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Ethylene as a Possible Mediator of Light- and Nitrate-Induced Inhibition of Nodulation of *Pisum sativum* L. cv Sparkle[1]

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ABSTRACT

Exposure of pea (*Pisum sativum* L. cv Sparkle) roots to light suppressed nodulation and induced an increase in ethylene production by roots. Dim light did not affect the number of infections per centimeter of root on the primary root, but most infections were blocked when the infection thread was in the epidermis or in the outer cortex. This is the same stage of infection on lateral roots that is blocked by exogenous ethylene. Silver, an inhibitor of ethylene action, increased nodule number on roots exposed to dim light. Exposure of pea roots to nitrate also suppressed nodulation and induced increased ethylene production by roots. However, induction of ethylene production from roots by nitrate was less than that induced by dim light. Nitrate decreased the number of infections per centimeter of lateral root. With 25 mM nitrate, about half of the infections that occurred were blocked in the epidermis or in the outer cortex. Silver did not reverse the inhibitory effects of nitrate on nodulation. Our data indicate that the inhibitory effect of light may occur via increased ethylene production, but they do not support the hypothesis that ethylene mediates nitrate control of nodule number. The possible role of ethylene in regulating nodule number should be studied in experiments in which light is excluded from the roots.

It is well documented that legumes control the number of their nodules. By an unknown systemic autoregulatory mechanism (2), the nodule number reflects the influence of soil constituents (e.g. nitrate, trace elements, water), environmental factors (e.g. shading, temperature), and stress (e.g. browsing, pesticides).

That nitrate inhibits nodulation was discovered in 1864 (14). How it does so must rank as one of the oldest questions of plant physiology (15). Nitrate affects several infection events, including the early stages of rhizobial binding, root hair curling, and infection thread formation (15).

Exogenous ethylene inhibits nodulation (6; our unpublished data), and several recent reports suggest that endogenous ethylene may have a role in regulating nodule number. The low-nodulating pea mutant E2 (*sym* 5) formed more nodules after treatment with inhibitors of ethylene formation or action (4). The infection process in E2 was blocked at the stage of inner cortical cell division (7). In another pea mutant E107 (*brz*), reduced nodulation seemed to be a secondary effect of stress brought on by excessive ion accumulation. Nodulation on this line was partly restored by Ag^+ (8), an inhibitor of ethylene action (1). Some strains of rhizobia caused the roots of *Vicia sativa* L. subsp. *nigra* to thicken, and nodule number decreased. The addition of AVG[2], an inhibitor of ethylene biosynthesis, restored the plant to normal (17), and it was proposed that the rhizobia induced the roots to produce excessive ethylene. AVG also increased the nodule number of alfalfa seedlings grown in plastic pouches (13).

That ethylene may mediate the suppression of nodulation by nitrate was proposed recently, because nitrate increased the ethylene production by alfalfa roots (11), and the nitrate inhibition of nodulation was apparently overcome by treatment with AVG (10).

Light, which promotes ethylene formation by pea root (3), also inhibits nodulation (5, 9). We have long used conical plastic pots ("Conetainers") in our research. Although those made of black plastic are opaque, we recently found that those made of white plastic were sufficiently translucent to permit inhibitory levels of light into the rhizosphere.

Higher plants form ethylene via l-aminocyclopropane-l-carboxylic acid, and that pathway is inhibited by AVG. However, legumes may also have alternate pathways. In epidermal cells of pea leaves and mung bean hypocotyls, ethylene is produced by a mechanism that is not enhanced by l-aminocyclopropane-l-carboxylic acid (12). Thus, studies with AVG may be insufficient to investigate the role of ethylene in nodulation. This is why we have used Ag^+, which is an inhibitor of ethylene action. A drawback to its use is phytotoxicity from excessive application (4).

To study whether light or nitrate regulated nodule number via increase in endogenous ethylene, intact pea (*Pisum sativum* L. cv Sparkle) roots were exposed to light or nitrate as a way of increasing ethylene production. In this study, we found no direct evidence that endogenous ethylene mediates nitrate regulation of nodule number. We suggest that previous research may have confounded the effects of light and nitrate on ethylene formation and nodule number.

