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Influence of Ethylene Produced by Soil Microorganisms on Etiolated Pea Seedlings

MUHAMMAD ARSHAD AND W. T. FRANKENBERGER, JR.*

Department of Soil and Environmental Sciences, University of California, Riverside, California 92521

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There is indirect evidence that soil microorganisms producing ethylene (C_2H_4) can influence plant growth and development, but unequivocal proof is lacking in the literature. A laboratory study was conducted to demonstrate the validity of this speculation. Four experiments were carried out to observe the characteristic "triple" response of etiolated pea seedlings to C_2H_4 microbially derived from L-methionine as a substrate in the presence or absence of Ag(I), a potent inhibitor of C_2H_4 action. In two experiments, the combination of L-methionine and *Acremonium falciforme* (as an inoculum) was used, while in another study the indigenous soil microflora was responsible for C_2H_4 production. A standardized experiment was conducted with C_2H_4 gas to compare the contribution of the microflora to plant growth. In all cases, etiolated pea seedlings exhibited the classical triple response, which includes reduction in elongation, swelling of the hypocotyl, and a change in the direction of growth (horizontal). The presence of Ag(I) afforded protection to the pea seedlings against the microbially derived C_2H_4. This study demonstrates that microbially produced C_2H_4 in soil can influence plant growth.

Ethylene (C_2H_4) is considered a plant hormone which can affect the plant at almost every phase of its stage of development. Despite its chemical simplicity, C_2H_4 is a potent regulator, affecting the growth, differentiation and senescence of plants at concentrations as low as 0.01 μl liter^{-1} (14). The effects of C_2H_4 have been observed in practically all aspects of plant growth and development, including seed germination (9), seedling growth (5), root growth (6), growth of leaves (12), stress phenomena (16), and ripening, aging, and senescence (4). The dramatic effect of C_2H_4, with its physiological action, on etiolated seedlings was the basis for its discovery by Neljubow in 1901 (11). Etiolated pea seedlings show a characteristic "triple" response to C_2H_4. This so-called triple response involves reduction in elongation, swelling of the hypocotyl, and a change in the direction of growth (8).

Ag(I) is a potent and specific inhibitor of C_2H_4 action (2). Its mechanism of action is believed to be interference with C_2H_4 binding (10). This inhibitor can be used as an experimental tool in probing the influence of exogenous C_2H_4 on plant growth and development. Beyer (2) studied the classical triple response of etiolated pea seedlings by exposing them to 0.25 μl of C_2H_4 liter^{-1} and treating them with various concentrations of Ag(I) as $AgNO_3$. Ag(I) applied foliarly effectively blocked the ability of exogenous C_2H_4 to elicit the classical triple response in etiolated peas.

C_2H_4 is reported to be synthesized by many species of bacteria and fungi (13). Agronomically, microbial production of C_2H_4 could have an impact on crop production under certain management conditions. Ethylene concentrations as low as 10 μg liter^{-1} can evoke plant responses, and concentrations of 25 μg liter^{-1} result in decreased fruit and flower development (13).

The primary objective of this study was to show that microbially produced C_2H_4 can affect plant growth by demonstrating the classical triple response of etiolated pea seedlings in the presence and absence of Ag(I).

* Corresponding author.

MATERIALS AND METHODS

A set of four laboratory experiments was conducted to demonstrate the influence of microbially produced C_2H_4 derived from L-methionine on intact etiolated pea seedlings in the presence or absence of Ag(I), a specific inhibitor of C_2H_4 action. Alaska peas (*Pisum sativum* cv. Alaska) were sown in 100-ml beakers containing either sand or soil and placed in airtight mason jars wrapped in green foil to provide "safe" green light (Fig. 1). Incubation was conducted in complete darkness throughout the experiments at 24 ± 3°C for up to 168 h. The treatments were applied by opening the jars after 72 h and continuing incubation for 96 h. Etiolated pea seedlings (72 h old) were foliarly treated with $AgNO_3$ solutions containing 0.1% Tween 20 as a surfactant. All seedlings not receiving $AgNO_3$ were treated with $NaNO_3$ (240 mg liter^{-1}) to account for the anion (NO_3^-) effect. Preliminary confirmation of L-methionine-derived C_2H_4 produced by *Acremonium falciforme* was made before the initiation of this study. *A. falciforme* is a soil fungus which is isolated on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) and which produces simple, awl-shaped, erect (orthotrophic) phialides (7). Under optimum conditions, C_2H_4 production by *A. falciforme* in cultures ranged from 790 to 1,361 nmol of C_2H_4 36 mg of mycelium^{-1} 72 h^{-1} (M. Arshad and W. T. Frankenberger, Jr., submitted for publication). The production of C_2H_4 from soil as a microbial metabolite in the presence of L-methionine was also confirmed at the end of incubation by gas chromatography analysis. Data regarding seedling length and diameter were recorded at the end of incubation.

