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Exogenous Ethylene Inhibits Nodulation of *Pisum sativum* L. cv Sparkle[1]

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ABSTRACT

Exogenous ethylene inhibited nodulation on the primary and lateral roots of pea, *Pisum sativum* L. cv Sparkle. Ethylene was more inhibitory to nodule formation than to root growth; nodule number was reduced by half with only 0.07 μL/L ethylene applied continually to the roots for 3 weeks. The inhibition was overcome by treating roots with 1 μM Ag^+, an inhibitor of ethylene action. Exogenous ethylene also inhibited nodulation on sweet clover (*Melilotus alba*) and on pea mutants that are hypernodulating or have ineffective nodules. Exogenous ethylene did not decrease the number of infections per centimeter of lateral pea root, but nearly all of the infections were blocked when the infection thread was in the basal epidermal cell or in the outer cortical cells.

Ethylene has been implicated as a regulator in many aspects of plant growth and development, response to stress, and senescence (14). Although several factors such as species, tissue type, and stage of development affect the plant response to ethylene, the concentrations of exogenous ethylene that produce visible symptoms are usually in the range of 0.1 to 1.0 μL/L. For example, the half-maximal response of growth inhibition of etiolated pea roots (*Pisum sativum* L.) was about 0.34 μL/L (4).

Exogenous ethylene was assumed to inhibit nodulation based on two reports (5, 6). When excised roots of *Phaseolus vulgaris* were incubated for 25 d in chambers flushed with air containing ethylene, nodule numbers were lowered to about 10% of control by 0.4 μL/L ethylene, a concentration that did not substantially inhibit root growth (6). However, there is no evidence that excised roots behave as roots on intact plants. Goodlass and Smith (5) passed air containing 10 μL/L of ethylene through the rhizosphere of greenhouse-grown, 4-week-old peas "Feltham First" for 7 weeks. The nodule number was less than that in the control (11 versus 38 nodules/plant), and the shoot dry weight was decreased by half. However, a concentration of 10 μL/L is higher than those usually reported for ethylene experiments, and it was added only at a plant age after most nodules have initiated. Moreover, the stage of nodule development blocked by exogenous ethylene is not known (14). Therefore, an examination of the effect of low ethylene concentrations on nodulation of intact plants seemed warranted, testing it on young plants at the stage when nodules are initiated.

We have been using the pea cv Sparkle and a family of its near-isogenic symbiosis mutants to study the formation and function of nodules (3, 8). In mutants at *sym*-5, there are few cortical cell divisions or nodule primordia in advance of the infection thread. However, nodules form (3, 7) if the roots are treated with inhibitors of ethylene formation (AVG)[2] (16) or action (Ag^+) (2). Another pea mutant E107 (*brz*) takes up excess ions and forms few nodules. Its nodule number is increased, though not to normal, by treatment of roots with Ag^+ (8). Thus, ethylene may be involved in decreasing nodulation of these mutants. Similarly, the inhibition of nodulation by light may be mediated by ethylene (13).

In this report, we establish that nodulation of intact plants is extremely sensitive to low concentrations of exogenous ethylene applied during the time of nodule initiation. We also determine the developmental stage at which exogenous ethylene inhibits nodule development on pea.

MATERIALS AND METHODS

Seeds were planted individually in coarse vermiculite in conical pots ("conetainers") that had 16 small holes in the upper 10 cm. Pea (*Pisum sativum*) cv Sparkle, its mutant line E135F (11), cv Rondo, and its mutant line nod-3 (9) were inoculated with *Rhizobium leguminosarum* bv. *viciae* strain 128C53. Sweet clover (*Melilotus alba*, U389) and soybean (*Glycine max* L. cv Ransom) were inoculated with *R. meliloti* strain 1021 and *Bradyrhizobium japonicum* strain 61A92, respectively. Plants were inoculated with rhizobia on planting or 2 DAP, and were subirrigated with nutrient solution containing 5 mM nitrate (10). Plants were grown under high-pressure sodium vapor and metal halide lamps (550 μmol m^{-2} s^{-1} PPFD) in a 16/8 h, 20/15°C light/dark regime.

