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EXPERIMENTAL ARTICLES

Effect of Substrate-Dependent Microbial Ethylene Production on Plant Growth[1]

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Abstract—Various compounds have been identified as precursors/substrates for the synthesis of ethylene (C_2H_4) in soil. This study was designed to compare the efficiency of four substrates, namely L-methionine (L-MET), 2-keto-4-methylthiobutyric acid (KMBA), 1-aminocyclopropane-1-carboxylic acid (ACC), and calcium carbide (CaC_2), for ethylene biosynthesis in a sandy clay loam soil by gas chromatography. The classic "triple" response in etiolated pea seedling was employed as a bioassay to demonstrate the effect of substrate-dependent microbial production of ethylene on plant growth. Results revealed that an amendment with L-MET, KMBA, ACC (up to 0.10 g/kg soil) and CaC_2 (0.20 g/kg soil) significantly stimulated ethylene biosynthesis in soil. Overall, ACC proved to be the most effective substrate for ethylene production (1434 nmol/kg soil), followed by KMBA, L-MET, and CaC_2 in descending order. Results further revealed that ethylene accumulation in soil from these substrates caused a classic "triple" response in etiolated pea seedlings with different degrees of efficacy. A more obvious classic "triple" response was observed at 0.15, 0.10, and 0.20 g/kg soil of L-MET, KMBA/ACC, and CaC_2, respectively. Similarly, direct exposure of etiolated pea seedlings to commercial ethylene gas also modified the growth pattern in the same way. A significant direct correlation (r = 0.86 to 0.97) between substrate-derived C_2H_4 and the classic triple response in etiolated pea seedlings was observed. This study demonstrated that the presence of substrate(s) in soil may lead to increased ethylene concentration in the air of the soil, which may affect plant growth in a desired direction.

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Key words: substrates, ethylene biosynthesis, soil, physiological response.

INTRODUCTION

The plant rhizosphere is a remarkable ecological environment as myriad microorganisms, capable of synthesizing various concentrations of phytohormones, are associated with the root systems of all higher plants. Many of these are highly dependent for their survival on preformed substrates (amino acids, organic acids, carbohydrates, nucleic acid derivatives, vitamins, and other growth substances) excreted by plant roots as exudates [1–3]. In turn, the soil microflora inhabiting the rhizosphere can cause dramatic changes in plant growth and development by contributing to the host plants' endogenous pools of phytohormones and/or by providing other services.

Ethylene is one of the established classes of phytohormones and is also a natural product of plant metabolism. The effects of ethylene have been observed in practically all aspects of plant growth and development, ranging from germination of seeds to senescence of various organs and in many responses to environmental stress [4–6]. Various compounds have been identified that promote ethylene concentration in the air of soil [7–9]. Since microorganisms can derive ethylene from a variety of compounds, it is highly likely that the presence of a substrate(s) in a rhizosphere can influence the production of physiologically active concentrations of ethylene.

Although studies have elucidated the role of exogenously applied ethylene gas and nonenzymatically ethylene releasing compounds (such as ethephon/ethrel) on various physiological processes of a plant, very little is still known about the influence of ethylene present in the air of soil on plant growth. It is likely that ethylene released by soil indigenous microorganisms from a suitable substrate(s) can act as a potential exogenous source in the rhizosphere for plant uptake and may create a physiological response.

Etiolated pea seedlings show a characteristic "triple" response exclusively to ethylene. This so-called classic "triple" response involves reduction in elongation, swelling of hypocotyl, and a change in the direction of growth [10]. Silver [Ag(I)] acts as specific inhibitor of ethylene action [11]. In the present study, this specific response was used as an experimental tool in probing the influence of exogenous ethylene released from specific substrates by rhizosphere microflora on plant growth and development. As far as we know, this is the first comprehensive study reporting the effects of

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ethylene released in soil amended with four different substrates on etiolated pea seedlings.