The first experiment characterized the influence of exogenous L-methionine-derived C_2H_4 from *A. falciforme* on etiolated pea seedlings. Sand (160 g) in beakers was sterilized by being autoclaved at 121°C for 2 h. Seeds were treated with 5% sodium hypochlorite for 10 min, washed, planted into the sand under aseptic conditions, and watered with sterile deionized water. Etiolated seedlings (72 h old) were then foliarly treated with five levels of filter-sterilized (0.22 μm) $AgNO_3$ solutions (0, 60, 120, 180, and 240 mg liter^{-1}).

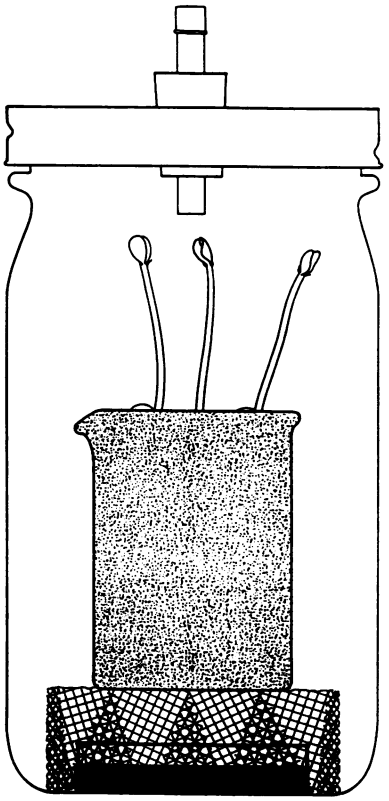

FIG. 1. Apparatus used to monitor the response of etiolated pea seedlings to microbially produced C_2H_4.

To maintain the *A. falciforme* culture and promote C_2H_4 production, we placed plates (15 by 60 mm) containing 15 ml of basal salt medium (containing, in milligrams per liter, the following: KH_2PO_4, 1,360; Na_2HPO_4, 2,130; $MgSO_4 \cdot 7H_2O$, 200; $CaCl_2 \cdot 2H_2O$, 700; $FeSO_4 \cdot 7H_2O$, 200; $CuSO_4 \cdot 5H_2O$, 40; $MnSO_4 \cdot H_2O$, 20; $ZnSO_4 \cdot 7H_2O$, 20; H_3BO_3, 3; $CoCl_2 \cdot 6H_2O$, 7; and $Na_2MoO_4 \cdot 2H_2O$, 4; the medium also contained 1.0 ml of concentrated H_2SO_4), 1.5% agar, 1.0% glucose, and 10 mM L-methionine (Sigma Chemical Co., St. Louis, Mo.) at the bottom of the jars (Fig. 1). The basal salt medium was sterilized by being autoclaved at 121°C for 15 min, and glucose and L-methionine solutions were sterilized separately by being filtered through 0.22-μm-pore membrane filters (type GS; Millipore Corp., Bedford, Mass.). These plates were covered with sieve lids (spacing, 1 mm) on which beakers with seedlings were placed. There were 12 seedlings per treatment.

To test for the production of C_2H_4 by *A. falciforme* directly from soil and its influence on etiolated pea seedlings, we sowed sterilized seeds (as described above) in sterile Hanford soil (coarse-loamy, mixed, nonacid, thermic Typic Xerorthent) which had been autoclaved at 121°C for 1 h three times on alternate days. There were seven treatments: (i) control, (ii) foliarly applied $AgNO_3$ (240 mg liter^{-1}), (iii) inoculation with *A. falciforme*, (iv) L-methionine (10 mM), (v) L-methionine (10 mM) and inoculation with *A. falciforme*, (vi) L-methionine (10 mM) and foliarly applied $AgNO_3$ (240 mg liter^{-1}), and (vii) L-methionine (10 mM), inoculation with *A. falciforme*, and foliarly applied $AgNO_3$ (240 mg liter^{-1}). For the L-methionine treatments, 5 ml of a 10 mM filter-sterilized (0.22 μm) solution of L-methionine was applied to soil with established 72-h-old etiolated seedlings. The soil was inoculated by adding 1 ml of a 10-day-old *A. falciforme* liquid culture grown in Sabouraud broth medium. Treatments not receiving L-methionine solution and/or inoculum were given equivalent amounts of sterilized water and/or medium. There were 10 seedlings per treatment.

