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MICROBIAL PRODUCTION OF ETHYLENE IN DESERT SOILS

The University of Arizona

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MICROBIAL PRODUCTION OF ETHYLENE IN DESERT SOILS

by

Hashim Mahmoud Babiker

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF SOILS, WATER AND ENGINEERING

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

WITH A MAJOR IN SOIL AND WATER SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

1983

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read
the dissertation prepared by Hashim Mahmoud Babiker
entitled Microbial Production of Ethylene in Desert Soils

and recommend that it be accepted as fulfilling the dissertation requirement
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TABLE OF CONTENTS

	<u>Page</u>
LIST OF FIGURES.....	vi
LIST OF TABLES.....	vii
ABSTRACT.....	viii
 CHAPTER	
1 INTRODUCTION.....	1
2 LITERATURE REVIEW.....	3
Effects of C ₂ H ₄ on Plant Growth.....	3
Effects of C ₂ H ₄ on Soil Microorganisms.....	4
Production of C ₂ H ₄ by Microorganisms.....	6
a. Bacteria.....	6
b. Fungi.....	7
Biochemistry of C ₂ H ₄ Production.....	8
a. Biochemistry of C ₂ H ₄ formation in plants....	8
b. Biochemistry of C ₂ H ₄ formation in fungi....	9
Factors Affecting C ₂ H ₄ Production.....	10
a. Effect of soil aeration.....	10
b. Effect of temperature.....	11
c. Effect of water potential.....	12
d. Effect of organic matter.....	12
e. Effect of organic amendments.....	12
3 MATERIALS AND METHODS.....	15
Ethylene Production in Soils.....	15
Gas Chromatography.....	16
Ethylene Production in Organic Amended Samples.....	17
Isolation of Soil Fungi.....	17
Ethylene Production by Pure Cultures of Fungi.....	18
Studies of Saline and Alkali Soils.....	20
4 RESULTS AND DISCUSSION.....	21
Ethylene Production in Desert Soils.....	21
Ethylene Production as Affected by Organic Compounds.	30
Ethylene Production as Affected by Salt Treatments...	39

TABLE OF CONTENTS -- Continued

	<u>Page</u>
Ethylene Production as Affected by Leaching of Saline Soils.....	41
Ethylene Production in Pure Cultures of Isolated Soil Fungi.....	42
Ethylene Production in Pure Cultures of Known Fungal Species.....	45
Ethylene Production in Sterilized Inoculated Soils...	48
5 SUMMARY AND CONCLUSIONS.....	52
APPENDIX A: Sample Calculation.....	57
APPENDIX B: A Typical Chromatogram.....	59
APPENDIX C: Collection Sites and Classification of the Soils Used.....	61
LITERATURE CITED.....	62

LIST OF FIGURES

Figure		Page
1	Ethylene production in three artificially salinized vineyard soils.....	23
2	Ethylene production in two coarse-textured non-saline range soils.....	24
3	Ethylene production in two saline non-sodic cultivated soils.....	25
4	Ethylene production in a Mount Lemmon soil (F).....	26
5	Ethylene production in three medium-textured non-saline cultivated soils.....	28
6	Ethylene production in a garden soil (L).....	29
7	Microbial C ₂ H ₄ production in a saline soil (A) as affected by organic compounds.....	31
8	Microbial C ₂ H ₄ production in a Mount Lemmon soil as affected by organic compounds.....	32
9	Microbial C ₂ H ₄ production in a garden soil as affected by organic compounds.....	33
10	Ethylene production in soils as affected by methionine, glucose and chloramphenicol.....	34
11	Microbial production of C ₂ H ₄ in a garden soil as affected by methionine.....	36
12	Microbial production of C ₂ H ₄ in four saline soils as affected by leaching.....	40

LIST OF TABLES

Table		Page
1	Selected properties of the soils used.....	22
2	Microbial production of C_2H_4 as affected by neutral and alkali salts.....	38
3	Ethylene production in pure cultures of isolated soil fungi grown on SDAM slants at 25°C.....	43
4	Ethylene production in pure cultures of known fungal species grown on SDAM slants at 25°C.....	46
5	Microbial C_2H_4 production in some special media.....	47
6	Microbial C_2H_4 production in sterilized inoculated soils.....	49

ABSTRACT

Ethylene (C_2H_4) production was monitored in twelve desert soils incubated moist at constant temperature for various incubation periods. In all but two soils with high organic matter content, C_2H_4 production was low. Statistical analysis showed a good correlation between organic matter content and C_2H_4 production. Minimum levels of C_2H_4 were observed in saline and sodic soils.

Adding ethanol, glucose, glycerol and methionine to soil samples significantly increased C_2H_4 formation. Methionine induced the highest level of C_2H_4 in all soils tested. Increased concentrations of methionine resulted in further significant increases in C_2H_4 production possibly indicating its role as a precursor for C_2H_4 . Chloramphenicol did not have a significant effect except in a saline soil suggesting that bacterial C_2H_4 production is of less significance in the other soils.

The addition of salts to the high C_2H_4 producing soils suppressed C_2H_4 production most likely because of a direct effect on C_2H_4 producing microorganisms through toxic salt levels, high osmotic pressure and/or increased pH.

Leaching of four saline soils and subsequent incubation resulted in significant increases in C_2H_4 in two soils. Ethylene producers, previously inhibited by salinity, were probably reactivated when the salts were removed.

A Fusarium isolate obtained from the highest C_2H_4 producing soil, produced the most C_2H_4 in pure culture followed by isolates belonging to the genera Aspergillus, Penicillium, Curvillaria, and Rhizopus. In a comparative study, a number of species, some of which were known to produce C_2H_4 , were tested in culture media. Nine species produced C_2H_4 in varying amounts of which Penicillium digitatum produced the highest concentration.

A sterilized saline soil produced significant C_2H_4 when inoculated with spores of Mucor hiemalis and the Fusarium isolate, 5 to 14 times that in non-sterilized soil probably indicating an originally low population of C_2H_4 producing organisms. The amounts of C_2H_4 produced in sterilized inoculated mollisol and garden soils were only a fraction of that produced in non-sterilized samples probably indicating the involvement of a number of species in the production of C_2H_4 in these soils.

CHAPTER 1

INTRODUCTION

Ethylene (C_2H_4) acts as a plant hormone initiating fruit ripening and regulating many aspects of plant growth and development. The physiological effects of the gas may occur at the parts per billion (ppb) level. It is now well established that C_2H_4 is formed in anaerobic microsites in soils as a result of microbial activity, and that high concentrations of the gas, up to 75 ppm, have been reported in soils. Such concentrations are high enough to inflict serious damage to plant roots, legume nodules and tuber crops.

Several reports have also shown that C_2H_4 affects microbial activity. It has been identified as a cause of fungistasis and to affect the activity of some bacteria and nematodes at levels usually encountered in soil atmosphere. This regulating effect of C_2H_4 would be of great practical importance in the biological balance of soil. Because of the importance of this gas in soil biology and soil plant relationships, the factors governing its production in soil need to be elucidated.

Many environmental factors affect C_2H_4 accumulation in soil, but O_2 concentration (Hunt et al., 1980), organic matter content (Goodlass

and Smith, 1978), temperature (Lindbert et al., 1979) and moisture content (Hunt et al., 1980) seem to be the most important. Other factors which need to be investigated include: soil reaction, salinity, sodicity and organic and inorganic soil amendments. Those latter factors are given much attention in this research.

The soil organisms responsible for the production of this gas are of prime importance. Hence much work has been done to identify the most important groups of microorganisms implicated, but no comparable studies have been done in the southwestern desert soils.

The main objectives of this study were:

1. to assess the C_2H_4 -producing potential of some Arizona desert soils having different properties;
2. to isolate the major fungal species from those soils which produce most C_2H_4 and to test them for the production of the gas in pure cultures and in sterilized soils;
3. to examine the effect of some organic compounds on the evolution of C_2H_4 ;
4. to investigate the effects of neutral and alkali salts on C_2H_4 formation in soil atmosphere.

CHAPTER 2

LITERATURE REVIEW

Ethylene, called "ethene" in the Geneva nomenclature, is the lightest olefinic hydrocarbon with a molecular formula $\text{H}_2\text{C}=\text{CH}_2$ and a molecular weight of 28.05 (Kniel, Winter and Stark, 1980). At ambient conditions it is a colorless, flammable gas with a slightly sweet odor. It is of the same density as nitrogen and about five times as soluble in water as is oxygen (Abeles, 1973). Besides its crucial importance in the petrochemical industry (Kniel et al., 1980), C_2H_4 is known to have significant regulatory effects on plants (Pratt and Goeschl, 1969). Ethylene, which is a natural product of plant metabolism, is also found to be produced in significant levels by soil microflora which prompted current research in this area.