MATERIALS AND METHODS

A sandy clay loam, and mixed hyperthermic ustic haplocalcids soil was used for assessing the substrate-dependent ethylene accumulation in soil. Surface soil samples (~0–15-cm layer) were collected, air-dried, and crushed to <2 mm (2 mm/10 mesh). The soil analysis revealed a pH of 7.8; electrical conductivity of saturated soil extract (ECe) was, 2.1 dS/m; cation exchange capacity was, 4.5 $cmol_c$/kg soil; and organic matter, 0.7%.

Ethylene biosynthesis in soil. Four substrates, namely L-methionine (L-MET), 2-keto-4-methylthiobutyric acid (KMBA) (Sigma, St. Louis, MO, USA), 1-aminocyclopropane-1-carboxylic acid (ACC) (ICN Biomedicals, Inc., Germany), and calcium carbide (CaC_2) (purchased locally) were assayed for the release of ethylene in the soil air. For ethylene measurement, 50 g of soil were added in a 460-ml glass bottle and treated with various concentrations (ranging from 0 to 0.2 g/kg soil) of L-MET, KMBA, and ACC applied as a solution (100 ml). Calcium carbide was applied as an encapsulated form. Each glass bottle was capped with mininert valves (Pierce Rockford, IL, USA), incubated in the dark at 30°C for a period of 168 h, and shook at 100 rpm. A control, for comparison, was kept containing just 100 ml of water to analyze the base level ethylene formation in soil from indigenous organic compounds. Similarly, experiments were repeated in sterilized soil (autoclaved at 121°C for 1 h) to know the biotic nature of ethylene production from various substrates. Solutions of L-MET, KMBA, and ACC were sterilized by passing through 0.22 μm filter (GS type). Calcium carbide was applied to sterilized soil in the encapsulated form. Experiments were run in three repetitions using a completely randomized design. After incubation, the ethylene concentrations were determined by gas chromatography, by withdrawing 1-ml gas samples from the headspace air above the soil suspension with a gas-tight glass hypodermic syringe, and the results were expressed as nmol C_2H_4/kg soil. The ethylene dissolved in the aqueous phase was not accounted for. The gas chromatograph (Shimadzu 4600) was equipped with a flame-ionization detector (FID) and a 2 m Porapak N (0.18–0.14 mm) column (Alltech Associates Inc., Deerfield, IL, USA). The column was operated isothermally at 70°C. Following conditions were used for operating the GC: sample size (1.0 ml), carrier gas (N_2) for 13 ml/min, H_2 flow for 30 ml/min, air flow for 300 ml/min, a detector temperature of 200°C, and an injector temperature of 120°C. Peak area and retention times for ethylene were compared to reference standards made by diluting 99.5% ethylene obtained from Matheson (Secaucus, NJ).

Time-dependent ethylene production in soil amended with optimal levels of substrates was also monitored. For this purpose, three substrates, namely, L-MET, KMBA, and ACC, were used at 0.10 g/kg of soil, while calcium carbide-dependent ethylene synthesis was determined at 0.20 g/kg soil. Each flask containing these substrates was incubated for 24, 48, 72, 96, 120, 144, and 168 h at 30°C under shaking conditions (100 rpm).

Effect of the air of soil ethylene derived from various substrates on etiolated pea seedlings (classic "triple" response bioassay). A series of laboratory experiments were conducted under controlled conditions (axenic) to demonstrate the effect of L-MET-, KMBA-, ACC-, and calcium carbide-derived ethylene produced by soil microorganisms on etiolated pea seedlings. Pea seeds (*Pisum sativum* cv. Mateor) were sterilized on the surface by dipping in 95% ethanol solution for 5 min and 0.02% mercuric chloride solution for 3 min, which were subsequently washed thoroughly with sterilized distilled water. These seeds were placed on sterilized filter paper in a Petri plate and incubated at 30°C for germination. Uniformly, pregerminated pea seedlings were transferred to 100-ml beakers filled with 100 g of soil amended with various concentrations (0, 0.05, 0.10, 0.15, and 0.20 g/kg soil) of L-MET, KMBA, ACC, or calcium carbide separately. Deionized distilled water was used as the control. These beakers were placed inside airtight jars (900 ml) wrapped in green foil to provide the "safe" green light. Incubation was in complete darkness throughout the experiment at 30°C for 7 days (168 h). There were three seedlings per beaker/treatment. Each treatment was replicated three times.

