containing 1 part peat-vermiculite-sand soil mix. As a control, one book container per block was inoculated with an equivalent amount of vermiculite. Seedlings were raised as previously described under seedling preparation.

Post-emergence Inoculation. - Three blocks of each age class of seedlings were inoculated with each isolate. Folding book containers were carefully opened to expose both sides of the root-soil core. An amount of inoculum equivalent to that added in the pre-emergence treatment (30 cc/seedling) was spread evenly over each side of the root system and the containers were closed and secured with tape. One book container per block was left uninoculated as a control.

Seedling Evaluation. -Six months after inoculation 24 randomly selected seedlings from each treatment were removed from the containers and their roots gently washed free of potting mix. Colonization of short roots by C. geophilum was estimated to the nearest 10% under 4X magnification. Oven-dry weight of roots and shoots was determined after 48 hr at 70°C.

RESULTS

For all three isolates of Cenococcum geophilum, inoculation of 1-, 2- and 3-month-old seedlings resulted in more mycorrhizae than inoculation at time of seeding (Fig. 1). Inoculation success ranged from a low of 6% for isolate A-166 at sowing time to as high as 98% for isolate A-145 at 2 months. In general, inoculation of 2-month-old seedlings was optimum for mycorrhiza formation. None of the inoculation treatments significantly stimulated growth compared to uninoculated controls.

Mycelial growth rates of each isolate were significantly different ($p=.01$) and ranked A-145 > A-161 > A-161 (Fig. 2). Isolate growth rate was correlated with success of pre-emergence inoculation at a $P = .20$ (Fig. 3).

DISCUSSION

Vegetative peat-vermiculite inoculum incorporated into the soil mix at time of seeding (pre-emergence) was used successfully for

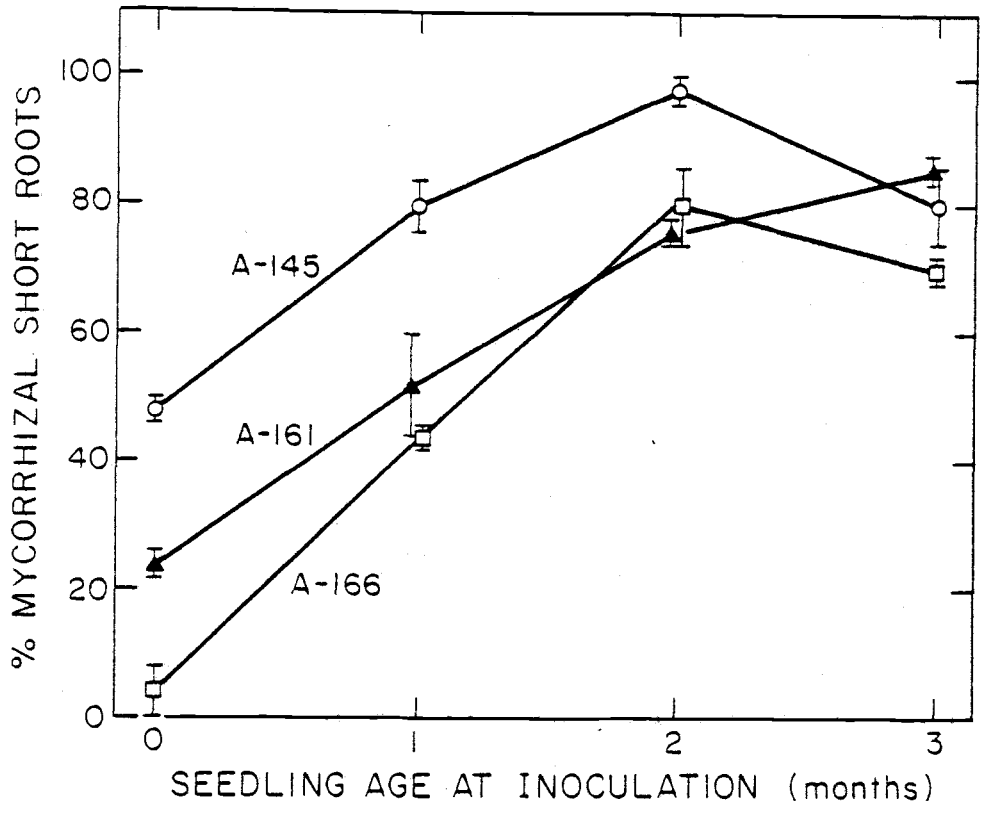

Figure 1. Effect of Douglas-fir seedling age at time of inoculation on ectomycorrhiza formation by three isolates of Cenococcum geophilum (A-145, A-161, A-166). Error bars represent the standard deviation of the mean of 3 replications of 8 observations.