Neljubow, in 1901, was the first to demonstrate that C_2H_4 regulated the growth and development of plants (Abeles, 1973). Since then research concerning this gas has been carried out by plant physiologists, horticulturalists, plant pathologists and soil microbiologists. The advent of gas chromatographic technique and its advancement through the years stimulated C_2H_4 research.

Effects of C_2H_4 on Plant Growth

The physiological effects of C_2H_4 on plant growth and development have been well documented and reviewed (Burg, 1962; Hansen, 1966;

and Pratt and Goeschl, 1969). The list of physiological effects of the gas includes: fruit ripening, senescence, abscission, breaking of dormancy, regulation of swelling and elongation, hypertrophy, promotion of adventitious roots, modification of root growth, inhibition of root hairs, epinasty, hook closure, inhibition of leaf expansion, exudation, and control of flower induction (Primrose, 1979). A variety of higher plants are affected by extremely low concentrations of C_2H_4 as low as 10 ppb (Primrose, 1979). It has been reported that root growth of cereals (Smith and Russell, 1969), of conifers (Lill and McWha, 1976), and root nodulation of leguminous plants (Grobelaar, Clarke and Haugh, 1971) were adversely affected at concentrations of 1 ppm or more.

Seed germination of many plant species may either be inhibited or stimulated by C_2H_4 (Olatoye and Hall, 1973). Hence C_2H_4 concentration has important effects on the establishment of crop plants and weeds. The injection of C_2H_4 into the soil is practiced to control witch weed (Striga asiatica). Eplee (1975) has shown that C_2H_4 at about 1 ppm triggered the germination of up to 90% of the weed seeds in the absence of their hosts. So the effects of C_2H_4 on plants can either be harmful or beneficial depending on the prevailing conditions and the concentration of the gas in soil atmosphere.

Effects of C_2H_4 on Soil Microorganisms

The first adverse effect of C_2H_4 on the activity of microorganisms has been discovered in fungi. The term fungistasis is applied to the inability of fungal propagules to germinate under normal soil condi-

tions unless exogenous nutrients are supplied (Dobbs, Hison and Bywater, 1960). Ethylene has been shown to be an inhibitor causing soil fungi-stasis (Smith, 1973, 1976a, 1976b). The germination and subsequent growth of propagules of Sclerotium, Helminthosporium, and other fungal species were inhibited by C_2H_4 . This inhibitory effect was reversed when C_2H_4 -free air was passed over the fungal growth (Smith, 1973). Five other fungal species, however, were relatively unaffected by considerably higher concentrations of C_2H_4 (Lynch, 1975). Soils high in organic matter produced most C_2H_4 but they required higher levels of C_2H_4 to show a fungistatic effect (Smith and Restall, 1971). Although many fungal species were shown to be sensitive to C_2H_4 , basidiomycetes and phycomycetes are more tolerant than others (Smith, 1976b). Some species, e.g. Agaricus bisporus, may even be stimulated by low levels of C_2H_4 (Smith, 1976b). These differences in sensitivity to C_2H_4 can be of great ecological importance in soil microbiology.

Although many actinomycetes are sensitive to C_2H_4 , they are much more tolerant than fungi (Smith, 1976b). Other bacterial species and some nematodes were also inhibited by C_2H_4 (Smith, 1973, 1976a, 1976b; Smith and Cook, 1974), although a quantitative determination of C_2H_4 effect on bacterial activity has not been successful (Smith, 1976b). Ethylene at concentrations up to 20 ppm did not produce measurable effects on bacterial activity which led Smith (1976b) to conclude that bacteria are not directly inhibited by C_2H_4 at the levels normally encountered in soils.

Production of C_2H_4 by Microorganisms

All experimental evidence points to soil microorganisms as the major producer of C_2H_4 in the soil atmosphere but the most important C_2H_4 -producer is still a matter of controversy (Smith, 1976a).

a) Bacteria. Smith and Restall (1971) suggested that facultative anaerobic bacteria were most likely the highest producers of C_2H_4 in soil. A few years later Smith and Cook (1974) proposed that the main production of C_2H_4 is by spore-forming bacteria in anaerobic microsites. Spore-formers were implicated because the gas was produced at temperatures fatal to most fungi and other bacteria. Most attempts, however, failed to indicate significant evolution of C_2H_4 by the spore-forming Clostridium and Bacillus species which casts a shadow of doubt over their importance. Experiments on the effect of water potential indicated that C_2H_4 was formed in relatively wet soils (-5 bars and wetter) but insignificantly at -15 bars or drier, suggesting that bacteria were more important than other soil flora (Smith and Cook, 1974). Anaerobic production of C_2H_4 was confirmed by high production of the gas in soils incubated under O_2 -free atmosphere.

Some bacterial species capable of producing C_2H_4 in pure culture were isolated (Primrose, 1976; Primrose and Dilworth, 1976). The bacterial genera identified as C_2H_4 -producers were Pseudomonas, Escherichia (Primrose and Dilworth, 1976), Citrobacter, Enterobacter, Erwinia, Klebsiella, Serratia, Aeromonas, and Arthrobacter (Primrose, 1976).

b) Fungi. Most studies so far have pointed out that fungi are an important biological source of C_2H_4 . In pure culture Mucor hiemalis and two yeast species produced high amounts of C_2H_4 when glucose and methionine were added to the medium (Lynch, 1975). Ilaq and Curtis (1968) reported that C_2H_4 was produced by various fungi. Aspergillus clavatus was found to produce the most C_2H_4 . Penicillium digitatum, the common mold of citrus, was also shown to produce significant levels of C_2H_4 in pure culture (Chou and Yang, 1973). Of 228 species of fungi tested, C_2H_4 was produced in about 25% of them at concentrations varying from 0.2 to over 500 ppm (Ilaq and Curtis, 1968). Ward, Turner and Osborne (1978) found that the mycelium of Agaricus bisporus in compost is responsible for C_2H_4 production. The list of fungi which produced C_2H_4 in vitro also includes Fusarium oxysporum (Swart and Kamerbeek, 1977; Graham and Linderman, 1980), Cenococcum geophilum, Hebeloma crustuliniforme and Laccaria laccata all of which are ectomycorrhizal fungi (Graham and Linderman, 1980), Alternaria solani, Ascochyta imperfecti, Aspergillus candidus, A. flavus, A. ustus, A. variicolor, Botrytis spectabilis, Cephalosporium gramineum, Chaetomium chlamaloides, Dematium pullulans, Hansenula subpelticulosa, Myrothecium roridum, Neurospora crassa, Penicillium corylophilum, P. luteum, P. patulum, Schizophyllum commune, Sclerotinia laxa, Scopulariopsis brevicaulis, Thamnidium elegans, Thielavia alata (Ilaq and Curtis, 1968) and Saccharomyces cerevisiae (Thomas and Spenser, 1978).

Biochemistry of C₂H₄ Production

Researchers have been trying to elucidate the biochemical pathway of C₂H₄ formation in both plant tissues and fungi with little success. Even though many compounds including amino acids, sugars, alcohols and organic acids have been identified as precursors of C₂H₄, their low rate of conversion in tracer experiments suggests that they are not the immediate precursors of C₂H₄ (Pratt and Goeschl, 1969).

a) Biochemistry of C₂H₄ formation in plants. Two pathways have been proposed for C₂H₄ biosynthesis in plant tissue: one involves the degradation of methionine and the other is associated with the breakdown of linolenic acid (Lieberman and Mapson, 1964). The conversion of peroxidized linolenic acid has only been demonstrated in model systems while the conversion of methionine to C₂H₄ has been shown in plant tissue as well (Lieberman, Kunishi, Mapson and Wardale, 1966). Propanal, the breakdown product of linolenic acid, was suggested by Lieberman and Kunishi (1967) as a precursor, but Baur and Yang (1969a) could not confirm this in apple tissue.

Evidence from tracer experiments has now shown that methionine is the most likely precursor of C₂H₄ in plant tissue (Abeles, 1973). Yang (1968) has shown that C₂H₄ is rapidly formed from methional or from α keto γ methyl thiobutyric acid (KMBA) in the presence of enzymes and cofactors. He suggested the following pathway: Methionine $\rightarrow$ KMBA $\rightarrow$ methional $\rightarrow$ C₂H₄.