For treatment with exogenous ethylene, conetainers were placed in 10-L chambers so that the roots were continually exposed to air containing ethylene (12). Air was scrubbed of ethylene by passage through Purafil (Purafil Inc., Atlanta, GA) and pumped to a four-outlet manifold. To three of the outlets, ethylene was added via micro valves (Nupro Co., Willoughby, OH) to give concentrations of approximately 0.1, 0.2, or 0.4 μL/L. The air flowed at 2.6 L/min through the 10-L chambers holding the conetainers. The ethylene

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[2] Abbreviations: AVG, aminoethoxyvinylglycine; DAP, days after planting; STS, silver thiosulfate.

Table I. *Effect of Air Flow in the Rhizosphere on Growth of Pea cv Sparkle*

Seeds were planted in "conetainers" placed in chambers. One chamber had 2.6 L/min air passing through it and the other chamber did not have air passing through it ("static"). Values are the mean (±SD) of seven plants (21 DAP) from one representative experiment.

Growth Parameter	Static	Air Flow	
Primary root length (cm)	31.0 ± 2.7	50.8 ± 5.3[a]	
Longest lateral root length (cm)	30.2 ± 2.3	42.9 ± 2.3[a]	
Nodules/plant	301 ± 51	306 ± 54	NS
Root dry weight (g)	0.31 ± 0.05	0.32 ± 0.07	NS
Shoot length (cm)	24.5 ± 2.0	24.8 ± 1.9	NS
Shoot dry weight (g)	0.71 ± 0.12	0.69 ± 0.15	NS

[a] Significant difference at the 5% level by *t* test comparison.

content in the chambers was monitored daily, and plants were harvested at 21 DAP. A "static" control had roots in a chamber with no air passing through it.

For silver treatment, 1, 10, and 100 μM STS (15) (1:4 M mixture of silver nitrate and sodium thiosulfate) was added to the subirrigant nutrient solution. Five milliliters of STS solution was also added to the top of each conetainer at 2-d intervals, except 2 DAP, when plants were inoculated.

At harvest, nodules on the upper 10-cm region of pea roots and on whole roots of sweet clover and soybean were counted. The length of the primary and the longest lateral roots, and the dry weight of roots and shoots were also recorded. In some trials, the uppermost 30 lateral roots were harvested individually from each of 10 plants in each ethylene treatment, and the nodules in each 1-cm segment were counted.

We did a "census" of infection development in segments of the 3rd and 14th lateral roots of plants at 21 DAP (7, 8). Longitudinal hand sections of 0.5-cm segments between 1 and 1.5 cm from the primary root were stained with toluidine blue O; the number of rhizobial infections and the stage of nodule development were observed microscopically. Results were recorded per centimeter of root. The total number of tertiary roots (as primordia, emerging, or emerged) were also counted.

RESULTS AND DISCUSSION

Flowing air through the rhizosphere must alter the composition of gases around the root, but it had no observable effect on nodulation. Except for the longer primary and lateral roots, growth and nodulation of pea cv Sparkle did not differ markedly in a no-ethylene flow-through root chamber compared to adjacent plants in a "static" rhizosphere (Table I). Sparkle normally formed most nodules on the first 5 cm of the lateral roots on the upper 5 cm of the tap root.

In preliminary experiments (data not shown), we found that continuous exposure of pea roots to 0.5 μL/L or more of ethylene completely abolished nodulation. Therefore, subsequent trials were done at three lower concentrations. In another trial, we found that *R. leguminosarum* on yeast mannitol agar continued to grow even if the Petri plates were left in the chambers containing ethylene at concentrations of 0.07, 0.22, and 0.45 μL/L. Therefore, the results we observed were probably plant and not rhizobia mediated.