To confirm the physiological action of substrate-dependent ethylene production in soil, an experiment was conducted with 0.10 g/kg of the soil of each substrate with and without silver nitrate. Silver nitrate (0.48 g/l) was applied to 3-day-old seedlings in the solution in the form of a foliar spray; the seedlings that were not treated with silver nitrate received a foliar application of sodium nitrate of the same strength to compensate the nitrate effect.

For further confirmation and comparison, etiolated pea seedlings were also exposed to four levels of commercial ethylene gas [0, 300, 600, 900, 1200 nmol per jar (900 ml)]. Seedlings were sown in autoclaved soil and watered with sterile water, and 99.5% commercial ethylene gas (Curtin Matheson Scientific, Inc., Secaucus, NJ) was injected into jars through a rubber septa to 2-day-old seedlings.

Statistical analyses. Standard error was calculated for ethylene production in soil from various substrates. Data regarding the effect of ethylene produced in soil on seedling length and stem diameter of etiolated pea seedlings were analyzed by applying a completely randomized design [12], and means were compared by Duncan's Multiple Range test [13]. Correlation between ethylene production in soil and the effect on growth of etiolated pea seedlings was also calculated [12].

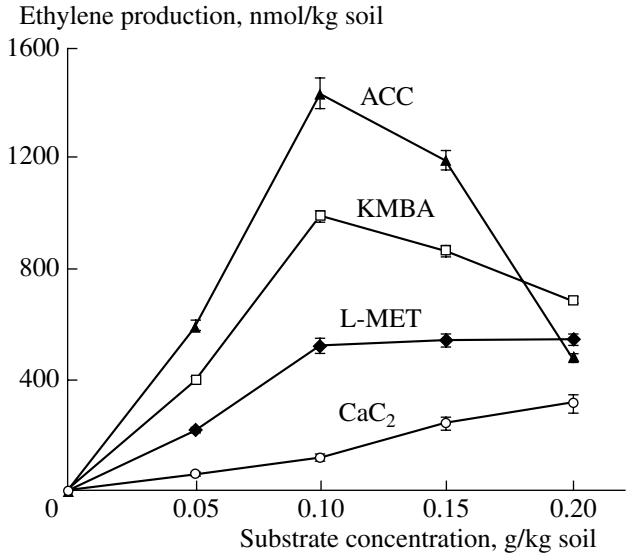

Fig. 1. Substrate (L-MET/KMBA/ACC/CaC_2)-dependent biosynthesis of ethylene in soil (average of three repeats). Fifty g of soil was amended with various levels of L-methionine (L-MET), 2-keto-4-methylthiobutyric acid (KMBA), 1-aminocyclopropane-1-carboxylic acid (ACC), and calcium carbide (CaC_2) and incubated at 30°C for 168 h under shaking of 100 rpm.

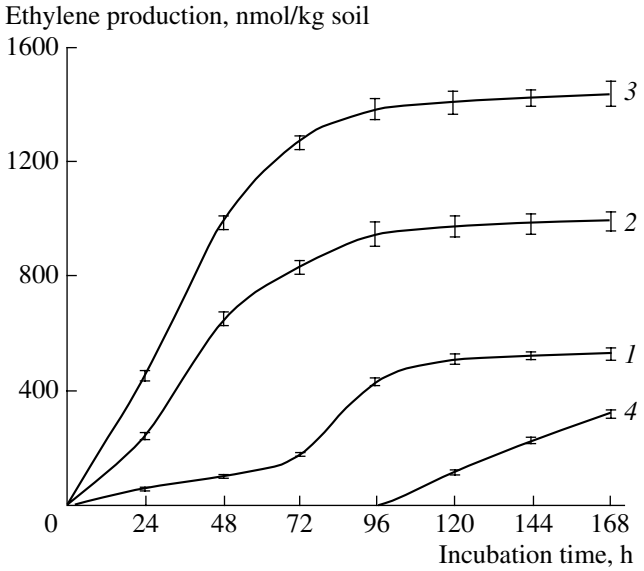

Fig. 2. Effect of the incubation time on substrate (L-MET/KMBA/ACC/CaC_2)-dependent biosynthesis of ethylene in soil. Fifty g of soil was amended with 0.10 g/kg of L-methionine (L-MET), 2-keto-4-methylthiobutyric acid (KMBA), and 1-aminocyclopropane-1-carboxylic acid (ACC), and 0.20 g/kg soil of calcium carbide (CaC_2).