The equation shows that carbons 3 and 4 were converted to C₂H₄. The L-isomer was found to be preferentially used over the D or DL-methionine (Baur and Yang, 1969b). Recently, Adams and Yang (1979) have identified 1-aminocyclo-propane-1 carboxylic acid (ACC) as an intermediate in the conversion of methionine to C₂H₄. They proposed the following sequence for C₂H₄ formation: Methionine → S adenosyl methionine (SAM) → ACC → C₂H₄. Yu, Adams and Yant (1979) further suggested that ACC synthase may be a key enzyme in C₂H₄ biosynthesis in plants.

b) Biochemistry of C₂H₄ formation in fungi. Penicillium digitatum has been a primary fungus on which biochemical studies were carried out. Growing the fungus in different labelled carbon media and measuring the relative rate of C₂H₄ evolution was the main approach used (Abeles, 1972). For the most part the rate of C₂H₄ synthesis followed the fungal growth curves, the highest rates of production were mostly associated with the time the mycelium growth was complete (Abeles, 1973).

The pathway of C₂H₄ formation in fungi which has also received experimental support is the dehydration of alcohol:

In Penicillium spp. the use of labelled $\text{CH}_3\text{-CH}_2\text{OH}$ resulted in the formation of labelled C_2H_4 . The list of other labelled compounds tested and shown to produce C_2H_4 by Penicillium include: glucose, alanine, glycine, aspartic acid, glutamic acid, serine, acrylic acid, propionic acid, glyoxylic acid, acetic acid, citric acid, fumaric acid, pyruvic acid and succinic acid (Abeles, 1972). The immediate precursor of C_2H_4 and its exact biochemical pathway in vivo are yet to be determined.

Factors Affecting C_2H_4 Production:

Ethylene production in soil is influenced by many environmental factors of which moisture content, organic matter content, O_2 level and temperature are especially important. A host of other factors may affect the production and accumulation of C_2H_4 in soil atmosphere.

a) Effect of soil aeration. The production of C_2H_4 takes place under anaerobic conditions in soil (Smith and Russel, 1969; Smith and Restall, 1971; Hunt, Campbell and Moreau, 1980). However, some investigators believe that anaerobiosis is only necessary for the provision of substrates required for the formation of C_2H_4 . When suitable substrates were provided, it was found that the production of C_2H_4 by Mucor hiemalis was stimulated by O_2 (Lynch, 1975). Lindberg, Granhall and Burg (1979) confirmed the previous notion of an inverse relationship between O_2 concentration and C_2H_4 formation. They also suggested anae-

robes or facultative anaerobes to be active in C_2H_4 production although they did not attempt to isolate the responsible organisms. A recent study by Hunt, Matheny, Campbell and Parsons (1982) showed that C_2H_4 can accumulate under both oxidative and reductive conditions, and that it is not greatly stimulated by batch interchange of oxidized or reduced suspensions of the soil samples. The requirement for anaerobic conditions seems to be agreed upon by most investigators but whether the C_2H_4 -forming process itself is anaerobic is still conjecture.

b) Effect of temperature. Several reports have indicated that the rate of C_2H_4 formation increases with temperature. Boiling and even autoclaving stimulated C_2H_4 formation (Lynch, 1975; Rovira and Vendrell, 1972). On the other hand, Smith and Cook (1974) reported that production of C_2H_4 was significantly reduced at temperatures above $80^\circ C$ and almost ceased after autoclaving. Sutherland and Cook (1980) detected C_2H_4 even when samples were treated with aerated steam at $80^\circ C$ but again autoclaving stopped the evolution of the gas during subsequent incubation at $35^\circ C$. Lindberg et al. (1979) have shown that no C_2H_4 was produced at or below $10^\circ C$. Increasing the temperature to $24^\circ C$ greatly enhanced C_2H_4 and further increase in incubation temperature to $37^\circ C$ resulted in an eight-fold increase in C_2H_4 level (Lindberg et al., 1979). This marked increase in C_2H_4 above $24^\circ C$ was thought to be due to the release of previously formed C_2H_4 adsorbed on organic matter, increased O_2 depletion, or cell lysis (Lynch and Harper, 1974).

c) Effect of water potential. Experiments done on the effect of water potential in C_2H_4 accumulation have generally indicated an increase in C_2H_4 production with a decrease in water potential. Highest values of C_2H_4 reported occurred at or near saturation (Lindberg, et al., 1979; Hunt et al., 1982). In fact, the need of high moisture content for significant production of C_2H_4 and the cessation of its production in dry soils (-15 bars or drier) was used as an argument by those who suggested bacteria as the main producers of C_2H_4 in soil (Cook and Smith, 1977; Smith and Cook, 1974; Smith and Restall, 1971).

d) Effect of organic matter. The evolution of C_2H_4 in soils was correlated with organic matter content of those soils (Hunt et al., 1982; Smith and Restall, 1971). Ethylene production was stimulated by the addition of cereal straw (Goodlass and Smith, 1978; Wainwright and Kawlenko, 1977; Yashida and Suzuki, 1975), chitin and cellulose (Wainwright and Kowlenko, 1977) and finely ground Bermuda grass residue (Hunt et al., 1980). Barley residues were found to be more effective than those of either cabbage or beans (Lynch, 1976). From these results we would expect our desert soils, which are usually low in organic matter content, to produce relatively low concentrations of C_2H_4 .

e) Effect of organic amendments. As has been mentioned earlier, the addition of methionine, which is believed to be a precursor of C_2H_4 in higher plants, stimulated C_2H_4 production (Lynch, 1972; Lynch and Harper, 1974). Many other organic compounds have also been tested as possible precursors of C_2H_4 in plant tissue and fungi. The list in-

cludes; ethionine, cystine, cysteine, N-formyl methionine, α ketoglutaric acid, L-norleucine (Primrose, 1976), ACC and SAM (Adams and Yang, 1979), propanol (Lieberman and Kunishi, 1967), methional, linolenic acid, KMBA, homoserine (Baur and Yang, 1969b) and a host of other sugars, amino acids, organic acids and alcohols already mentioned (Abeles, 1972).

Several other soil amendments and conditioners have also been tested. The fungicides Captan and Thiram increased C_2H_4 in soil over one and two weeks incubation (Wainwright and Kowalenko, 1977). The herbicide 2, 4-D (2, 4-dichloro-phenoxy acetic acid) resulted in a small increase in C_2H_4 . The same result was obtained with N-serve, a nitrification inhibitor. Paraquat, Carbofuran and Endosulfan did not have any effect but lime ($CaCO_3$) significantly increased C_2H_4 production (Wainwright and Kowalenko, 1977).

Sutherland and Cook (1980) examined the effects of antibiotics and other compounds on C_2H_4 production. They reported that chloramphenicol and novobiocin (two antibacterial agents) inhibited C_2H_4 production but the antifungal agent cycloheximide did not. Sodium cyanide and sodium azide also suppressed C_2H_4 production. On the other hand, chlorogenic acid and EDTA (ethylene diamine tetra acetic acid) stimulated C_2H_4 production in soil (Sutherland and Cook, 1980).

Other soil factors which could affect C_2H_4 production especially in desert soils include salinity, sodicity and soil reaction. The ef-

fects of these factors have not been examined previously and hence special attention was given to them in this study.

CHAPTER 3

MATERIALS AND METHODS

Twelve surface soils (0 to 15 cm) were collected from various locations in Arizona. These soils represent some of the major soil types in the state and include a wide range of physical and chemical properties. They were: two natural desert soils, D and K, from the Santa Rita Experimental Range; a mountain-top soil, F, from Mount Lemmon; a garden soil, L, collected from a Tucson home; three artificially salinized soils (A, B and C) supplied by Dr. Arbabzadeh-Jolfaee, originally collected from the Safford Experiment Station Vineyard; Five irrigated soils, E and J were obtained from Yuma Valley Experiment Farm, and G, H and I collected from the U of A Farm at Marana, Campbell Avenue Farm, Safford Experiment Station, respectively. Of the last five soils, E and I were naturally saline. These soils represented six textural classes and had organic matter contents varying from 0.4 to 11.2% and pH values ranging from 5.0 to 8.3 (Table 1). For further information on the soils, see Appendix C.