Continuous ethylene application to the root zone decreased plant growth as well as nodule number of Sparkle (Fig. 1). Total nodule number in this trial was decreased by about half, from 306 ± 54 to 145 ± 47, by 0.07 μL/L ethylene and was further decreased by 0.22 μL/L ethylene. At 0.45 μL/L ethylene, nodules were almost completely absent. In several trials with Sparkle, the ethylene concentration required to lower nodule number by half was about 0.1 μL/L. Moreover, nodule number on the primary root was decreased from 5 to nearly 0 by 0.07 μL/L ethylene. Nodulation on lateral roots in the upper 5-cm region, where most nodules normally developed by 21 DAP, was also almost completely blocked even by 0.07 μL/L ethylene.

Figure 2 shows the average number of nodules in each 1-cm segment of the uppermost 30 lateral roots. Ethylene at 0.08 μL/L almost abolished nodulation in the first few centimeters. The nodules recorded were formed mostly on younger parts of the lateral roots (between 5 and 10 cm). Thus, the inhibitory effect was less pronounced in regions beyond 5 cm from the primary root. This indicates that the sensitivity of nodulation to exogenous ethylene differs in different regions of the root.

Nodulation was much more sensitive to exogenous ethylene than was root growth (Fig. 1). At 0.45 μL/L, ethylene

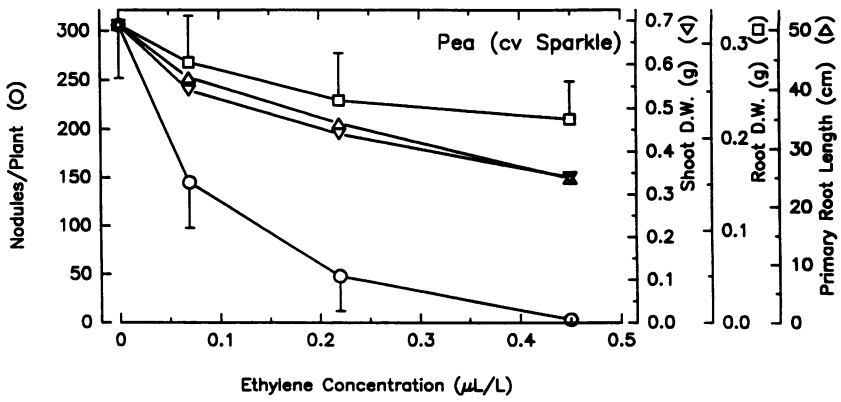

Figure 1. Effects of different concentrations of exogenous ethylene on nodule number (○), shoot dry weight (▽), root dry weight (□), and primary root length (△) of Sparkle at 21 DAP. Values are the mean (±SD) of seven plants from one representative experiment. Bars are 1 SD for nodule number and root dry weight. The magnitudes of SD for primary root length and shoot dry weight were similar to those for root dry weight.

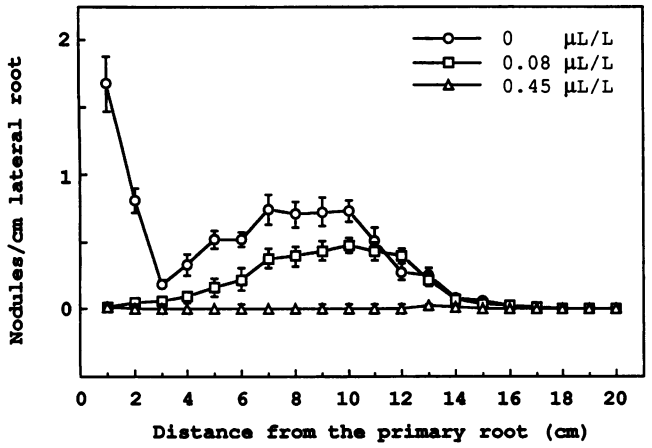

Figure 2. Effect of exogenous ethylene on nodule distribution on lateral roots. Values are the mean (±SE) of the 30 uppermost lateral roots per plant from 10 plants from each treatment.

almost abolished nodulation, but decreased root dry weight and the primary root length from 0.32 ± 0.07 to 0.22 ± 0.04 g (31%) and from 50.8 ± 5.3 to 24.8 ± 3.1 cm (51%), respectively. This concentration of ethylene is slightly higher than the apparent half-maximum concentration (0.34 μL/L) that inhibited root growth of etiolated pea seedlings (4). Reduction of shoot dry weight was similar to that of the primary root length.