MATERIALS AND METHODS

Growth Conditions

Seeds of *Pisum sativum* L. cv Sparkle were individually planted in coarse vermiculite in 2.4- × 16.5-cm conical pots

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

("Conetainer") made of white low-density polyethylene (Ray Leach Conetainer Nursery, Canby, OR). Plants were subirrigated with nutrient solution containing 5 mM nitrate and grown under high-pressure sodium vapor and metal halide lamps (550 μmol m^{-2} s^{-1} PPFD) in a 16-h/8-h, 20/15°C day/night regimen (4). Nutrient volumes were maintained by adding fresh solution at 2-d intervals and by replacement with fresh solution weekly. Plants were inoculated with *Rhizobium leguminosarum* bv *viciae* 128C53 at the time of planting or at 2 DAP for seedlings treated with Ag^+.

Treatment with Light or Nitrate

The roots were exposed to different levels of light by growing plants individually in three types of pots. White conetainers were wrapped in aluminum foil ("dark") or used as received ("dim light"). The transparency of these conetainers was about 25% across the light spectrum (Fig. 1). Pots of similar dimensions (2.2 [i.d.] × 16.5 cm) were made of plexiglass tubing, a clear plastic that transmits about 95% of the light ("bright light"). Only some of the pea roots grew in direct contact with the conetainer walls. Most of the root was within the vermiculite substrate and presumably received only a portion of the light that penetrated the conetainer wall. When roots were gently pulled out of the conetainer, the portions of the roots contacting the conetainer wall were visible on the surface of the rooting substrate. Those portions of roots that were thus exposed to dim or bright light had become thicker and greenish. For some experiments, nodules on roots in contact with the walls were counted first before all the nodules on the entire roots were counted. The stage of nodule development was studied with the primary root because it was more sensitive to light than the lateral roots.

To study the effects of nitrate, all plants were grown in wrapped (dark) conetainers and subirrigated with the defined nutrient solution (4) containing various concentrations of nitrate. Nodule number and the stage of nodule development were measured 21 DAP as described previously (7, 8). Longitudinal hand sections of 0.5-cm segments from 2 to 2.5 cm (measured from the cotyledonary node) of the primary root (light experiment) or from 1 to 1.5 cm (measured from the primary root) of the 3rd and 14th lateral roots (nitrate experiment) were stained with toluidine blue O. The "census" of rhizobial infections and the stage of nodule development were tabulated as before (7, 8).

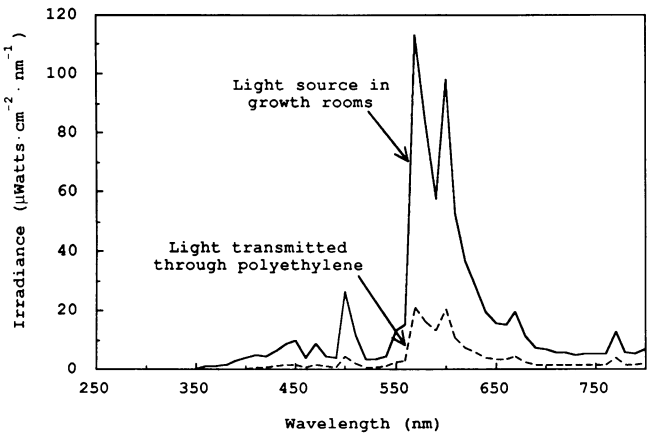

Figure 1. Light penetration through the low-density white polyethylene of the conetainer wall. The irradiance was measured at 10-nm intervals with an Optonics Laboratory model 752 Spectro calibrator. The light source was the metal halide and sodium vapor lamps in the light room. To measure light absorption, a single layer of the conetainer wall was placed over the sensor. The light intensity in the light room at plant height was 550 μmol m^{-2} s^{-1} PPFD.

Table I. *Effect of Inoculation on Ethylene Production*

Plants were inoculated with rhizobia at the time of planting, and ethylene production by 3-cm-long primary root tips was measured 5 DAP. Values are the means ± SE of 10 samples pooled from two independent experiments.

Treatment	Dark	Dim Light
	pmol·g fresh $wt^{-1}\cdot h^{-1}$	
Noninoculation	26.9 ± 2.0	45.7 ± 2.8
Inoculation	28.3 ± 3.2	45.4 ± 4.6

Ethylene Production

To measure ethylene production by the young root, three 3-cm-long primary root tips of 5-d-old plants were harvested into 5-mL vials and incubated for 3 h in the dark at room temperature. The ethylene accumulated was measured by GC (flame ionization detector).

Treatment with Ag^+

For Ag^+ treatment, plants were subirrigated with the nutrient solution containing 1 or 5 μM STS (16). An additional 5 mL of STS was applied on top of the vermiculite of each conetainer at 2-d intervals except the day of inoculation. In a previous experiment, the inhibitory effect of 0.45 μL/L of exogenous ethylene on nodulation was overcome by 1 μM STS without any visible adverse effects (K.H. Lee and T.A. LaRue, unpublished data).

