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Nodule Formation Is Stimulated by the Ethylene Inhibitor Aminoethoxyvinylglycine[1]

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ABSTRACT

Previous researchers found that formation and function of nitrogen-fixing nodules on legume roots were severely inhibited by addition of exogenous ethylene. Nodule formation by *Rhizobium meliloti* on *Medicago sativa* was stimulated twofold when the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) was added with the inoculum. Stimulation of nodule formation by AVG showed a similar concentration dependence as inhibition of ethylene biosynthesis, suggesting that the primary action of AVG is the inhibition of ethylene biosynthesis. When AVG was added 2 to 3 days after inoculation, the number of nodules formed was still increased. On a per plant basis, however, the average nitrogen fixation was unchanged by AVG treatment and was independent of nodule number.

Nitrogen-fixing nodules are formed on the roots of host legumes by infection with a compatible bacterial species of *Rhizobium* or *Bradyrhizobium*. The development of the nodule is an ordered process which requires a complex exchange of signals between the two symbionts. Host cells are induced to proliferate and establish nodule meristems. Bacteria penetrate through an infection thread into the root cortex and are released into host cells. The released rhizobia or bradyrhizobia cells are surrounded by a host-derived membrane and differentiate into dinitrogen-fixing bacteroids. The plant appears to be the primary regulator of nodulation. The host regulates the number of nodules formed, the maturation of those nodules, and the nitrogen fixation of the nodules dependent upon available nitrogen. The number of nodules formed and the rate of nitrogen fixation are greatly decreased in the presence of fixed nitrogen (reviewed in ref. 21).

The plant hormone ethylene negatively affects nodule growth and function. Grobbelaar *et al.* (16) showed that nodulation was decreased by 90% on bean explants treated with 0.4 ppm ethylene and was completely inhibited at higher concentrations of ethylene. Likewise, Goodlass and Smith (14) demonstrated that addition of 10 ppm ethylene decreased nodule formation on pea by 70% and on clover by 44%. They also showed that ethylene decreased the rates of nitrogen fixation to only 10 and 30% of the controls for pea and clover, respectively.

Given the adverse effects of exogenous ethylene on nodulation, we determined whether inhibition of ethylene biosynthesis would enhance nodulation and nitrogen fixation. To inhibit ethylene biosynthesis, we chose AVG[2] (2), an inhibitor of the enzyme 1-aminocyclopropane-1-carboxylic acid synthase which converts *S*-adenosyl methionine to 1-aminocyclopropane-1-carboxylic acid—the immediate precursor of ethylene (1, 5). We report that the presence of AVG stimulates nodule formation but does not result in increased nitrogen fixation.

MATERIALS AND METHODS

Bacterial Strains and Cultures

Rhizobium meliloti strain 2011 was maintained on and cultured in YEMG (5.0 g mannitol, 5.0 g sodium gluconate, 0.5 g yeast extract, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 1.6 g $CaCl_2 \cdot 2H_2O$ per L at pH 6.8) at 30°C. Inocula for plants were prepared by diluting log phase cultures (A_{600} = 0.3–0.6) with sterile water to a concentration of 10^7 cells/mL. Rhizobia cell numbers were determined by viable cell counts.

Plant Material

Medicago sativa (alfalfa) cv AS-R3 from Asgrow Seed was used in all experiments. Seeds were surface sterilized by treatment in 70% ethanol for 30–60 min followed by 20 min in 10% $Ca(OCl)_2$. Seeds were rinsed thoroughly with several changes of sterile distilled H_2O, imbibed in distilled H_2O over night, and transferred in groups of five to plastic growth pouches (Northrup King Seed Co., Minneapolis, MN). The growth pouches had been previously sterilized with ethylene oxide and wetted with 10 mL of sterile Jensen's nitrogen-free medium (22). Plants were maintained in a growth chamber at 70 to 80% RH 23°C in light for 16 h, and 21°C in dark for 8 h. Pouches were watered with sterile water as required during the incubation period.