The ethylene sensitivity of nodulation on peas was not cultivar specific; in a single trial, similar results were seen with cv Rondo (Fig. 3A). Thus, 0.07 μL/L ethylene decreased the nodule number of cv Rondo from 116 ± 46 to 61 ± 29, and 0.45 μL/L ethylene blocked the nodulation almost completely. Sweet clover was also similarly inhibited; again, nodulation in this species was more sensitive to exogenous ethylene than was root growth (Fig. 3B). Ethylene decreased the dry weight of shoot and root and primary root length of soybean similarly to the other species (Fig. 3C). Nodulation of soybean, however, was not affected by exogenous ethylene; nodule numbers did not change significantly with 0.45 μL/L ethylene. The sensitivity of the pea cultivars and sweet clover, thus, appears to be similar to that of excised bean roots (6). Differences in the initial timing of ethylene application might explain why our data (with less than 0.5 μL/L) differ so much from those obtained with 10 μL/L required to suppress nodulation in greenhouse-grown peas that were exposed to ethylene from only 28 DAP (5).

The mutant line cv Sparkle E135F, homozygous for *sym* 13, forms small, ineffective nodules that senesce early (11). Nodule formation was also sensitive to exogenous ethylene (Fig. 4A); few macroscopically visible nodules formed even at 0.06 μL/L. The mutant line cv Rondo nod-3, homozygous for *nod* 3, hypernodulates (9). It formed about 3 times as many nodules as its parent "Rondo." The effect of ethylene on this mutant (Fig. 4B) parallels the inhibition observed on its parent (Fig. 3A). In both mutant lines tested here, ethylene was more inhibitory to nodulation than to plant growth.

Inhibitory effects of exogenous ethylene on growth and nodulation could be reversed by the addition of silver, an inhibitor of ethylene action, although the most effective concentration of silver was slightly different among growth parameters (Table II). For example, addition of 1 μM STS to roots treated with 0.45 μL/L of ethylene made the length of the primary root and root dry weight similar to those of the control lacking ethylene in the rhizosphere and restored nodulation. Addition of 10 μM STS also increased the primary root length, but was inhibitory to nodulation. This may be due to the different ethylene sensitivity of different organs or to the phytotoxicity of silver, which was evident at 100 μM STS.

At what developmental stage does exogenous ethylene block nodulation? In regions where nodules normally form, on both of the lateral roots treated with three concentrations of ethylene, the number of total infections/cm in roots were similar and were not significantly different from that of the no-ethylene control. On the control, there were 10.4 ± 1.6 and 7.6 ± 1.0 infections/cm on the 3rd and 14th laterals, respectively. On roots exposed to 0.45 μL/L ethylene, the number of infections was 9.6 ± 2.3 and 8.4 ± 1.5, respec-