TABLE 1. Protection by Ag(I) of etiolated pea seedlings against L-methionine-derived C_2H_4 produced by *A. falciforme*

Treatment	Seedling length (cm)	Seedling diam (mm)
Control (untreated)	8.29 cd[a]	2.19 a
$NaNO_3$ (240 mg liter^{-1})	9.66 d	2.18 a
L-Methionine (10 mM)	3.95 a	3.27 c
L-Methionine (10 mM) + $NaNO_3$ (240 mg liter^{-1})	4.39 a	3.16 c
L-Methionine (10 mM) + $AgNO_3$ (60 mg liter^{-1})	4.61 a	3.02 c
L-Methionine (10 mM) + $AgNO_3$ (120 mg liter^{-1})	6.26 b	2.58 b
L-Methionine (10 mM) + $AgNO_3$ (180 mg liter^{-1})	7.02 bc	2.38 ab
L-Methionine (10 mM) + $AgNO_3$ (240 mg liter^{-1})	8.44 cd	2.24 a

[a] Values followed by the same letter were not significantly different at the 0.05 level according to the Duncan multiple-range test.

To monitor production of C_2H_4 by the indigenous soil microorganisms, and its impact on etiolated pea seedlings, we used six treatments, including two levels of L-methionine (5 and 10 mM) with or without $AgNO_3$ (240 mg liter^{-1}), a control, and foliarly applied $AgNO_3$ alone. L-Methionine solution (5 ml) was applied to nonsterile Hanford soil containing 72-h-old etiolated seedlings. Deionized water (5 ml) was applied to the treatments not receiving L-methionine. There were six seedlings per treatment.

To make a comparison among the responses of etiolated pea seedlings to C_2H_4 applied as a gas and C_2H_4 microbially derived from L-methionine, we used six treatments, including five levels of C_2H_4 gas (0, 1, 5, 10, and 50 nmol liter^{-1}) and a combination of 50 nmol of C_2H_4 liter^{-1} and foliarly applied $AgNO_3$ (240 mg liter^{-1}). Seeds were sown in autoclaved sand and watered with sterile deionized water, and 99.5% C_2H_4 (Curtin Matheson Scientific, Inc., Secaucus, N.J.) was injected into the jars through rubber septa. There were 10 seedlings per treatment.

RESULTS

Influence of exogenous C_2H_4 derived from *A. falciforme* on etiolated pea seedlings. Table 1 shows that etiolated pea seedlings were influenced by L-methionine-derived C_2H_4 production by *A. falciforme* in the presence of various levels of $AgNO_3$. It is obvious that the maximum and significant reduction in seedling length occurred when the inoculum and substrate (L-methionine) were applied in the absence of $AgNO_3$ (Fig. 2). Comparison of this treatment with the control indicated the physiological action of C_2H_4 on etiolated pea seedlings. An increase in $AgNO_3$ concentration resulted in increased seedling length, thereby decreasing the L-methionine-derived C_2H_4 effect. At 240 mg of $AgNO_3$ liter^{-1}, the seedling length was almost equal to that of the control, indicating complete protection against C_2H_4. The seedling lengths in the control and in the $NaNO_3$ (240 mg liter^{-1}) and $AgNO_3$ (240 mg liter^{-1}) treatments were not significantly different. There was also no difference between treatment with L-methionine alone or in combination with $NaNO_3$, indicating that there was no anion (NO_3^-) effect.

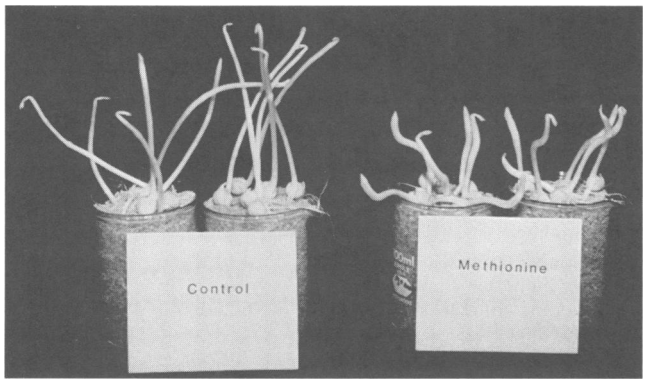

FIG. 2. Response of etiolated pea seedlings to exogenous L-methionine-derived C_2H_4 produced by *A. falciforme* in the absence of $AgNO_3$.