Nodulation Assays

Seedlings were grown for 5 d. The positions of the root tip and the smallest emerging root hairs were noted on the pouch

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[2] Abbreviation: AVG, aminoethoxyvinylglycine or L-α-amino-γ-(2′-aminoethoxy)-β-butenoic acid.

before inoculation. Plants in each pouch were inoculated with 1 mL of *R. meliloti* 2011 at 1×10^7 cells/mL. The aqueous volume of a wet pouch is approximately 10 mL, so AVG (Sigma, St. Louis, MO) was added in the 1 mL inoculum at 10-fold the desired final concentration. The number and relative location of nodules on the main root were determined 8 d after inoculation because additional nodules were not observed after 8 d. Because plant growth in pouches is variable, only plants whose roots grew to the bottom of the pouch were scored for nodulation. For experiments in which AVG was added after inoculation, roots were inoculated with bacteria as above, and AVG was added in 1 mL of distilled H_2O at the indicated times.

Inhibition of Ethylene Biosynthesis by AVG

Plants were grown in plant growth pouches for 5 d. On the fifth day, AVG was applied to experimental plants to final concentrations of 0.1, 1.0, and 10 μM with a distilled H_2O control. At various times after treatment, 20 plants of like treatment were placed into a glass test tube (15 × 150 mm), and the tubes were stoppered with gas tight septum stoppers and incubated for 2 h. Ethylene accumulation was determined on a 2 m × 1/8 inch activated alumina 60/80 column in a Varian Gas Chromatograph 3700 with a flame ionization detector. Moles of ethylene were estimated by comparison to an ethylene standard.

Measurement of Nitrogenase Activity

Acetylene reduction assays were performed essentially according to Hardy *et al.* (17). For each assay, 10 plants were placed in a 15 × 150 mm test tube and stoppered. Pure acetylene gas was added to 4%. Samples (100 μL) were removed at 5, 10, and 15 min and analyzed for ethylene as described above. The reduction of acetylene remained linear over this period, and rates were computed by linear regression. All r values were ≥0.95.

RESULTS

Stimulation of Nodule Formation

Addition of AVG to the alfalfa inoculum enhanced nodule formation in a concentration dependent manner (Table I). As little as 1 μM AVG significantly increased the number of nodules formed, and maximal enhancement was achieved at 10 μM with the number of nodules formed being twice that of controls. The average number of nodules formed in the presence of 20 μM AVG was always less than the number formed at 10 μM, but the differences were not always statistically significant. When AVG was added at 50 and 100 μM, the plants showed stunted root growth and chlorosis of the leaves and were therefore not scored for nodulation.

Table I. *Enhancement of Nodule Formation by AVG*

AVG Concentration	No. of Nodules[a]
μM	*No. plant*$^{-1}$
0.0	5.1a
0.5	5.9a
1.0	7.1b
2.0	9.3c
10.0	11.4d
20.0	9.7c

[a] Values are means of 68 to 100 plants. Means followed by a different letter are significantly different (0.01 confidence level) by Duncan's new multiple-range test.

Inhibition of Ethylene Biosynthesis in Alfalfa

To determine the concentration dependence of AVG on inhibition of ethylene biosynthesis in our experimental system, we measured ethylene accumulation of control and AVG treated plants at 6, 24, and 120 h after addition of the inhibitor. Concentrations of AVG which significantly stimulated nodule formation (1 to 10 μM) inhibited ethylene biosynthesis by at least 50% (Table II). Inhibition of ethylene biosynthesis by AVG was rapid, having full effect within 6 h of application. Concentrations of 10 μM AVG continued to inhibit ethylene biosynthesis by 64% 5 d after application.

Spatial Distribution of Nodules

Legume root cells are only transiently responsive to *Rhizobium* infection. Mature portions of the alfalfa root, *i.e.*, those above the zone of root hair growth do not respond to rhizobia (4). The observed enhancement of nodule formation by AVG (Table I) could be accounted for either by formation of more nodules in the responsive zone or formation in nonresponsive zones. To distinguish between these two alternatives, we analyzed nodule distribution on control and AVG-treated roots. Nodules formed in the same regions of the root regardless of treatment. Therefore, treatment with AVG did not alter the zone of responsiveness to rhizobia (Fig. 1).