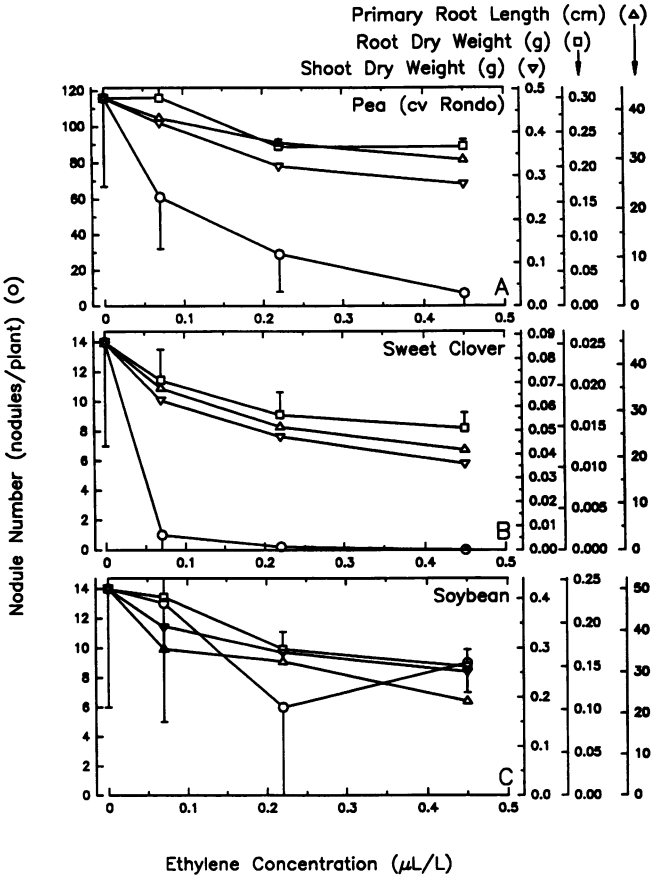

Figure 3. Effects of different concentrations of exogenous ethylene on nodule number (○), shoot dry weight (▽), root dry weight (□), and primary root length (△) of pea Rondo (A), sweet clover (B), and soybean (C). The plants were grown for 21 d in the same chambers. Values are the mean (±SD) of seven plants. Bars are 1 SD for nodule number and root dry weight. The magnitude of SD for primary root length and shoot dry weight was similar to those for root dry weight.

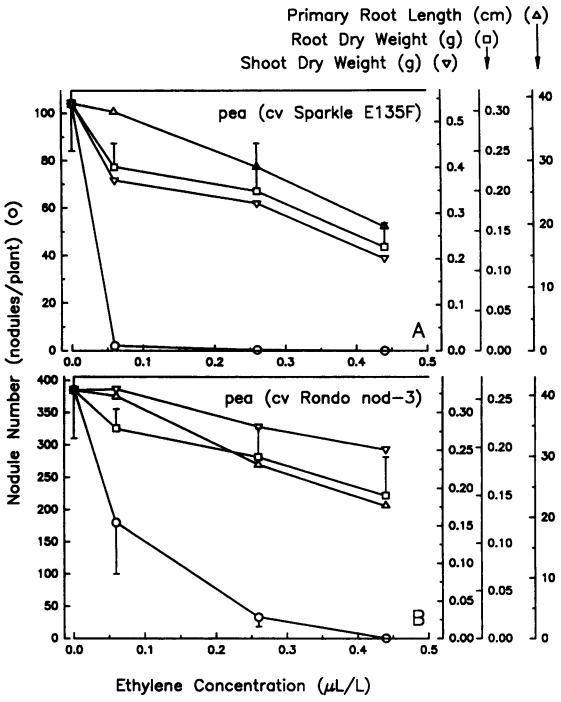

Figure 4. Effects of different concentrations of exogenous ethylene on nodule number (○), shoot dry weight (▽), root dry weight (□), and primary root length (△) of pea cv Sparkle E135F (A) and cv Rondo nod-3 (B). The plants were grown for 21 d in the same chambers. Values are the mean (±SD) of seven plants. Bars are 1 SD for nodule number and root dry weight. The magnitude of SD for primary root length and shoot dry weight was similar to those for root dry weight.