Fresh soil samples collected from the field in plastic bags were stored moist at 4°C. Prior to use, the samples were air-dried at room temperature for 24 to 48 hours and then passed through a 2mm sieve.

Ethylene Production in Soils

To assess the ability of the soils to produce C_2H_4 , 50g samples contained in 150 ml serum bottles (wrapped with aluminum foil) were in-

cubated in the dark after bringing the samples to field capacity (approximated by the moisture content held at 1/3 bar tension) with deionized water. The bottles were fitted with air-tight rubber stoppers (Wheaton Scientific, 13 x 20 mm) to facilitate gas sampling. Samples were incubated at 30°C. Five replicates were used throughout this study and the appropriate controls were included in each incubation experiment.

Gas samples were withdrawn from the head space of the bottles into Vacutainers (Monoject, 16 x 100mm) with a double-headed needle, at various incubation periods ranging from six hour to 15 days. This study was done to determine the time(s) when maximum levels of C_2H_4 accumulated. Subsequently, a seven-day incubation period was adopted for soil samples and used for the rest of the study because preliminary results have shown that maximum C_2H_4 accumulation occurred after seven days of incubation in most soils used.

Gas Chromatography

One-milliliter samples were withdrawn from the Vacutainers using 1 ml gas-tight syringes (Precision Sampling Corp. Series A-2 "Pressure-Lok") and injected into a gas chromatograph (Varian 3700) equipped with a flame ionization detector. A 2m x 1/8" (80-100) Porapak N stainless steel column was used. Nitrogen was the carrier gas and the apparatus was operated isothermally at 80°C. The peak area and retention time were compared with a standard containing 959 ppm C_2H_4 in N_2 (Scott Specialty Gases, San Bernadino, CA). The limit of detection of this system

was found to be 1.57 p moles of C_2H_4 in a 1 ml gas sample. A sample calculation is shown in Appendix A.

Ethylene Production in Organic Amended Samples

Three levels of glycerol, ethanol and L methionine were tested in three soils. The effect of L-methionine, glucose and chloramphenicol was tested in the same soils in a factorial type experiment. These experiments were run in 15 ml disposable tubes (VWR Scientific Inc. disposable culture tubes 16 x 150 ml) stoppered with the same septa used on the incubation bottles. The compounds tested were added in aqueous solutions. Five-gram samples were wetted with the aqueous solution to bring its moisture content to 40% (dry weight basis). The samples were incubated at 30°C in the dark for seven days and C_2H_4 was assayed as before.

The effect of L-methionine concentration on C_2H_4 formation was assessed in the garden soil in 15ml glass tubes. Concentrations of 0, 0.25, 0.50, 1.00, 2.00 and 4.00 mg/g were added and the samples incubated for seven days at 30°C.

Isolation of Soil Fungi

Two soils which produced the highest levels of C_2H_4 , i.e. soils F and L, were chosen to isolate prospective C_2H_4 -producing fungi.

A modified Sabourauds dextrose agar medium (the commercial medium was amended with 5 g/liter L-methionine which is thought to be a C_2H_4 precursor and 50 mg/liter chloramphenicol to suppress bacterial growth) was inoculated with various dilutions of soil and the plates in-

cubated at 22°C in the dark for five days. Based on appearance of the colonies, different isolates were subcultured in fresh medium to obtain pure cultures of the isolates. Identification of the isolates was based on observation of colony characteristics, fruiting bodies, spores and mycelial characteristics as seen in slides and slide culture preparations. Several media were used to grow the isolates for identification and storage, i.e. Sabourauds dextrose and Sabourauds maltose media, brain-heart infusion agar, Czapeck-Dox agar and malt agar. Each isolate was grown at 22°C on slant culture tubes and stored at 4°C for further use.

Ethylene Production by Pure Cultures of Fungi

To determine C₂H₄ production by the various isolates, three different methods were evaluated (Lynch and Harper, 1974; Considine, Flynn and Patching, 1977; Chalutz and Lieberman, 1977). The method of choice was a modification of that used by Considine, et al. (1977):

The test isolates were grown on slant culture tubes stoppered with cotton wool and incubated in the dark at 22°C until the mycelium covered the surface of the medium (a solution of 0.5% L-methionine sterilized by passing through a millipore filter, was aseptically added to the warm Sabourauds dextrose agar medium before pouring 10 ml portions into sterile tubes. A sterile 1% glucose solution was added to the brain-heart infusion medium together with the 0.5% L-methionine solution). The tubes were then stoppered with alcohol sterilized rubber septa (stoppers were immersed in a 70% alcohol solution acidified with

0.1N HCl for 24 hours and dried under a sterile hood for two hours (Lynch and Harper, 1974)) and incubated for 48 hours at 25°C in the dark before gas samples were taken directly from the culture tubes for C₂H₄ assay. Uninoculated slants were included as controls.

Mucor hiemalis, Geotrichum sp. and Candida vartiovaari (American Type Culture Collection #26035, 32345 and 32346, respectively) and 14 other known species of non-pathogenic fungi: Absidia spinosa, Aspergillus nigrans, Geotrichum candidum, Hansenula saturnus, Mortierella isabellina, Penicillium digitatum, Rhizopus arrhizus, Rhizopus stolonifer, Rhodotorula sp., Scopulariopsis brevicaulis, Schizosaccharomyces octosporus, Streptomyces sp., Zygorhynchus sp. (Courtesy of Dr. Sinski, Microbiology Dept., U of A), and Saccharomyces cerevisiae purchased at local stores) were also evaluated as to their ability to produce C₂H₄ in pure cultures.

The saline soil (A) and the two highest C₂H₄-producing soils, F and L, were autoclaved for two hours on three consecutive days, amended with a sterile solution of 1% glucose and 0.5% methionine and inoculated with the isolate, i.e. Fusarium sp., which produced the highest level of C₂H₄. Mucor hiemalis, propagated on a synthetic medium (Lynch and Harper, 1974), was also used on these soils for comparative purposes. Inoculation was done by harvesting the spores with sterile water from a solid culture medium (malt agar contained in 250 ml conical flasks). The turbidity of the suspension was measured in a spectrometer (Spectro-

nic-20) and a spore concentration of 10^4 /ml was used as an inoculum (Lynch and Harper, 1974).

Studies on Saline and Alkali Soils

Saline and alkali (sodic) soils usually produce little or no C_2H_4 (Haverland and Pepper, 1981). To investigate the effect of neutral and alkali salts, the following experiments were carried out:

About 150 to 200 g samples of each of four saline soils; (A, B, C and E) were suspended in deionized water and shaken for two hours. The suspension was filtered in Buchner funnels with the aid of a suction pump. The electrical conductivity (Ec) was measured (Solu Bridge Soil Tester). The washing was repeated to remove most salts (until the Ec dropped to about 0.2 mmhos/cm). Leached samples were then air-dried, crushed, sieved (<2mm) and rewetted for incubation. Unwashed subsamples were also wetted to field capacity and incubated and analyzed for C_2H_4 production.

Two soils, previously identified as high C_2H_4 producers (soils F and L), and the saline soil A were treated with 0.04, 0.2 and 0.4 meq/g concentrations of NaCl, NaOH and KOH in 15 ml disposable tubes. After seven days incubation at 30°C, C_2H_4 concentration was determined and soil pH was measured in all samples in a 1:2 soil/water ratio using a Leeds and Northrup pH meter.

CHAPTER 4

RESULTS AND DISCUSSION

Ethylene Production in Desert Soils

Ethylene production was monitored in twelve desert soils for incubation periods ranging from 6 to 360 hours in the dark at 30°C (Figs. 1 to 6). All soils, with the exception of the Mount Lemmon soil (F) and the garden soil (L), produced a maximum amount of C_2H_4 of less than 120 p moles/g soil. The artificially salinized soils; A, B, and C (Fig. 1), which also contained considerable amount of exchangeable sodium (about 14%), produced the lowest concentration of C_2H_4 with soil B being the poorest. Although soils E and I were also saline, with soil E having the highest E_c (Table 7), they produced appreciably higher levels of C_2H_4 (Fig. 3) than soils A, B and C. The relatively elevated C_2H_4 levels in soils E and I are likely to be due to their low exchangeable sodium (Table 1). In addition, soils E and I were naturally saline soils, and likely contained a microbial population which had evolved to be less sensitive to salinity than the microbial population in the artificially salinized soils.