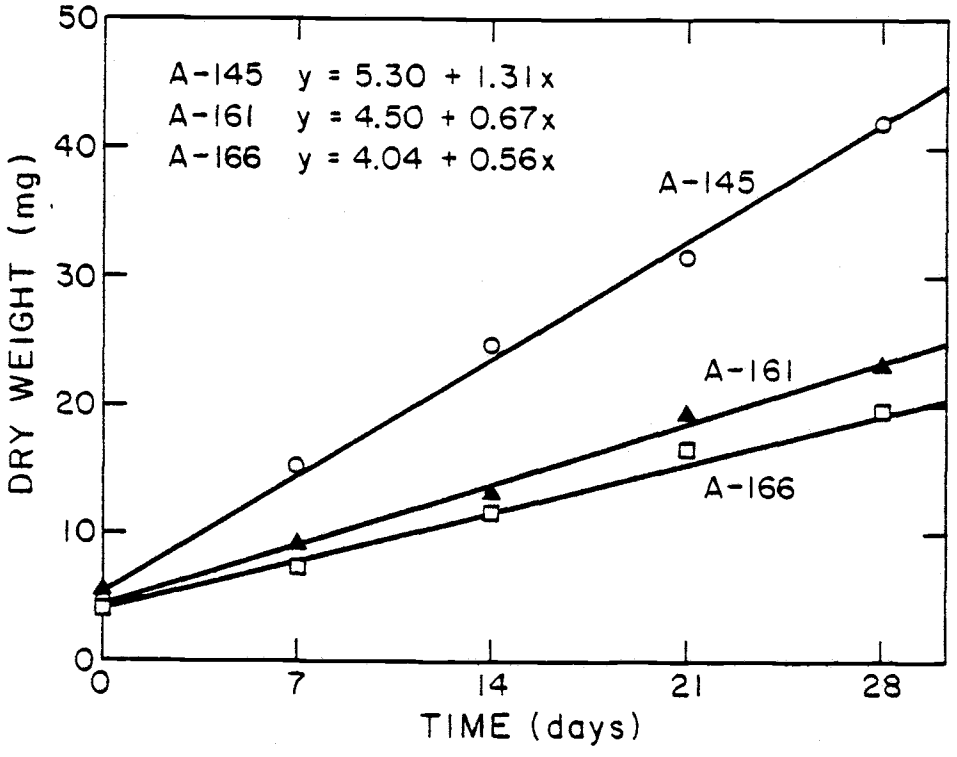

Figure 2. Growth rate of three isolates of Cenococcum geophilum (A-145, A-161, A-166) in modified Melin-Norkrans liquid medium. Slopes differ significantly at the .01 level using the general linear test for equality of two regression lines.