Delayed Addition of AVG

To determine the stages of nodule development affected by AVG, AVG was added at various times following inoculation. AVG added at the time of inoculation increased the number of nodules formed, but addition of AVG 48 h postinoculation gave even greater stimulation of nodule formation (Table III). Significant stimulation was observed even when AVG was

Table II. *Inhibition of Ethylene Biosynthesis following Addition of AVG*

AVG Concentration	Ethylene Biosynthesis Rate at Time after Addition of AVG[a]:		
	6 h	24 h	120 h
μM	*pmol* C_2H_4 *plant*$^{-1}$ h^{-1}		
0.0	6.7a	7.1a	6.0a
0.1	4.1b	6.8a	
1.0	3.4b	2.9b	
10.0	2.9b	2.1b	2.5b

[a] Values are means of four replicates with 20 plants per replicate. Means followed by a different letter are significantly different from other values within a time interval (0.01 confidence level) by Duncan's new multiple-range test.

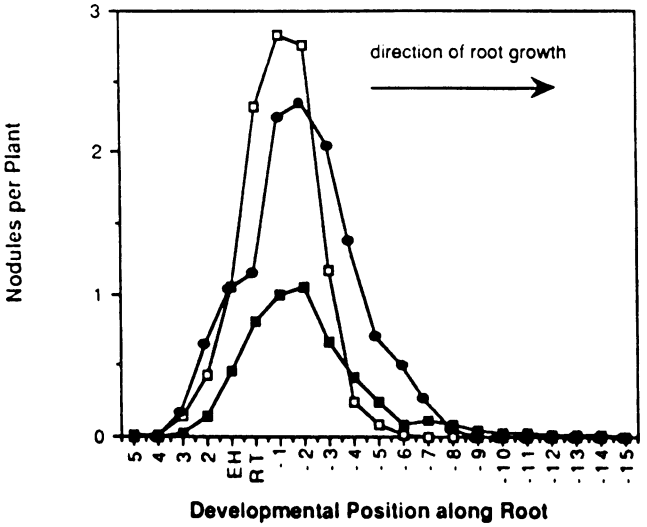

Figure 1. Distribution of nodules formed on the roots of control plants (■) and on plants exposed to 10 μM AVG at time of inoculation (□) and at 48 h postinoculation (●). At the time of inoculation, the root tip (RT) and the smallest emerging root hairs (EH) were marked. To standardize for differences in root growth, the distance from the RT to EH marks was taken as a unit of measure for each root. Nodule positions along the root are displayed in these units. Roots grow 2 to 4 developmental units (8–16 mm) in a 24 h period.

Table III. *Effects of Delayed Addition of AVG on Nodulation*

Time of Addition of 10 μM AVG	No. of Nodules[a]
h postinoculation	*No. plant^{-1}*
	6.1a
0	10.5c
12	10.4c
24	11.4cd
48	12.5d
72	8.7b

[a] Plants were scored for nodule number 8 d postinoculation. Values are means of 49 to 108 plants. Means followed by a different letter are significantly different (0.01 confidence level) by Duncan's new multiple-range test.

added 72 h post-inoculation. The position of nodule formation along the root remained the same regardless of the time of addition of AVG (Fig. 1).

Acetylene Reduction Assays

The average nitrogenase activity per plant as measured by acetylene reduction was the same for control and 10 μM AVG-treated plants throughout the period of nodule development examined (Table IV). This was despite the fact that AVG-treated plants had twice as many nodules (Table I). Nodules on AVG-treated plants were noticeably smaller than nodules on control plants such that excision of nodules for mass determination was not possible.

Table IV. *Measurement of Nitrogenase Activity by Acetylene Reduction*

Postinoculation	Reduction of Acetylene to Ethylene[a]	
	Control	10 μM AVG-Treated
d	*pmol C_2H_4 plant^{-1} min^{-1}*	
14	357	520
16	785	497
21	522	432

[a] No statistically significant differences (0.01 confidence level) were found between control and AVG-treated plants of similar age with Student's *t*-test.