Soil D, a non-saline sandy soil, and soil H, a non-saline loamy soil also produced low C_2H_4 (Fig. 2 and Fig. 5). Soils D and H are medium to coarse-textured with low organic matter contents (Table 1). Figure 2 also shows C_2H_4 production in soil K which is also a sandy loam

Table 1. Selected properties of the soils used.

Soil	pH 1:2 soil/water ratio	Saturation Extract				* N ppm	+ P	Organic Matter	Moisture at 1/3 bar tension	Mechanical Analysis			Textural Class
		Ec _e mmhos/cm	Na meq/L	K meq/L	ESP --%--					Sand %	Silt	Clay	
A	7.7	8.5	47.2	1.1	13.1	21.4	3.5	0.8	37	22	36	42	Clay
B	7.9	7.0	41.5	0.8	13.2	173.4	3.8	0.9	32	27	35	38	Clay loam
C	8.3	5.1	16.7	0.2	14.0	71.1	3.5	0.7	33	35	34	31	Clay loam
D	7.6	0.4	0.7	0.1	0.7	7.0	0.9	0.6	5	85	11	4	Loamy sand
E	7.4	15.9	27.2	2.4	3.6	295.3	1.7	0.5	28	28	46	26	Silt loam
F	5.0	0.2	0.3	0.3	0.7	2.4	0.3	11.2	23	63	28	9	Sandy loam
G	7.9	0.8	3.7	0.5	2.9	13.7	3.7	0.6	27	31	35	34	Clay loam
H	8.0	1.0	1.8	0.9	0.1	29.8	9.2	0.4	18	45	44	11	Loam
I	6.6	7.7	7.8	1.2	0.7	7.5	0.9	1.2	18	57	30	13	Sandy loam
J	7.8	2.6	12.2	0.7	5.5	23.3	1.5	1.1	35	9	38	53	Silty clay
K	6.5	0.6	0.7	0.5	0.6	10.1	1.4	0.4	8	75	17	8	Sandy loam
L	7.4	0.6	2.5	0.6	1.5	8.4	16.4	5.4	19	62	27	11	Sandy loam

* From CO₂ extraction. Technicon reduction of nitrate reported as N. To convert N to NO₃ multiply N X 4.4.

+ CO₂ extraction and orthophosphate determination (Technicon). To convert P to PO₄ multiply P by 3.1.

Figure 1. Ethylene production in three artificially salinized vineyard soils.

Figure 2. Ethylene production in two coarse-textured non-saline range soils.

Figure 3. Ethylene production in two saline non-sodic cultivated soils.

Figure 4. Ethylene production in a Mount Lemmon soil (F).

and similar to soil D in land use and organic matter content, yet its ethylene producing pattern is significantly higher than soil D. A possible explanation for this is the slightly acidic pH of soil K which is probably more favorable to C_2H_4 producing microorganisms in this soil.

Two soils which can be categorized separately from all the others are Soils F and L (Figs. 4 and 6). Their organic matter contents were 11.2 and 5.4%, respectively, and their C_2H_4 production patterns are characteristically higher than the rest of the soils which suggests a strong influence of organic matter content of the soil on its ability to produce C_2H_4 . In fact, statistical analysis showed good correlation between organic matter content and C_2H_4 production with an r^2 of 0.85. This is in accordance with the results of Smith and Restall (1971) and Hunt et al. (1982).

Both the garden soil and the Mount Lemmon soil started producing C_2H_4 after only six hours incubation whereas for all other soils, at least a 24-hour lag period was necessary before any detectable C_2H_4 was produced. This lag period was probably important in establishing an anaerobic environment necessary for C_2H_4 accumulation. To start with, the soils are most likely to have small anaerobic pockets where C_2H_4 forms but because they were initially incubated in an aerobic environment, C_2H_4 is likely to be oxidized as soon as it is formed (Cornforth, 1975). After 24 hours incubation, the O_2 concentration decreases in this closed system and C_2H_4 oxidation is likely to be at a minimum and so detectable C_2H_4 increases can be measured. In case of Soils F and L,

Figure 5. Ethylene production in three medium-textured non-saline cultivated soils.

Figure 6. Ethylene production in a garden soil (L).

C_2H_4 formation was probably in excess of its oxidation because of their high organic matter contents and perhaps higher C_2H_4 producing populations.

Soil J (Fig. 5), a silty clay non-saline cultivated soil, is significantly higher in C_2H_4 production than the rest of the low organic matter soils. A higher C_2H_4 producing population is a possible explanation. Figure 5 also shows C_2H_4 production in a Pima clay loam (G) which is also non-saline nonsodic and shows higher level of C_2H_4 than the saline soils with the exception of Soil I. Note also, that in all soils except the two high organic matter soils, C_2H_4 production was essentially at a maximum after seven days incubation.

Lynch and Harper (1980) referred to coarse-textured soils and those low in organic matter as "substrate deficiency soils" with respect to C_2H_4 production. According to this categorization, all soils used, again with the exception of the Mollisol (F) and the garden soil (L), can be considered substrate deficient, and soils F and L are substrate-rich soils.

Ethylene Production as Affected by Organic Compounds

Three different levels of L-methionine, glycerol and ethanol were added as amendments to soils A, F and L. The effect of 0.2, 1.0 and 2.0 $\mu\text{g/g}$ soil of each of these compounds in Soil A is shown in Figure 7. All treatments showed significantly increased C_2H_4 relative to the control, but in case of glycerol and ethanol, there was no difference in C_2H_4 production with increasing concentration of the added organic ma-

Figure 7. Microbial C_2H_4 production in a saline soil (A) as affected by organic compounds.

Figure 8. Microbial C_2H_4 production in a Mount Lemmon soil as affected by organic compounds.

Figure 9. Microbial C_2H_4 production in a garden soil as affected by organic compounds.

Figure 10. Ethylene production in soils as affected by methionine, glucose and chloramphenicol.

1: control; 2: glucose, 2 mg/g soil; 3: methionine, 2 mg/g soil;
4: glucose + methionine; 5: glucose + methionine + chloramphenicol

terial. L-methionine, however, even at the low concentration of 0.2 mg/g, produced significantly greater C_2H_4 concentrations than at all levels of glycerol and ethanol. Increasing the concentration of L-methionine to 1.0 mg/g resulted in a further significant increase in C_2H_4 , but at a concentration of 2.0 mg/g of the amino acid, there was no further significant improvement in C_2H_4 production. In Soil F (Fig. 8), only L-methionine resulted in a significant increase in C_2H_4 . Here again the effect of 1 mg concentration of the amino acid was significantly greater than the lowest concentration. The results shown by Soil F are more or less repeated in Soil L (Fig. 9) with the exception of a significant increase in C_2H_4 at the two higher levels of glycerol.

These results indicate that L-methionine may be a precursor of C_2H_4 in these soils. Increases in C_2H_4 observed with glycerol and ethanol are probably due to these compounds acting as energy sources thereby increasing the heterotrophic microbial population active in C_2H_4 production. Soil A, which is perhaps the poorest in carbon and energy sources showed similar but significant increases at all three levels of glycerol and ethanol (Fig. 7) while these compounds had no significant enhancing effect on C_2H_4 production in the carbon richer soils F and L (Figs. 8 and 9) with the exception of the two high levels of glycerol in Soil L.

Figure 11 shows the effects of a wider range of concentrations of methionine in C_2H_4 production in the garden soil. Increasing the concentration of methionine from 0.25 to 1.0 mg/g significantly increased C_2H_4 production. This increase leveled off at concentrations equal to

Figure 11. Microbial production of C_2H_4 in a garden soil as affected by methionine.

or greater than 2.0 mg/g soil possibly because of enzyme saturation. These results are in contrast to those reported by Hunt et al. (1980) and Sutherland and Cook (1980) who reported that the addition of methionine had no effect on the production of C_2H_4 in their soils. Several other reports, however, agree that methionine significantly increased C_2H_4 production (Chalutz and Lieberman, 1977; Lynch, 1972; Lynch and Harper, 1974). It is evident, then, that methionine behaves differently in different soils.

Goodlass and Smith (1978) reported that ethanol promoted C_2H_4 production but they did not suggest ethanol as a precursor for C_2H_4 . The effect of glycerol in this study, is similar to that of ethanol and probably suggests their role is as a carbon and energy source rather than as substrate for C_2H_4 .