RESULTS

Biosynthesis of ethylene in soil. Results regarding the biosynthesis of ethylene in soil derived from L-MET, KMBA, ACC, and calcium carbide are presented in Fig. 1. Ethylene accumulation in soil was substrate- and concentration dependent, and no ethylene was detected in unamended soil (control). In the case of L-MET, KMBA, and ACC, concentration up to 0.10 g/kg of soil had significant stimulatory effects on ethylene production (ranging from 525 and 1434 nmol/kg soil), and a further increase in substrate concentration resulted in low levels of ethylene in soil. However, in soil-amended with calcium carbide, a gradual increase in ethylene (ranging from 58 to 315 nmol/kg soil) accumulation was observed with increasing levels of calcium carbide (from 0.05 to 0.20 g/kg soil). Comparison of substrates revealed that amendment of the soil with ACC resulted in the maximum production of ethylene in soil which was followed in descending order by KMBA, L-MET, and CaC_2.

Ethylene was not detected in sterilized (autoclaved) soil amended with a filter (0.22 μm) sterilized solution of ACC, KMBA, and L-MET. In the case of calcium carbide, copious amount of acetylene (C_2H_2) was detected but ethylene was not found.

Time-dependent ethylene synthesis, in soil amended with various substrates, is evident from Fig. 2. Ethylene synthesis was recorded in soil amended with L-MET, KMBA, and ACC just after 24 h of incubation, which continued for the 96 h of incubation and then leveled off. In case of L-MET, ethylene production was low up to 72 h, and then a rapid increase was observed during the next 24 h. However, ethylene was not detected in calcium carbide-amended soil during the 96 h incubation, and afterwards there was a gradual increase in the soil-air ethylene till the termination of incubation.

Effect of soil-air ethylene derived from various substrates on etiolated pea seedlings. After confirming substrate-dependent ethylene biosynthesis in soil, experiments were conducted in a growth chamber under axenic conditions, to assess the physiological response of etiolated pea seedlings exposed to ethylene produced by soil indigenous microorganisms from the added substrates (L-MET, KMBA, ACC, and CaC_2). Results revealed that the application of a substrate to the soil resulted in a significant reduction in seedling length, with a swelling of the stem and horizontal growth of etiolated pea seedlings (Table 1). In the case of L-MET, the effect on the length/diameter of pea seedlings was more pronounced, beyond 0.15 g L-MET/kg of soil, than that observed at low concentrations; however, the effect of 0.15 and 0.20 g L-MET/kg of soil was nonsignificant with each other. L-MET application caused a reduction in etiolated pea seedling with a length up to 75%, while the stem diameter increased up to 99% compared with the control (no L-MET). An amendment of soil with KMBA showed a more obvious response at 0.10 g/kg of soil in etiolated pea seedlings and significantly reduced the seedling length (74%) and increased the stem diameter (79%), compared with unamended soil. Like KMBA, the most prominent classic "triple" response was also observed at 0.10 g ACC/kg of soil, compared to other concentrations of ACC. A decrease in seedling length and

Table 1. Effect of different substrates (L-MET/KMBA/ACC/CaC_2) on the growth of etiolated pea seedlings (average three repeats, 3 × 3 seedlings)