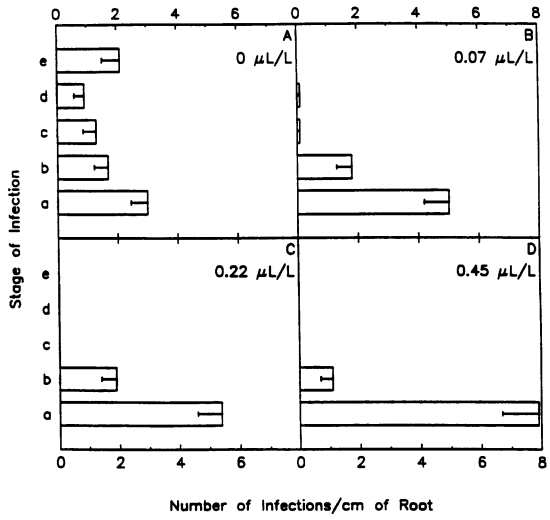

Figure 5. Effect of different concentrations of exogenous ethylene on the developmental stages of infection on lateral roots of Sparkle at 21 DAP. The developmental stages of infection were classified as follows: a, infection thread in epidermis; b, infection thread in outer cortex; c, infection thread in inner cortex and cell divisions in advance of it; d, nodule primordium or emerging nodule; and e, emerged nodule. Each column indicates the mean number (±SE) of infections at each stage. Each panel shows the results pooled from the 3rd and 14th lateral roots, counted down from the cotyledon, of 10 plants.

tively. This indicates that exogenous ethylene did not inhibit the very early stages of nodule development, such as colonization of rhizobia on the root hair or formation of an infection thread and its initial growth. The average number of total tertiary roots, sum of all stages, was not significantly decreased by ethylene. On the third lateral, there were 2.2 ± 0.8 tertiary roots/cm on the control and 3.2 ± 0.3 roots/cm on roots treated with 0.45 μL/L of ethylene. However, a census of the stages of infection showed that the infections in ethylene-treated plants were blocked when the thread was either in the basal part of the epidermal cell or in the outer cortex (Fig. 5). Very seldom did infection threads penetrate to the inner cortex, and nodule primordia did not appear with the two highest ethylene concentrations. The penetration of infection thread from epidermis into the outer cortex seems most sensitive to exogenous ethylene.

The effect of exogenous ethylene on infection of Sparkle is similar to that observed in the pleiotropic mutant E107 (*brz*) (8). That mutant demonstrates stress symptoms, presumably from excessive accumulation of toxic metals, and nodulation is decreased. In E107, most infections are blocked when the thread is in the basal epidermal cell (8).

In another pea mutant E2 (*sym* 5), the infection is blocked at a later stage; the infection threads progress to the inner

Table II. *Effect of Silver (Ag^+) on Peas Treated with Exogenous Ethylene*

Plants were grown in chambers where roots were continuously exposed to 0 or 0.45 μL/L of ethylene for 3 weeks. Ag^+ (as STS) was added to the substrate as specified. Values are the mean (±SD) of seven plants from one representative experiment.

Ethylene	Ag^+	Nodule No.	Primary Root	Root Dry Weight
μL/L	*μM*	*nodules/plant*	*cm*	*g*
0	0	114 ± 54	26.7 ± 3.0	0.25 ± 0.08
0	1	157 ± 61	25.6 ± 3.5	0.25 ± 0.06
0.45	0	9 ± 4	19.5 ± 0.7	0.17 ± 0.04
0.45	1	119 ± 63	24.9 ± 2.0	0.21 ± 0.05
0.45	10	38 ± 22	41.5 ± 3.9	0.22 ± 0.07
0.45	100	12 ± 9	21.0 ± 3.6	0.14 ± 0.02
$LSD_{0.05}$		47	3.3	0.06

cortex, but nodule primordia seldom appear (7). Treatment with silver, however, increased the cell divisions and promoted initiation of nodule primordia of E2. On *V. sativa* subsp. *nigra*, which formed few nodule primordia with certain rhizobial strains, nodulation was also restored by application of the ethylene biosynthesis inhibitor AVG (17). Therefore, the absence of cell divisions in the inner cortex under high exogenous ethylene levels suggests that the onset of cell division also can be a target of inhibitory effects of ethylene in Sparkle. This possibility is also supported by the fact that ethylene inhibits cell division (1).

We have demonstrated for the first time that exogenous ethylene inhibits nodulation on young intact plants. The sensitivity to ethylene is different on different regions of the root. There is still no direct evidence that endogenous ethylene is involved in regulating nodulation. If ethylene is a regulator of nodule number, it may act at more than one stage of nodule development: at the passage of the infection thread from the epidermal cell into the cortex and at the initiation of the nodule primordium.

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