TABLE 2. Influence of L-methionine-derived C_2H_4 produced by *A. falciforme* on etiolated pea seedlings grown in autoclaved soil

Treatment[a]	Seedling length (cm)	Seedling diam (mm)
Control	6.23 b[b]	1.97 ab
$AgNO_3$ (240 mg liter^{-1})	7.57 b	1.87 a
Inoculation with *A. falciforme*	6.67 b	2.07 b
L-Methionine (10 mM)	7.22 b	2.10 b
L-Methionine (10 mM) + inoculation with *A. falciforme*	2.58 a	2.44 c
L-Methionine (10 mM) + $AgNO_3$ (240 mg liter^{-1})	6.11 b	1.93 ab
L-Methionine (10 mM) + inoculation with *A. falciforme* + $AgNO_3$ (240 mg liter^{-1})	5.77 b	2.07 b

[a] Samples that did not receive $AgNO_3$ received $NaNO_3$ (240 mg liter^{-1}).
[b] Values followed by the same letter were not significantly different at the 0.05 level according to the Duncan multiple-range test.

It is evident from Table 1 that the precursor, L-methionine without $AgNO_3$, increased the seedling stem diameter significantly over those in the control and in the $NaNO_3$ (240 mg liter^{-1}) treatment. Stem diameter decreased with increasing $AgNO_3$ concentration, and at 240 mg liter^{-1}, it was comparable to that in the control.

Figure 3 depicts the growth pattern of etiolated pea seedlings exposed to the fungal metabolite generated by *A. falciforme*. Seedlings displayed the classical triple response, with a reduction in elongation, swelling of the hypocotyl, and horizontal growth upon L-methionine enrichment. However, the seedlings did not respond to C_2H_4 upon application of high [$AgNO_3$] levels.

Influence of L-methionine-derived C_2H_4 produced by *A. falciforme* on etiolated pea seedlings in autoclaved soil. The application of both L-methionine and *A. falciforme* to soil reduced seedling length and increased the stem diameter significantly, as compared with all other treatments (Table 2). L-Methionine provided to the roots in the autoclaved soil had no significant effect on the etiolated seedlings, as compared with the control. The same was true with inoculation of *A. falciforme* alone. These results led to the view that it was a metabolite of L-methionine released by the fungus to which pea seedlings showed the response. Since the response was similar to the classical triple response, we believe that this metabolite was most likely C_2H_4 derived from L-methionine. This hypothesis was further supported by the results of a combined treatment with L-methionine, *A. falciforme*, and $AgNO_3$ (240 mg liter^{-1}): seedling length and stem diameter were comparable to the control, indicating that protection was afforded by Ag(I) against C_2H_4. Furthermore, C_2H_4 was detected in the headspace by gas chromatography analysis.

Influence of C_2H_4 produced by indigenous soil microorganisms on etiolated pea seedlings. After we confirmed that L-methionine itself did not have any influence on etiolated pea seedlings, we applied the amino acid to nonsterile soil to observe its effects as a precursor of C_2H_4 produced by the indigenous soil microflora. The control had a slight effect on the etiolated pea seedlings when compared with the $AgNO_3$ treatment (Table 3). This result could possibly have been due to the residual level of C_2H_4 generated upon decomposition of the soil organic matter. The autoclaved soil and $AgNO_3$ treatment were not significantly different (Table 2). L-Methionine application to nonsterile soil resulted in a significant reduction in seedling length, with swelling of the stem and