Substrate*, g/kg soil	L-MET		KMBA		ACC		CaC_2	
	seedling length, cm	seedling diameter, mm	seedling length, cm	seedling diameter, mm	seedling length, cm	seedling diameter, mm	seedling length, cm	seedling diameter, mm
Control (no substrate)	11.50 a**	0.95 d	11.20 a	1.01 c	11.00 a	0.93 d	11.30 a	0.93 d
0.05	4.70 b	1.41 c	3.90 b	1.49 b	3.30 c	1.64 b	7.30 b	1.27 c
0.10	3.46 c	1.70 b	2.90 d	1.81 a	2.89 d	1.88 a	5.96 c	1.43 bc
0.15	2.92 d	1.89 a	2.98 d	1.75 a	3.51 c	1.51 b	3.58 d	1.57 b
0.20	2.96 d	1.90 a	3.28 c	1.73 a	7.60 b	1.19 c	3.30 d	1.79 a

* 100 g of soil in a jar was amended with different concentrations of substrates and incubated in the dark at 30°C for 168 h.
** Values followed by different letters in a column were significantly different ($P \le 0.05$), using Duncan's multiple range test.

increase in stem diameter up to 74 and 102%, respectively, were observed in ACC amended soil. There was a gradual increase in length and decrease in stem diameter of etiolated pea seedlings beyond 0.10 g ACC/kg of soil. Calcium carbide also significantly affected the growth pattern of etiolated pea seedlings, as well as a more pronounced classic "triple" response was observed at higher concentration of calcium carbide (0.20 g/kg soil). Calcium carbide application significantly reduced seedling length (up to 71%) and increased the stem diameter (up to 92%), compared with the untreated control. Overall ACC was the most effective substrate, at all concentrations, in creating the classic "triple" response in etiolated pea seedlings, compared to other substrates (L-MET, KMBA, or CaC_2).

Direct exposure of etiolated pea seedlings, exposed to commercial ethylene gas caused a significant reduction in seedling length (up to 80%) and an increase in stem diameter (up to 77.4%), with more horizontal growth (Table 2). A more pronounced classic "triple" response was observed at 1200 nmol of ethylene gas. The application of ethylene gas beyond 1200 nmol had a drastic suppressing effect on etiolated pea seedlings, and seedlings could not survive (data not shown).

Table 2. Direct influence of commercial ethylene gas on the growth of etiolated pea seedlings (average of three repeats, 3 × 3 seedlings)

$[C_2H_4]$, nmol/l	Seedling length, cm	Seedling diameter, mm
Control	10.96 a*	0.93 d
300	5.93 b	1.24 c
600	5.46 b	1.43 b
900	5.20 b	1.49 ab
1200	2.20 c	1.65 a

* Values followed by different letters in a column were significantly different ($P \le 0.05$), using Duncan's multiple range test.

The application of silver nitrate protected the etiolated pea seedlings against soil-air ethylene derived from L-MET/KMBA/ACC/CaC_2, as the seedling length and stem diameter were comparable to unamended controls (data not shown). Figure 3 showed that the application of silver nitrate mimicked the effect of calcium carbide-dependent ethylene in soil. A similar effect of Ag(I) on the classic "triple" response was also observed in the case of commercial ethylene gas.

Regression analyses revealed a direct significant correlation ($r = 0.91$ [L-MET], 0.88 [KMBA], 0.86 [ACC], and 0.92 [CaC_2]) between substrate-derived $[C_2H_4]$ and the decreased length of etiolated pea seedlings (Table 3). Similarly, correlation ($r = 0.97$ [L-MET], 0.97 [KMBA], 0.87 [ACC], and 0.95 [CaC_2]) was also significant between the substrate-derived $[C_2H_4]$ and the increase in stem diameter of etiolated pea seedlings. There was also a significant linear relationship between the directly injected commercial $[C_2H_4]$ gas, the decrease in seedling length ($r = 0.92$) and the increase in the stem diameter ($r = 0.95$) of etiolated pea seedlings.

DISCUSSION

This study compares the potential of four different substrates for the possible production of ethylene in soil and its influence on the growth of etiolated pea seedlings. It was found that the addition of substrates (L-MET, KMBA, ACC, or calcium carbide) had concentration-dependent stimulatory effect on ethylene synthesis in soil, which implies that these compounds serve as precursors of ethylene in soil. Ethylene was not detected in the sterilized soil amended with the filter-sterilized solution of L-MET, KMBA, or ACC, which further implies that the synthesis of ethylene is primarily a biochemical reaction. Several workers reported that many fungi and bacteria are capable of producing ethylene from L-MET via the KMBA pathway [14–16]. Similarly, KMBA- or ACC dependent biosynthesis of ethylene in soil has also been reported by other workers [9, 17–19].