All experiments were repeated at least twice, and representative data are presented.

Table II. *Effect of Light on Ethylene Production and Nodulation*

Ethylene production by 3-cm-long root tips of 5-d-old primary root (n = 10) and nodule numbers on 3-week-old, primary and lateral roots (n = 7) exposed to different light conditions were measured as described in the text. Values are means.

Root Environment	Ethylene Production	Nodules on	
		Primary root	Lateral roots
	pmol·g fresh $wt^{-1}\cdot h^{-1}$	*nodules/plant*	
Dark	36.0	25.9	330
Dim light	69.8	5.1	224
Bright light	87.5	4.3	161
$LSD_{0.05}$	15.1	11.9	59

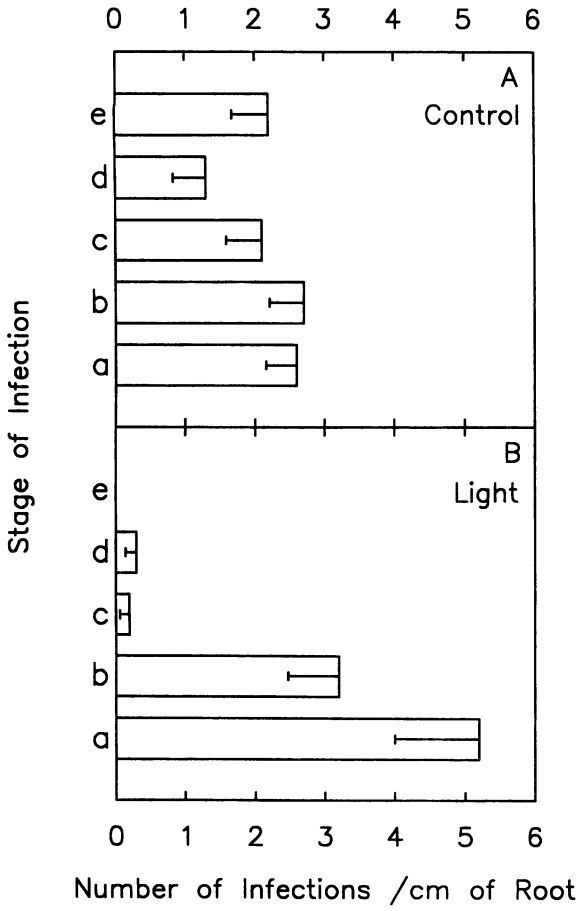

Figure 2. Effect of dim light in the rhizosphere on the developmental stage of infection on the primary root of Sparkle. The developmental stage of infection was classified as follows: a, infection thread in epidermis; b, infection thread in outer cortex; c, infection thread in inner cortex and cell 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 from the primary root of 20 plants. A, Results from roots in dark pots; B, results from roots in translucent plastic pots. The number of total infections per centimeter of root were 10.9 ± 0.8 and 8.9 ± 1.7 for the dark and the dim light treatments, respectively.

RESULTS AND DISCUSSION

Effect of Light

Ethylene production by 5-d-old, 3-cm-long primary root tips was independent of the presence of the rhizobia, both in darkened roots and roots grown in dim light (Table I). Thus, Sparkle pea is unlike *V. sativa* L. subsp. *nigra*, in which ethylene production was apparently stimulated by *R. leguminosarum* (17).

Table II shows that light in the rhizosphere increases ethylene production by 5-d-old roots; the two levels of light increased ethylene production by 90 and 140%, respectively, over production by roots grown in darkened pots. Measured 21 DAP, nodule number decreased with increasing levels of light (Table II). Whereas the primary root in darkened pots formed about 30 nodules, primary roots in vermiculite in translucent or transparent pots formed few nodules. Nodule number on the lateral roots was also decreased, although not as much, by dim light and was further decreased by bright light. Nodulation on the primary root thus seems more sensitive to light than does nodulation on lateral roots.

On the primary root, the total number of infections per centimeter of root was similar between the control (10.9 ± 0.8) and the dim light treatment (8.9 ± 1.7) (Fig. 2). The pattern of developmental stages of infections in dim light treatment was similar to what we observed on lateral roots treated with exogenous ethylene (K.H. Lee and T.A. LaRue, unpublished data); most infections were stopped in the epidermis or in the outer cortex (Fig. 2). This is in agreement with the observation of Grobbelaar et al. (5) that light on isolated bean roots is inhibitory at some time after the infection but before the nodule is macroscopically visible.