Figure 10 shows the influence of glucose and methionine separately, together, and in combination with chloramphenicol on C_2H_4 production in Soils A, F and L. Glucose at a concentration of 2 mg/g increased C_2H_4 significantly in all three soils. L-methionine at the same rate resulted in significantly higher levels of C_2H_4 in Soil A and Soil F, but not in Soil L. Glucose plus methionine, however, was not significantly different from the methionine treatments in all three soils. It was expected that, at least in Soil A, glucose plus methionine would yield the highest ethylene. It is possible, however, that the addition of glucose not only stimulated C_2H_4 production, but also enhanced the growth of heterotrophs that utilize C_2H_4 .

Table 2. Microbial production of C_2H_4 as affected by neutral and alkali salts.

Soil	Salt added	Salt Concentration meq/g soil	pH 1:2 soil/water ratio	C_2H_4 P moles g^{-1} 7days $^{-1}$
Vine orchard saline soil (A)	Control	0.00	7.7	9
	NaCl	0.04	7.5	8
		0.10	7.3	8
		0.20	7.4	8
	NaOH	0.04	8.2*	7
		0.10	9.1*	9
		0.20	10.0*	15
	KOH	0.04	8.5*	8
		0.10	9.1*	8
		0.20	10.1*	12
Mount Lemmon Soil (F)	Control	0.00	5.0	1055
	NaCl	0.04	4.8	643*
		0.10	4.9	482*
		0.20	4.9	488*
	NaOH	0.04	6.3*	1143
		0.10	6.8*	541*
		0.20	7.5*	664*
	KOH	0.04	6.3*	1202
		0.10	6.9*	751*
		0.20	7.6*	549*
Garden Soil (L)	Control	0.00	7.5	1016
	NaCl	0.04	7.4	1036
		0.10	7.4	1027
		0.20	7.3	880*
	NaOH	0.04	7.9*	860*
		0.10	8.3*	561*
		0.20	8.8*	429*
	KOH	0.04	8.1*	766*
		0.10	8.5*	480*
		0.20	9.0*	407*

*Significant at the 5% level.

The chloramphenicol treatment reduced the C_2H_4 production only in Soil A which may indicate that bacteria play a more important role in C_2H_4 production in this soil than in either Soils F or L. Sutherland and Cook (1980) reported that chloramphenicol inhibited C_2H_4 production in their soils indicating a bacterial role in C_2H_4 production. Although a bacterial role cannot be ruled out in our soils, it is probably of less importance than fungi.

Ethylene Production as Affected by Salt Treatments

Sodium chloride, sodium hydroxide and potassium hydroxide were added to Soils A, F and L at the rates of 0, 0.04, 0.1 and 0.2 mg/g soil. The amount of C_2H_4 accumulating in a seven-day incubation period and soil pH determined in a 1:2 soil/water ratio are shown in Table 2.

In Soil A, which is already saline (Table 1), further addition of these salts did not produce significant changes in the levels of C_2H_4 . Although C_2H_4 production was at a minimum to begin with, adding more salt did not inhibit C_2H_4 completely as was expected and the gas production remained the same or it increased slightly at the highest level of alkali added. The significant pH increase may have affected C_2H_4 production chemically rather than biologically.

In Mount Lemmon soil (F) all three treatments of NaCl significantly reduced C_2H_4 . The reduced C_2H_4 is most likely due to the direct inhibition of C_2H_4 producing microorganisms by the salt. In both alkali treatments, however, the lower level of 0.04 mg/g slightly increased C_2H_4 production. This increase was not significant but may have occurred

Figure 12. Microbial production of C_2H_4 in four saline soils as affected by leaching.

because of a more favorable environment due to increased pH. This may have affected bacterial C_2H_4 production in this soil. Both higher alkali levels decreased the evolution of C_2H_4 significantly most likely because of sodium toxicity, high osmotic pressure and/or increased pH, all of which are likely to affect microbial activity.

In the garden soil (L) a high concentration of NaCl was necessary to significantly reduce C_2H_4 production, but all the alkali treatments decreased C_2H_4 significantly. The large increase in pH could be an accentuating inhibitory factor.

Ethylene Production as Affected by Leaching of Saline Soils

The salts in four saline soils (A, B, C and E) were washed until the E.C. dropped to about 0.2 mmhos/cm. The soils were incubated together with non-washed soils and C_2H_4 production was assessed after a seven-day period. Figure 12 shows the effect of salt leaching on C_2H_4 production.

In Soils A and E, a significant increase in C_2H_4 resulted from leaching. An increased C_2H_4 production in both B and C was also detected though it was not statistically significant. These results indicate that removing the salts in Soils A and E created a better environment for C_2H_4 production. The microorganisms producing C_2H_4 were probably there in these saline soils but were previously inhibited by the salt. Washing the salts removed the inhibiting factor and the microbes actively produced C_2H_4 . In case of Soils B and C, when salts were added to these soils (Soils A, B and C were artificially salinized), they

might have destroyed the C_2H_4 producing population and hence salt leaching did not improve C_2H_4 production in these soils. In the natural saline soil (E), C_2H_4 producing species more tolerant of salt may have evolved.

These experiments on the effect of salt, therefore, indicate an inhibiting effect on C_2H_4 production in our soils. They also suggest that C_2H_4 may be produced by salt sensitive species but further experiments are necessary to determine the specific salt effects and survival studies of the C_2H_4 producing species under salt stress.

Ethylene Production in Pure Cultures of Isolated Soil Fungi

Fourteen fungal isolates belonging to nine genera were isolated from the two highest C_2H_4 producing soils (F and L). Mean C_2H_4 concentrations produced on SDAM slants are shown on Table 3. A Fusarium species produced the highest level of C_2H_4 , more than all the C_2H_4 produced by five other isolates belonging to the genera Aspergillus, Penicillium, Curvillaria and Rhizopus. Eight other isolates tested did not produce significant C_2H_4 concentrations (Table 3).

Previous reports have implicated species belonging to the genera Aspergillus (Ilag and Curtis, 1968) Fusarium (Swart and Kamerbeek, 1977; Graham and Linderman, 1980) and Penicillium (Chalutz et al., 1977), but neither Curvillaria nor Rhizopus spp. were reported. Swart and Kamerbeek (1977) found that all Fusarium species and formae speciales they examined produced C_2H_4 in variable amounts but F. oxysporum f. sp. tulipae produced the most. High C_2H_4 production was also reported in F.

Table 3. Ethylene production in pure cultures of isolated soil fungi grown on SDAM slants at 25°C.

Isolate #	Genus	Ethylene P mole/sample/48 hours
F-4	<u>Fusarium</u>	833
F-8	<u>Aspergillus</u>	293
F-28	<u>Penicillium</u>	232
L-21	<u>Curvularia</u>	147
L-3	<u>Penicillium</u>	75
F-17	<u>Rhizopus</u>	64
F-20	<u>Aspergillus</u>	N.S.
L-7	<u>Aspergillus</u>	N.S.
F-2	<u>Penicillium</u>	N.S.
F-22	<u>Mucor</u>	N.S.
L-27	<u>Rhizopus</u>	N.S.
F-30	<u>Cladosporium</u>	N.S.
F-31	<u>Alternaria</u>	N.S.
L-19	<u>Chaetomium</u>	N.S.

F and L refer to soil from which the fungus has been isolated.
N.S. = Not significant

Values are means of 5 replicates.

oxysporum f. sp. pini (Graham and Linderman, 1980). So it was not surprising that our Fusarium (F-4) isolate was a high C_2H_4 producer. Not only in SDAM did Fusarium produce the highest level of the gas, but also in BHIA (Table 5). Compared to Mucor hiemalis which is believed by Lynch and Harper (1974) to be a high C_2H_4 producer, Fusarium produced more than 11X the amount of C_2H_4 produced by M. hiemalis in SDAM. Differences in C_2H_4 production by the latter fungus in different media (Tables 4 and 5) could be explained on basis of differences in the composition of the media, pH and the rate of mycelium growth. It was reported that even single-spore isolates of the same fungus vary widely in C_2H_4 production (Swart and Kamberbeek, 1977).

Second in importance in C_2H_4 production were the Aspergillus (F-8) and Penicillium (F-28) isolates. Notice that all three highest C_2H_4 producing isolates were obtained from the highest C_2H_4 producing soil, the Mollisol (Soil F). Another Penicillium isolate (L-3) and a Curvularia isolate (L-21) were obtained from the garden soil (L) and come next in C_2H_4 producing ability in vitro. In the literature, several aspergilli have been reported to produce C_2H_4 . The most important is Aspergillus clavatus which produced over 500 ppm C_2H_4 (Ilaq and Curtis, 1968). Penicillium digitatum has been used as a research tool in C_2H_4 biochemistry because of its known ability to produce high C_2H_4 (Chalutz et al., 1977). Other Penicillium spp. that produced C_2H_4 in vitro include P. corylophilum, P. patulum and P. luteum (Ilaq and Curtis, 1968).