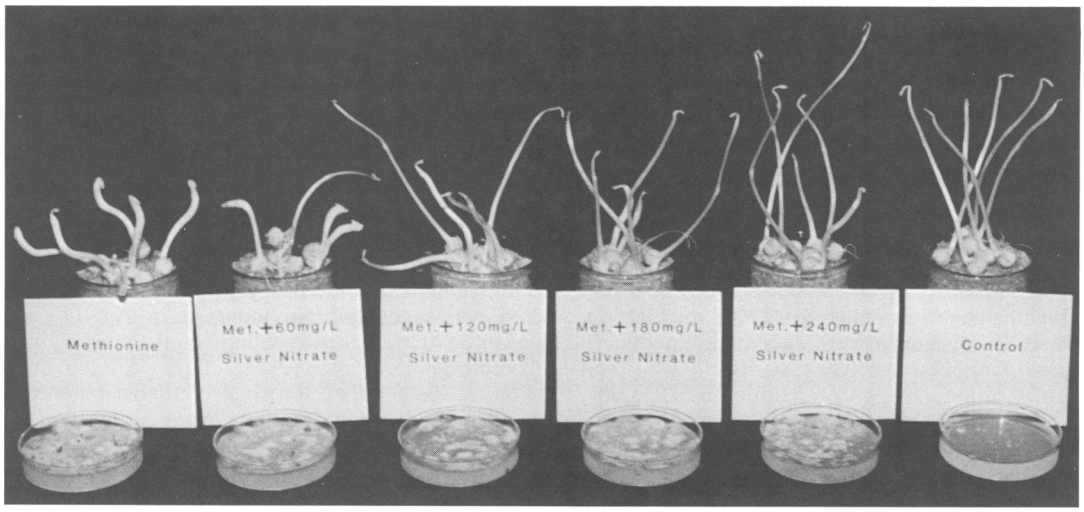

FIG. 3. Response of etiolated pea seedlings to exogenous L-methionine (Met.)-derived C_2H_4 produced by *A. falciforme* in the presence of $AgNO_3$.

TABLE 3. Influence of L-methionine-derived C_2H_4 produced by indigenous soil microflora on etiolated pea seedlings

Treatment[a]	Seedling length (cm)	Seedling diam (mm)
Control	6.56 b[b]	1.87 a
$AgNO_3$ (240 mg liter^{-1})	13.50 d	1.93 ab
L-Methionine (5 mM)	5.14 ab	2.49 c
L-Methionine (5 mM) + $AgNO_3$ (240 mg liter^{-1})	11.10 c	2.06 ab
L-Methionine (10 mM)	3.90 a	2.75 d
L-Methionine (10 mM) + $AgNO_3$ (240 mg liter^{-1})	10.10 c	2.11 b

[a] Samples that did not receive $AgNO_3$ received $NaNO_3$ (240 mg liter^{-1}).
[b] Values followed by the same letter were not significantly different at the 0.05 level according to the Duncan multiple-range test.

horizontal growth as described in the classical triple response to C_2H_4 (Table 3). The effect was more pronounced at a high L-methionine concentration, 10 mM, than at a low one, 5 mM. $AgNO_3$ application protected the seedlings against the L-methionine-derived C_2H_4 released by the indigenous microflora.

Direct influence of C_2H_4 on etiolated pea seedlings. Figure 4 shows the effect of C_2H_4 applied as a gas on 72-h-old etiolated pea seedlings. Comparison with the control revealed that C_2H_4 depressed seedling length and increased the stem diameter significantly, producing more horizontal growth. A more pronounced classical triple response was observed when increasing C_2H_4 concentrations were applied (Table 4). $AgNO_3$ (240 mg liter^{-1}) inhibited this response. Application of C_2H_4 gas beyond 50 nmol retarded seedling growth completely.

TABLE 4. Direct influence of C_2H_4 on etiolated pea seedlings

C_2H_4 concn (nmol liter^{-1})[a]	Seedling length (cm)	Seedling diam (mm)
0 (control)	11.30 d[b]	2.01 a
1	9.24 d	2.15 ab
5	6.72 c	2.38 bc
10	3.66 b	2.61 c
50	2.50 a	3.37 d
50 (plus $AgNO_3$ at 240 mg liter^{-1})	10.07 d	2.08 ab

[a] Samples that did not receive $AgNO_3$ received $NaNO_3$ (240 mg liter^{-1}).
[b] Values followed by the same letter were not significantly different at the 0.05 level according to the Duncan multiple-range test.

DISCUSSION

The results obtained in this study demonstrated the influence of L-methionine-derived C_2H_4 produced by *A. falciforme* on etiolated pea seedlings. The specific anti-C_2H_4 properties of Ag(I) confirmed that C_2H_4 was the fungal metabolite responsible for the observed effects. Moreover, the culture was incubated outside of the root zone, and thus a gaseous product being derived from L-methionine in the medium affected the growth of the pea seedlings. Inhibition of the physiological action of C_2H_4 by Ag(I) is in good accordance with the findings of Beyer (2), Beyer et al. (3), and Smith and Hall (15).