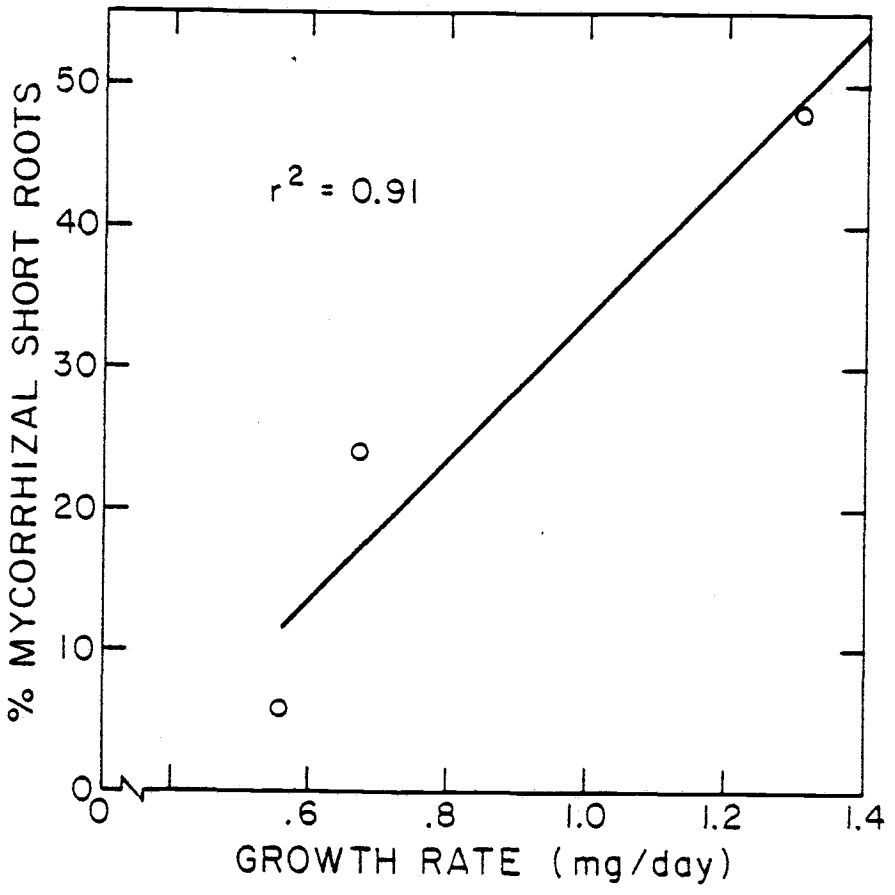

Figure 3. Correlation between growth rate of Cenococcum geophilum isolates in vitro and ectomycorrhiza formation of Douglas-fir from inoculation at time of seeding.

inoculation of containerized loblolly pine with Pisolithus tinctorius (Marx and Barnett 1974; Ruehle and Marx 1977). In these reports, mycorrhiza formation by P. tinctorius ranged from 20 to 80% in commercial peat-vermiculite soil mixes fertilized at low nutrient levels. In a similar soil mix, pre-emergence inoculation of Douglas-fir with Cenococcum geophilum isolate A-145 resulted in nearly 50% mycorrhization; however, with isolates A-161 and A-166 mycorrhiza formation was less than 25%.

Inoculation success was significantly increased by post-emergence inoculation. This inoculation method apparently facilitated the interaction between inoculum and short roots, the sites for mycorrhizal fungus colonization. Mycorrhization increased with seedling age at inoculation up to 2 months old, indicating that short roots must be present to maximize inoculum potential. When short roots were immediately available for colonization, all isolates had approximately the same potential for forming mycorrhizae.

For pre-emergence inoculation, mycorrhizal fungus inoculum potential apparently dropped significantly from the time of inoculation at seeding until short roots were formed. The loss of inoculum potential appeared to be correlated with isolate growth rate in vitro. Isolates A-161 and A-166, which grew much slower than isolate A-145, were ineffective in pre-emergence inoculation. Slow growth rate of C. geophilum has been suggested as one reason for its poor

performance in a nursery inoculation of loblolly pine (Marx and others 1978).

Trappe (1977) mentioned several other isolate characters in addition to growth rate which might affect mycorrhiza-forming potential of vegetative mycelial inoculum: growth habit of isolates in peat-vermiculite medium, variation in temperature and moisture tolerance, and interactions with other soil microorganisms. Bowen and Theodorou (1979) found that colonization of Pinus radiata roots by ectomycorrhizal fungi was either inhibited, stimulated, or unaffected by selected soil bacteria.

Our results indicate that C. geophilum is adaptable to pre-emergence inoculation of containerized Douglas-fir. However, the variation in inoculation success among the isolates tested lends support to the concept of screening multiple isolates of a species for evaluation of their suitability in wider scale inoculation and outplanting studies (Trappe 1977).

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