DISCUSSION

Action of AVG

Previous research had demonstrated that ethylene severely inhibited the formation of nodules on legume roots by *Rhizobium* (11, 14, 16). As shown in this study, AVG-induced decreases in ethylene biosynthesis were correlated with a stimulation of nodule formation in alfalfa (Table I). While there are inherent problems with inhibitor studies, there are several reasons to believe that the primary effect of this inhibitor is on ethylene biosynthesis. AVG is an effective and highly specific inhibitor of ACC synthase with a low I_{50} (2×10^{-6} M (2). The concentration of AVG which inhibited ethylene biosynthesis in this study and others (2) was the same as that required to increase nodule number. It should be noted that we measured whole plant ethylene. This underestimates the extent of ethylene biosynthesis inhibition in the root. Other plant enzymes inhibited by AVG such as β-cystathionase, are far less sensitive to AVG and have I_{50} values of about 1×10^{-4} M (X Ruan, NK Peters, unpublished observations). Such high concentrations of AVG were indeed toxic to the plants and drastically inhibited root growth.

Although inhibition of ethylene biosynthesis is most likely the primary effect of AVG on the physiology of the host, we do not know how this stimulates nodule formation. Ethylene is known to have many effects on plant development including inhibition of cell division, DNA synthesis, and hook expansion (3), and induction of phytoalexin and extensin biosynthesis (13). Each of these effects of ethylene is antagonistic to the formation of nodules. One important process in nodule formation is the stimulation of cortical cell divisions and establishment of a nodule meristem. In alfalfa, cortical cell divisions commence within 12 h of inoculation (12). By inhibiting ethylene biosynthesis, cell division would proceed without the inhibitory control by endogenous ethylene. This is not really a stimulation of cell division and meristem formation; it is merely a lack of inhibition.

Rhizobia infection itself may induce ethylene biosynthesis by the host plant. Inoculation of *Vicia sativa* subsp. *nigra* with *R. leguminosarum* biovar *viciae* causes roots to grow abnormally forming thicker and shorter roots (24). Under these conditions, nodules form only at points of lateral root emergence and not by root hair infection. This abnormal root and nodule development is suppressed by application of AVG, suggesting that ethylene is involved in the process (24).

Rhizobia invade the host root, yet the bacteria do not

normally elicit a host defense response in effective associations. There are several examples, however, in which the bacteria elicit a defense-like response from the host in ineffective associations (23), and the elicitation of the response can be the result of single bacterial mutations (10). Since ethylene is a key messenger in host defense responses, inhibition of ethylene biosynthesis could thus play a role in the ability of the bacteria to circumvent a defense response.

AVG Treatment and Feedback Regulation

The host regulates both the formation and activity of nodules. Split root experiments have demonstrated that the plant regulates the formation of new nodules through a systemic negative feed-back mechanism (6, 19, 20). Although treatment with AVG allows more nodules to form, nodules form in the normally responsive zones of the root (Fig. 1). No new portions of the root are made responsive to rhizobia with AVG treatment, demonstrating that negative feedback regulation is not affected. The spatial distribution of nodules on AVG-treated plants is more restricted than on control plants (Fig. 1 developmental positions −4 to −10), suggesting that feedback suppression is stronger in AVG-treated plants.

Plant mutants which do not properly regulate nodule formation have been isolated (7, 8, 15, 18). These plant regulatory mutants form two- to fourfold more nodules than the parent line (8, 15, 18) but vary in their nitrogenase activity relative to the number of nodules formed. In some cases, the nitrogenase activity is proportionally increased with the number of nodules (15). In other cases, the nitrogenase activity is the same per plant independent of nodule number (9). In the case of the AVG-treated plants, nitrogen-fixation did not increase with increased nodulation. This disparity between a greater number of nodules and no increase in nitrogenase activity is most likely a result of the nodules on AVG-treated plants being smaller and less developed. The failure of nodules on AVG-treated plants to develop to normal size may be a result of the plant's negative feedback regulation or a limitation of growth in pouches.

CONCLUSIONS

Treatment with AVG stimulates nodule formation, and nodules form in normally responsive zones of the root. This enhancement occurs even when AVG is added 3 d after inoculation. Increased nodulation does not result in an increase of nitrogen fixation.

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