The importance of substrate-inoculum interactions in plant growth was demonstrated. L-Methionine and inoculation with *A. falciforme* independently did not influence the growth of etiolated pea seedlings in autoclaved soil, but when they were combined, the seedlings showed a response similar to the classical triple response (Fig. 5). Ag(I) eliminated this response. These results indicate that L-methionine was not utilized as a precursor in the roots of etiolated pea seedlings. However, when L-methionine was applied to nonsterile soil, the indigenous microflora produced C_2H_4, causing the triple response.

We have also shown that the synthesis of C_2H_4 by *A. falciforme* does not follow the same pathway as the biosynthesis of C_2H_4 from methionine in higher plants, since it cannot derive C_2H_4 from the intermediate, 1-aminocyclopropane-1-carboxylic acid (Arshad and Frankenberger, submitted). However, a comparison of results obtained in all four experiments confirmed that microbially produced C_2H_4 derived from L-methionine yields a response very similar to that obtained by the direct use of C_2H_4 gas. The response obtained with 10 mM L-methionine in the first three experiments was almost the same as that obtained by the application of 10 nmol of C_2H_4 gas. The actual concentration of C_2H_4 found at the end of incubation was 19.0 ± 2.7 nmol. The results obtained with L-methionine-derived C_2H_4 produced by soil microflora are in conformity with the findings of Babiker and Pepper (1), who reported that the addition of L-methionine to soil significantly stimulated C_2H_4 production. However, as far as we are aware, this is the first study

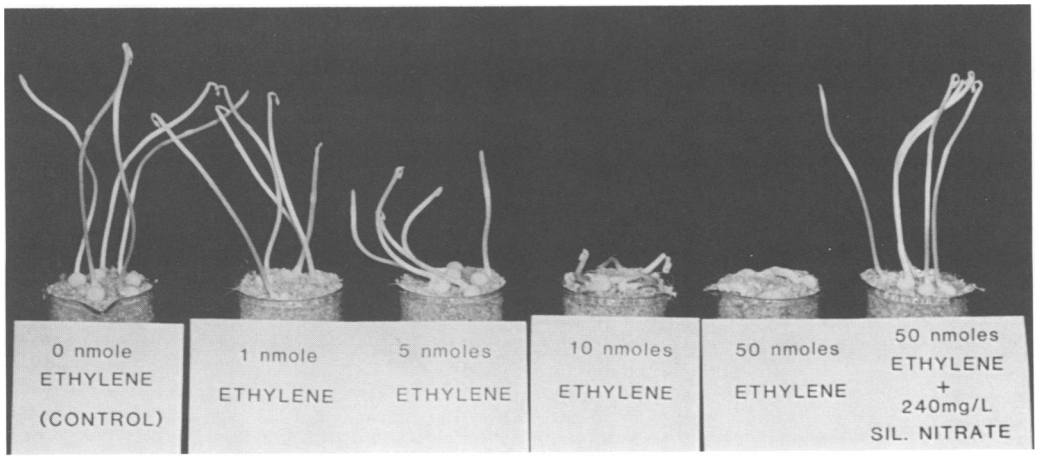

FIG. 4. Response of etiolated pea seedlings to C_2H_4 gas. SIL., Silver.

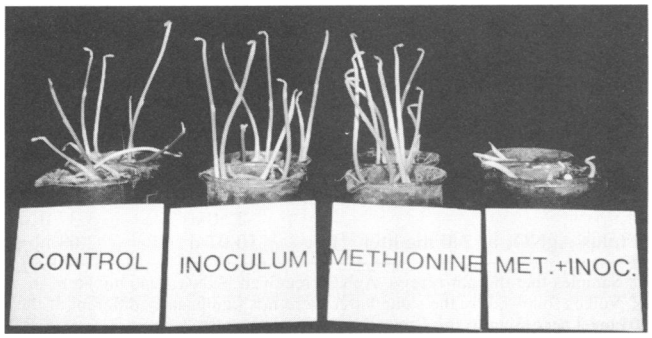

FIG. 5. Response of etiolated pea seedlings to the interaction between L-methionine and inoculated *A. falciforme* in sterile soil.

to show the unequivocal direct influence of microbially produced C_2H_4 on plant growth.

ACKNOWLEDGMENTS

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