Ag^+, at 1 or 5 μM, restored the total number of nodules on roots grown in dim light to the amount on dark-grown roots (Table III). The greatest increase in nodule number was on the lateral roots, including the zones of the root that grew in contact with the conetainer wall. Nodulation on the primary root, which seemed more sensitive to dim light, did not respond to Ag^+ as much as that on lateral roots.

Effect of Nitrate

Increasing nitrate was associated with increased ethylene production by 5-d-old roots (Fig. 3). Nitrate at 25 and 50 mM increased ethylene production of the dark-grown root tips over that of 5 mM treatment by 48 and 78%, respectively. It should be noted, however, that the increase of ethylene by nitrate was less than that achieved by growing roots in dim light in translucent pots (Table II).

At 21 DAP, nodule numbers on both primary and lateral roots were affected by the levels of nitrate (Fig. 3). Plants grown with 5 mM nitrate formed nodules most abundantly, and those grown with 0, 10, or 25 mM nitrate formed fewer

Table III. *Effect of Ag^+ on Nodulation of Roots Grown in Dark or Dim Light*

In two trials, plants were grown for 3 weeks with or without STS. Nodules were counted as described in the text. Values are the means of seven plants for each representative experiment.

Light	Ag^+	Nodules on Lateral Roots Contacting Wall	Nodules on	
			Primary root	Lateral roots
	μM		*nodules/plant*	
Dark	0	31	32	183
Dark	1	30	39	209
Dim light	0	4	10	138
Dim light	1	14	13	219
$LSD_{0.05}$		8	12	39
Dark	0	—[a]	23	408
Dark	5	—	52	321
Dim light	0	—	6	288
Dim light	5	—	15	428
$LSD_{0.05}$		—	12	80

[a] —, Not counted.

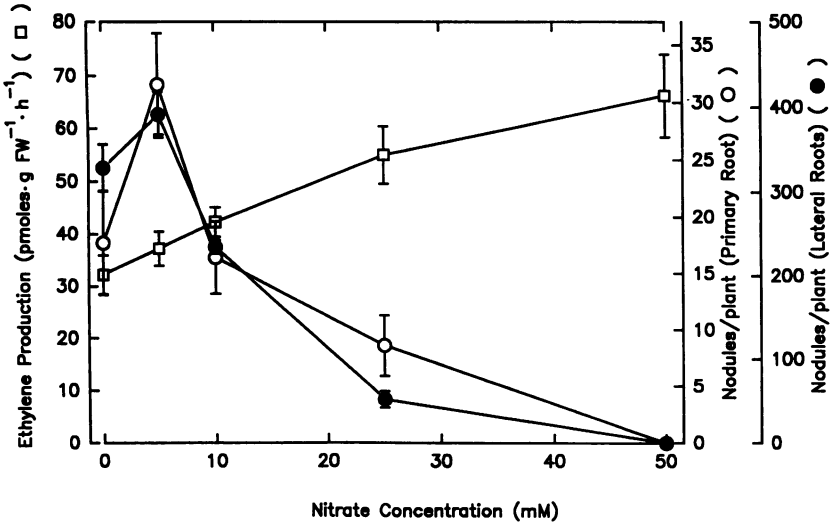

Figure 3. Effect of nitrate on ethylene production and nodulation of Sparkle. Ethylene production (n = 8) was measured in 5-d-old, 3-cm-long root tips. Nodulation (n = 7) of the primary and lateral roots was measured at 21 DAP. Values are the means ± SE. FW, Fresh weight.