Although our isolates produced significant concentrations of C_2H_4 which could account for the C_2H_4 produced by the respective soils from which they were isolated, it must be borne in mind that the conditions in a soil environment are completely different from a culture medium. Nutrient status, aeration, presence of a mixed microbial population, and competition determine which species produce C_2H_4 and how much of the gas will accumulate. In a mixed soil population, C_2H_4 degrading and C_2H_4 absorbing organisms are present so in effect we are measuring the net C_2H_4 production. Another important factor which is not accounted for in this study is C_2H_4 production by bacteria which could be very significant.

We notice that there are great differences in C_2H_4 producing ability at the genus level (Table 3), and as Swart and Kamerbeek (1977) had found, there are even differences within the same fungal species.

Ethylene Production in Pure Cultures of Known Fungal Species

In a comparative study, fungal species some of which are known to produce C_2H_4 , were also evaluated on SDAM slants (Table 4). The highest level of C_2H_4 was produced by Penicillium digitatum. This agreement with previous observations that this fungus is an important C_2H_4 producer (Jacobsen and Wang, 1968; Chalutz, et al., 1977). Still the C_2H_4 produced by the Fusarium isolate was much higher. A Mortierella sp. produced significantly high levels of C_2H_4 , even higher than Geotrichum sp., Candida vartiovaari and M. hiemalis. The latter fungus did poorly in this medium (only 72 pmoles/sample) but produced much greater C_2H_4

Table 4. Ethylene production in pure cultures of known fungal species grown on SDAM slants at 25°C.

Fungal Species	Ethylene P mole/sample/48 hours
<u>Penicillium digitatum</u>	536
<u>Mortierella sp.</u>	408
<u>Geotrichum sp. (ATCC)</u>	339
<u>Rhizopus nigricans</u>	265
<u>Candida varii</u> (ATCC)	182
<u>Rhodotorula sp.</u>	179
<u>Trichoderma sp.</u>	85
<u>Mucor hiemalis (ATCC)</u>	72
<u>Scopulariopsis brevicaulis</u>	41
<u>Streptomyces sp.</u>	N.S.*
<u>Absidia spinsa</u>	N.S.
<u>Schizosaccharomyce octosporus</u>	N.S.
<u>Saccharomyces cerevisiae</u>	N.S.
<u>Rhizopus arrhizus</u>	N.S.
<u>Hansenula saturnus</u>	N.S.

*N.S. = not significant

Values are means of 5 replicates.

Table 5. Microbial C₂H₄ production in some special media.

Fungal species	Medium	Ethylene p moles/sample/48 hours
<u>Mucor hiemalis</u> (ATCC)	Lynch & Harper medium*	370
<u>Fusarium</u> F4	Brain heart infusion agar	590
<u>Saccharomyces</u> <u>cerevisiae</u> *	Sobaurand's dextrose broth	410

*Lynch and Harper (1974).

*Incubation done in 150 ml serum bottles at 25°C.

All media are amended with 1 mg methionine per milliliter.

(Table 5) in a synthetic medium described by Lynch and Harper (1974). Rhizopus nigricans, Rhodotorula sp., Trichoderma sp. and Scopulariopsis brevicaulis all produced significant C_2H_4 levels (Table 4).

Species that did not produce significant C_2H_4 when grown on SDAM were: Streptomyces sp., Absidia spinosa, Schizosaccharomyces octosporus, Saccharomyces cerevisiae, Rhizopus arrhizuys and Hansenula saturnus. Saccharomyces cerevisiae, however, when grown in Sabourauds dextrose broth amended with 1 mg methionine/ml in shaken 150 ml serum bottles produced significant C_2H_4 concentration (Table 5). Thomas and Spencer (1978) reported that S. cerevisiae cells absorb some of the C_2H_4 in the culture bottle head space. This may explain why C_2H_4 was not detected in SDAM slants. It may also suggest that C_2H_4 might have been produced and reabsorbed by the thalli of those fungi reported here as non-producers. The ability of these fungi to absorb C_2H_4 can be studied by growing them and introducing known concentrations of C_2H_4 and incubating in a closed system then remeasuring C_2H_4 . This is important to know because net C_2H_4 accumulation is equal to production minus degradation and absorption.

Ethylene Production in Sterilized Inoculated Soils

The high C_2H_4 producing Fusarium isolate together with Mucor hiemalis, which has been reported as an important C_2H_4 producing fungus (Lynch and Harper, 1974), were used as inoculum in three sterilized soils. Table 6 shows C_2H_4 production in this experiment. We notice the

Table 6. Microbial C₂H₄ production in sterilized inoculated soils.

Soil	Inoculant	C ₂ H ₄ P mole/g/7 day
A	Control	8
	<u>M. hiemalis</u>	52**
	<u>Fusarium sp.</u>	137**
F	Control	37
	<u>M. hiemalis</u>	12
	<u>Fusarium sp.</u>	219**
L	Control	14
	<u>M. hiemalis</u>	42**
	<u>Fusarium sp.</u>	120**

**Significant at the 1% level.

Incubation temperature was 30°C.

low C_2H_4 in autoclaved controls relative to non-sterilized soils and inoculated samples indicating that C_2H_4 is of biological origin.

The saline soil, A, which was a very poor C_2H_4 producer when non-sterilized (Figure 1), produced significant levels of C_2H_4 when inoculated with either Fusarium or M. hiemalis. This probably indicates that the soil was lacking in efficient C_2H_4 producing species. This may seem to be contradicted by the results obtained in the leaching experiment but the increase in C_2H_4 production in leached soils was probably brought about by salt sensitive species which were reactivated when the salts were removed. Also, in the leaching study, even though significant increases occurred following leaching, overall C_2H_4 levels were still low (less than 40 p moles/g). In this experiment, the inoculated organisms appear to enhance C_2H_4 levels from 6 to 17 times in all soils. Another important factor is the elimination of competition in these sterile soils which in essence reduces one level of stress. This will also eliminate any C_2H_4 absorbing or degrading species.

Mucor hiemalis did not produce significant C_2H_4 in Soil F. In fact the C_2H_4 in the uninoculated controls was higher, though not significantly so, than in the samples inoculated with M. hiemalis. This could mean that M. hiemalis mycelium reabsorbs the C_2H_4 it produces and this may explain why it did poorly in pure culture. In any case these results rule out M. hiemalis as an important C_2H_4 producer in the soils tested. Again, the Fusarium sp. produced significant C_2H_4 in both F and L but the level of C_2H_4 was only a fraction of that found in nonsteri-

lized soils. This probably indicates that C_2H_4 production is a contribution of more than one fungal species, but a bacterial role in C_2H_4 production is also possible. Similar results were obtained by Sutherland and Cook (1980). Their explanation was that they might have failed to isolate the more important C_2H_4 producing species, a requirement for two or more microorganisms for C_2H_4 production, as in symbiotic methane production, the destruction of a C_2H_4 precursor in soil by autoclaving or a failure to provide the proper conditions for C_2H_4 production in autoclaved soil. Some or all of these reasons might have been operative in our experiment.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Cumulative C_2H_4 production was monitored in twelve different desert soils incubated moist at constant temperature for various incubation times. Gas samples collected from the bottles head-space were analyzed using gas chromatography.

Ethylene production was low in all but two soils with high organic matter. Statistical analysis showed a fairly good correlation between organic matter contents and C_2H_4 production with an r^2 of 0.85. Minimum levels of C_2H_4 were observed in three artificially salinized soils which contained high exchangeable sodium indicating a negative influence of salt on C_2H_4 production. Two naturally saline soils produced higher C_2H_4 concentrations possibly because of low exchangeable sodium or because of a more salt tolerant ethylene producing microorganism. Other non-saline, non-sodic soils which included range soils and cultivated soils ranging in texture and chemical properties but all with low organic matter contents (1.2% or less), produced a maximum C_2H_4 of less than 120 pmoles/g after a seven-day incubation period. In comparison, the two high organic matter soils accumulated maximum levels of C_2H_4 of 1321 pmoles g^{-1} (a garden soil with 5.4% organic matter) and 3675 pmoles g^{-1} (a mollisol with 11.2% organic matter). The garden soil continued to produce C_2H_4 up to 10 days after which C_2H_4 production significantly

decreased but the mollisol was actively producing C_2H_4 even after 15 days incubation. Also, these two soils showed no lag period in C_2H_4 production unlike all other 10 soils.