Fig. 3. Response of etiolated pea seedlings to calcium carbide-derived ethylene in soil in the presence and absence of silver nitrate (an inhibitor of ethylene action).

Ethylene synthesis was concentration-dependent, and concentrations beyond 0.10 g/kg of the soil of KMBA/ACC had inhibitory effects on ethylene biosynthesis. In the case of L-MET, production of ethylene leveled off beyond 0.10 g/kg soil. Higher concentration of L-MET/KMBA/ACC might be toxic to the soil microorganisms involved in ethylene synthesis or inhibit the activity of enzyme(s) involved in ethylene generation through a substrate inhibition mechanism. This premise supports the findings of Frankenberger and Phelan [18] who reported that the ACC-dependent production of ethylene was inhibited beyond a concentration of 10 mM. A time-based study revealed that KMBA and ACC almost followed the same pattern for ethylene synthesis, while in the case of L-MET, ethylene synthesis was very low during the initial 72 h, and then it picked up. Since KMBA and ACC are immediate precursors of ethylene, both substrates followed the same pattern, while L-MET is first converted to either ACC or KMBA and then to ethylene, so this could be the reason that during the initial 72 h of incubation there was a poor synthesis of ethylene.

Contrary to L-MET/KMBA/ACC, the concentration (from 0.05 to 0.20 g/kg soil) of calcium carbide increases, caused a gradual increase in ethylene accumulation in soil. Ethylene was not released in sterilized soil amended with calcium carbide; large amount of acetylene was generated in calcium carbide-amended sterilized soil. Likewise, ethylene could not be detected during the first 96 h of incubation, although it increased gradually afterwards. It is likely that calcium carbide (after reacting with water) is first decomposed into acetylene via nonenzymatic (abiotic) transformation, and then acetylene is biochemically (enzymatically) converted into ethylene [20], resulting in a gradual increase in the production of ethylene in soil.

Results of the trials conducted on etiolated pea seedlings demonstrated that the application of various substrates to soil significantly affected seedling growth and created the classic "triple" response in etiolated pea seedlings; the degree of the response changed with kind and concentration of the substrate. Overall, ACC/KMBA created a more pronounced classic "triple" response at low concentration compared to L-MET or calcium carbide, which might be due to relatively more ethylene production at this concentration. Moreover, a significant direct correlation was found between $[C_2H_4]$ produced from all substrates and the decrease in seedling length and swelling in the stem diameter of etiolated pea seedlings. The response was exactly the same to that observed in the case of the direct application of commercial ethylene gas. This implies that indigenous soil microflora produced ethylene from added substrates (Fig. 1), which affected the growth

Table 3. Correlation between $[C_2H_4]$-derived from different substrates and the growth of etiolated pea seedlings

C_2H_4 derived from substrate	*r* value	
	seedling length	seedling diameter
L-MET	0.19*	0.97*
KMBA	0.88*	0.97*
ACC	0.87*	0.87*
CaC_2	0.93*	0.95*

* Significant at $P \le 0.05\%$.

pattern of etiolated pea seedlings. This premise is supported by the observation that the application of Ag(I), a known inhibitor of ethylene action, eliminated the affect of substrates on etiolated pea seedlings. Arshad and Frankenberger [21] also reported that ethylene derived from MET by *Acremonium falciforme* influenced the growth of etiolated pea seedlings. Similarly, Russian workers have also claimed that calcium carbide improved the growth and yield of crops, by enriching the rhizosphere with ethylene formed from microbial reduction of acetylene released from calcium carbide upon its reaction with water [7, 22]. In brief, the results of this study provided direct evidence in support of the hypothesis that microbial produced ethylene from a suitable substrate in the rhizosphere could influence the growth and development of a plant.

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