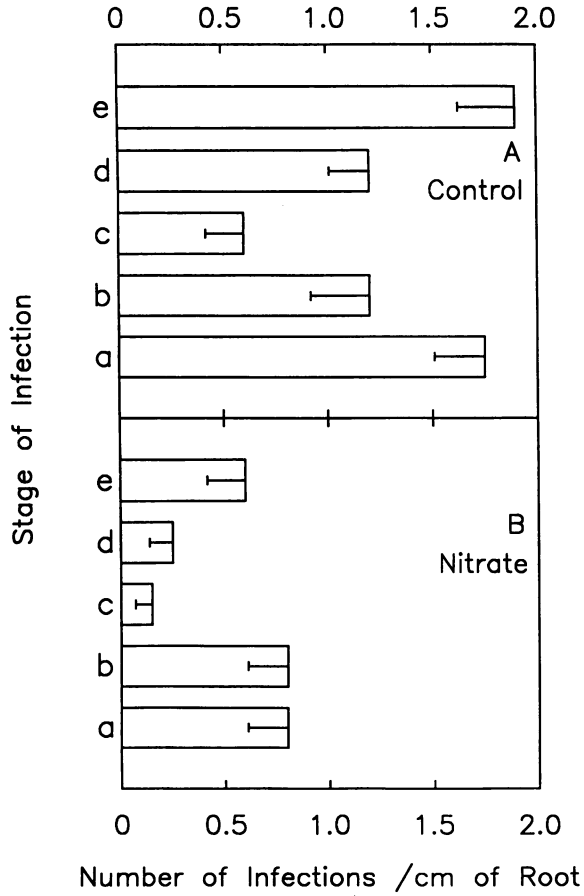

Figure 4. Effect of nitrate in the rhizosphere on the developmental stage of infection on the lateral roots of Sparkle. The developmental stage of infection was classified as in Figure 2. Each column indicates the mean number (±SE) of infections at each stage from the 3rd and 14th lateral roots of 20 plants. A, Results from the control; B, results from the 25 mM nitrate treatment. The total number of infections are 6.7 ± 0.4 and 2.6 ± 0.3 for the control and the nitrate treatments, respectively.

nodules. At 50 mM, nitrate completely blocked nodulation on both primary and lateral roots. Inhibition of nodulation by 10 and 25 mM nitrate was about 48 and 73% for the primary root and was about 40 and 87% for lateral roots, respectively. Nodulation on the primary and lateral roots was thus similarly sensitive to nitrate. Overall, the positive relationship between nitrate level and ethylene production, and the negative relationship between nodulation and ethylene production above 5 mM nitrate (Fig. 3), was similar to the recent report that suggested the possible involvement of ethylene in the nitrate inhibition of nodulation of alfalfa (10).

On the other hand, nitrate exerted its main inhibitory effect at a different stage of nodule development than did dim light or exogenous ethylene. The number of total infections on lateral roots during 25 mM nitrate treatment (2.6 ± 0.3) was fewer than that of the control (6.7 ± 0.4), indicating that the main inhibitory effect of nitrate occurred at early stages of infection, before thread formation. The census showed that about half of the infections that formed were stopped in the epidermis or in the outer cortex (Fig. 4). This agrees with previous reports that nitrate inhibits the early stages of infection (15).

Table IV. *Effect of Ag^+ on Nodulation of Roots Grown with High Nitrate*

Roots were continuously exposed to 5 or 25 mM nitrate for 3 weeks with or without STS (1 μM). Nodules were counted 21 DAP. Values are the means of seven plants for one representative experiment.

NO_3^-	Ag^+	Nodules on		Total Nodule
		Primary root	Lateral roots	
mM	μM		nodules/plant	
5	0	32	183	214
5	1	39	209	248
25	0	11	20	30
25	1	24	32	56
$LSD_{0.05}$		13	27	29

Ag^+ increased the nodule number only on the primary root exposed to 25 mM nitrate but failed to restore nodulation on the lateral roots to control levels (Table IV). This is in contrast with its effect on plants treated with exogenous ethylene, when nodulation was normalized by 1 μM STS treatment (K.H. Lee and T.A. LaRue, unpublished data). Therefore, although nitrate does increase ethylene production by roots (10, 11) (Fig. 3), there is no supporting evidence that this is the regulator of nodule number.

What is of concern, however, is that dim light reduces nodulation and is even more potent than nitrate in stimulating ethylene production. In the published experiments cited earlier, roots may have been exposed to light. The *V. sativa* roots in the experiments of Zaat et al. (17) were in liquid medium in test tubes. The alfalfa seedlings observed by Peters and Crist-Estes (13) were in clear plastic growth pouches. The alfalfa seedlings treated with nitrate by Ligero et al. (10, 11) were in test tubes, and their ethylene production was measured in tubes or vials. If roots in those experiments were exposed to light, the effect of AVG may have been a result of lowering the production of light-induced ethylene.

Specifically, the possibility that nitrate acts via induced ethylene production to regulate nodule number is not supported by our experiment. Nitrate blocked nodulation at an earlier stage than did exogenous ethylene (K.H. Lee and T.A. LaRue, unpublished data; Fig. 4), and the effect of nitrate was not reversed by Ag^+ (Table IV). Of general interest, we show that even low levels of light are inhibitory to nodulation. Many researchers, including us, have found it convenient to count nodules on roots grown in transparent or translucent chambers or pouches. In the future, studies of the regulation of nodule number should be conducted on dark-grown roots.

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