The influence of organic compounds on C_2H_4 production was examined in three soils. Three levels of glycerol, ethanol and methionine were tested. All treatments showed significant increase in C_2H_4 in a saline sodic soil but no significant differences in C_2H_4 were detected with different levels of glycerol and ethanol. Ethylene produced in methionine treatments was significantly higher than the two other compounds and increased significantly with higher methionine concentrations possibly indicating its role as a precursor for C_2H_4 . In two high C_2H_4 producing soils significant increases were only detected with methionine treatments and the high levels of glycerol suggesting that ethanol and glycerol acted as carbon and energy sources.

Glucose and methionine were examined separately, together and in combination with an antibacterial agent, chloramphenicol, in three soils. Methionine was more effective than glucose but the two compounds together did not increase C_2H_4 significantly from the separate addition of either compound. Chloramphenicol reduced C_2H_4 production only in the saline sodic soil possibly by killing C_2H_4 producing bacteria which apparently are not important in the other two soils.

The addition of saline and alkali solutions to soils had an inhibiting effect on C_2H_4 production in the two high C_2H_4 producers. Further addition of salt to the saline soil did not produce significant

differences in C_2H_4 production. Sodium hydroxide and potassium hydroxide behaved similarly producing significant increases in pH and reducing C_2H_4 production. The decrease in C_2H_4 production is most likely due to a direct effect on the C_2H_4 producing population through toxic salt levels, high osmotic pressure and/or increased soil reaction (in case of the alkali treatments).

Leaching of four saline soils and subsequent incubation resulted in significant increases in C_2H_4 production in two soils. These soils probably contained C_2H_4 producing microorganism which were inhibited by the salt. Removing the salts reactivated the organisms and C_2H_4 was produced. In the two other soils (which were artificially salinized) adding salts probably irreversibly inhibited C_2H_4 producing microbes.

The most frequent fungal species were isolated from the two high C_2H_4 producing soils. Ethylene production was assayed in pure cultures of these isolates and other known fungal species in Sabouraud dextrose agar slants amended with 1 mg/dl methionine. A Fusarium sp. isolated from a Mount Lemmon soil produced the highest C_2H_4 in pure culture followed by isolates belonging to the genera Aspergillus, Penicillium, Curvillaria and Rhizopus. Eight other isolates did not produce detectable C_2H_4 . There were obvious differences in C_2H_4 production in species belonging to the same genus. Penicillium digitatum, reported in the literature as an important C_2H_4 producer, produced a high level of C_2H_4 in pure culture, second only to Fusarium sp. Significant C_2H_4 was also produced by Mortierella sp., Geotrichum sp. (ATCC), Rhizopus nigricans,

candida vartiovaari (ATCC), *Rhodotorula* sp., *Trichoderma* sp., *Mucor hiemalis* (ATCC) and *Scopulariopsis brevicaulis*. *Mucor hiemalis* did poorly in SDAM, but produced much higher levels of ethylene in a synthetic medium described by Lynch and Harper (1974). *Saccharomyces cerevisiae* did not produce significant C_2H_4 in SDAM slants but produced high levels in a liquid medium amended with methionine. Six other fungal species produced no significant C_2H_4 in SDAM slants. Of all the soil isolates and the known fungal species 52% produced significant C_2H_4 in pure cultures.

Three sterilized soils were inoculated with the spores of *M. hiemalis* and *Fusarium* sp., amended with sterile solution of glucose and methionine and assayed for C_2H_4 . The saline soil, one of the lowest C_2H_4 producers, produced significant C_2H_4 with both inoculants. *M. hiemalis* induced C_2H_4 five times that in non-inoculated non-sterilized samples. *Fusarium* species produced almost 14 times the C_2H_4 produced in non-sterile soil. This suggests that this soil was lacking in efficient C_2H_4 producers, so the low C_2H_4 production in this soil was probably the result of low C_2H_4 producing population, low substrate level and saline conditions affecting the sensitive C_2H_4 producers.

Inoculating a mollisol with *M. hiemalis* resulted in lower C_2H_4 production than the control samples. This can be accounted for by C_2H_4 being reabsorbed by the fungus mycelium which may explain why this fungus produced low C_2H_4 in pure culture. *Fusarium* sp. produced high C_2H_4 in all three soils but the amount it produced was only a fraction of

what the soils produced when they were incubated without sterilization. This possibly means that C_2H_4 production in soil is a contribution of various microbial species.

This research does not rule out the importance of bacteria in C_2H_4 production in our desert soils, hence further research is needed to investigate their importance. The effect of plant roots on C_2H_4 production is another important area of research.

APPENDIX A

SAMPLE CALCULATION

Ethylene standard used contained 959 ppm C₂H₄.

Volume of standard used was 25 ul.

Barometric pressure in laboratory was estimated as 700 mm of mercury.

Average total units of standard (peak height x attenuation) = 572.

1 ml pure C₂H₄ = 1000 ul/ml = 10⁶ ppm.

∴ 1 × 10⁶ ppm = 10³ ul.

i.e. 1 ppm = 10⁻³ ul.

$$\frac{959 \times 25 \text{ ul}}{572 \times 1000} = 0.042 \text{ ppm/unit}$$

$$0.042 \times 1000 = 42 \text{ uul/unit}$$

To convert to umoles use the equation $PV = nRT$

$$\text{where: } P = \text{pressure in atmospheres} = \frac{700}{760} \text{ atmospheres}$$

$$V = \text{Volume; } n = \text{number of moles; } R = \text{gas constant} = 0.082$$

and $T = \text{temperature in Kelvin.}$

If $V = 1 \text{ ul}$ & temperature = 300°K, then $n = 0.037 \text{ umoles}$

$$\therefore 1 \text{ umole} = 26.7 \text{ ul}$$

$$\text{and } 42126.7 = 1.57 \text{ umole/unit}$$

$$= 1.57 \text{ pmole/unit.}$$

$$\frac{\text{Total units}}{1 \text{ c.c. injection}} \times 1.57 = \text{pmole/injection}$$

$$\text{pmole/injection} \times \text{head - space volume} = \text{pmole/sample.}$$

$$\text{To relate } \frac{\text{p mole per sample}}{\text{oven-dry weight of sample}} = \text{pmole g}^{-1}$$

To relate pmoles and ppm:

$$\text{If } 0.042 \text{ ppm} \equiv 1.57 \text{ ppm}$$

$$\text{Then } 1 \text{ ppm} \equiv 37.4 \text{ pmoles}$$

APPENDIX B

A TYPICAL CHROMATOGRAM

APPENDIX C

SITES AND CLASSIFICATION OF THE SOILS USED

Soils A, B and C were collected from the vineyard of the University of Arizona Branch Experiment Station at Safford. All three soils belong to a Pima clay loam variant i.e. fine-silty, mixed, thermic, Typic Torrifuvents. Soil I, collected from the same station (Site #4), is coarse-loamy, mixed, thermic Typic Torrifuvents.

Soils D and K were both obtained from Santa Rita Range Experiment Station. The former soil is a Comoro sandy loam classified as coarse-loamy, mixed, thermic, Typic Torrifuvents and the latter is a Sonoita sandy loam belonging to the coarse-loamy, mixed, thermic Typic Haplargids.

Soil E, a Pimer silt loam (Site #7) and Soil J, a Holtville silty clay (Site #4) were both collected from Yuma Valley Experiment Station and are classed as fine-silty, mixed (calcareous) hyperthermic, Typic Torrifuvents and clayey over loamy, montmorillonitic (calcareous), hyperthermic Typic Torrifuvents, respectively.

Soil F was collected from Mount Lemmon and is classified as a Typic Haploborolls.

Soil G, a Pima clay loam, was collected from the University of Arizona Farm at Marana and is classified as fine-silty, mixed (calcareous), thermic Typic Torrifuvents.

Soil H, a Gila loam from Campbell Avenue Farm, is a coarse-loamy, mixed (calcareous) hyperthermic Typic torrifluvents.

Soil L, a Cave soil collected from a home garden just north of the University of Arizona campus, is classified as loamy, mixed, thermic, shallow Typic Paleorthids.

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