Plant Physiol. (1991) 97, 1221–1225
0032-0889/91/97/1221/05/$01.00/0

Received for publication April 23, 1991
Accepted July 15, 1991

Nitrate Inhibition of Nodulation Can Be Overcome by the Ethylene Inhibitor Aminoethoxyvinylglycine[1]

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ABSTRACT

Previously, we reported (a) a positive correlation between the nitrate concentrations in growth medium and ethylene evolved from uninoculated and inoculated alfalfa (*Medicago sativa*) roots and (b) a negative correlation between ethylene evolution and nodulation. Here, we report that the inhibitory effect of NO_3^- on nodulation of alfalfa can be eliminated by the ethylene inhibitor aminoethoxyvinylglycine (AVG). This effect was probably related to the strong inhibition (90%) of ethylene biosynthesis caused by AVG in these inoculated and NO_3^--treated roots. These results support our hypothesis that the inhibitory effect of NO_3^- is mediated through the phytohormone ethylene. A possible role of endogenous ethylene in the autoregulation of nodulation also is discussed. AVG at 10 micromolar significantly ($P < 0.05$) increased total nitrogenase activity (acetylene reduction) in 2.5 and 5 millimolar NO_3^--fed plants probably as a result of the very high stimulation of nodulation.

The formation of a nitrogen-fixing root nodule is the consequence of a series of interactions between host plant and microsymbiont. The process is regulated by internal plant factors, which are termed autoregulation factors (5, 24) and environmental factors, of which NO_3^- is a major component in the inhibition of both nodulation and N_2 fixation (8, 28). The effect of nitrate is exerted soon after inoculation, inhibiting both initial cortical cells divisions and infection thread formation (21). Moreover, an interaction between NO_3^- and the autoregulation signal during NO_3^- inhibition has been suggested in soybean (11, 21).

Ethylene is likely to be an important natural regulator of root growth and may affect many aspects of root development and nodule formation. Exogenously supplied ethylene strongly inhibits nodulation and N_2 fixation (13, 15) and root growth (14, 15). During root infection by *Rhizobium*, the ethylene evolution rate increases markedly, leading to dramatic changes in root development (18, 29). However, treatment with AVG[2] at the time of inoculation significantly inhibits ethylene biosynthesis and stimulates nodule formation in alfalfa (23).

Enhanced ethylene evolution rates in uninoculated as well as in inoculated alfalfa roots in the presence of NO_3^- have been reported (19). A negative correlation between ethylene evolution and nodulation rates was also observed. We proposed that the negative effect of NO_3^- on nodulation may have been mediated through ethylene. The aim of this study was to determine whether inhibition of ethylene biosynthesis by the ethylene inhibitor AVG would increase nodulation in the presence of NO_3^-.

MATERIALS AND METHODS

Plant Culture

Medicago sativa (alfalfa) cv Aragón (Ramiro Arnedo, La Calahorra, Spain) seeds were surface sterilized in 2.5% (w/v) $HgCl_2$ for 10 min, thoroughly rinsed in distilled water, and germinated in Petri dishes at 25°C in the dark. Seedlings were grown aseptically in a growth chamber as described before (19) in 20 × 200-mm test tubes (five plants per tube) with mineral solution (27) supplemented with KNO_3. Plants (10 d old) were inoculated with 1 mL per tube (approximately 10^9 cells, viable cell counts) of a suspension of *Rhizobium meliloti* GR4 as described elsewhere (19).

Nitrate and AVG Treatments

The interactions of four concentrations (2.5, 5, 10, and 20 mM) of NO_3^- as KNO_3 with four concentrations (0, 1, 10, and 20 μM) of AVG were studied. Nitrate was added to the solution before planting so that the plants grew in NO_3^- from the beginning. Because 2 mM NO_3^- was shown not inhibit nodulation in alfalfa (18) and the use of plants grown in low nitrate has been recommended (8, 28), 0 mM NO_3^- was not included in the present study. Solutions were balanced for cations and anions with KCl. AVG was dissolved in distilled water and sterilized by filtration. Dilutions were obtained such that addition of 0.5 mL per tube (containing 10 mL of medium) gave the desired concentration. AVG was applied immediately after inoculation, and 0.5 mL per tube of distilled water was added to plants not treated with AVG.

Nodulation Assays

For each treatment, 14 growth tubes with five plants growing uniformly, as judged by their roots reaching the bottom

[1] Financial support was obtained by grant No. PB87–0869 and BT87–0003 from Dirección General de Investigación Científica y Tecnológica.

[2] Abbreviations: AVG, aminoethoxyvinylglycine or L-α-amino-γ-(2′-aminoethoxy)-β-butenoic acid; ARA, acetylene reduction activity.

of the tube, were scored. The time course of nodulation was followed by counting nodule number per tube at 7, 10, 14, and 19 d after inoculation.

Inhibition of Ethylene Biosynthesis by AVG

Plants were grown, inoculated, and treated with AVG as above. Only plants grown in 10 mM NO_3^- were used, and ethylene biosynthesis (as ethylene accumulation in time) was measured at 48 and 96 h after AVG addition by GC as described before (19) with some modifications. Fifteen whole plants (from three test tubes) were incubated for 4 h in gas-tight septum-stoppered vials (30 mL) containing a strip of wet filter paper to maintain humidity. From preliminary experiments in which plants were separated into root and shoot, it was determined that, in our experimental system, ethylene was almost entirely produced by the root. Thus, results were obtained from whole plants to avoid possible artifacts as a response to injury.

Nitrogenase Activity

Plants grown in 2.5 and 5 mM NO_3^- as described above were used to quantify the effect of AVG on total nitrogenase activity. Acetylene reduction assay by GC was as described elsewhere (18). Gas samples from vials were removed after 5, 10, and 15 min incubation for ethylene analysis. Nitrogenase was expressed as μmol C_2H_4 produced per tube (five plants) per h. Nodules in this system were too small to be picked and weighed.

RESULTS

Effect of Nitrate and AVG on the Nodulation Rate

Nitrate and AVG strongly affected nodulation rates of alfalfa roots in a concentration-dependent manner (Fig. 1). By day 19 after inoculation, nodulation had plateaued in all treatment conditions with no appreciable increase in nodule appearance after this time (data not shown). Similar results have been found for soybean (5).

As nitrate concentrations were increased, the appearance of nodules was delayed, and concomitantly nodulation rate slowed, resulting in a sharp decrease in the number of nodules per plant. All of these effects were highly significant ($P < 0.01$) (Fig. 1).

In contrast, AVG enhanced nodule formation on alfalfa roots at all NO_3^- concentrations assayed, with the effect being most pronounced at higher NO_3^- concentrations. In plants grown on 2.5 and 5 mM NO_3^-, as little as 1 μM AVG significantly increased the number of nodules formed by day 7 after inoculation on alfalfa roots, whereas 10 and 20 μM AVG strongly reduced nodule appearance by days 7 and 10 (Fig. 1). The effects were different, however, for plants fed 10 mM or 20 mM NO_3^-. Only slight or no enhancement of nodule number (by day 7) was observed with 1 and 10 μM AVG. Furthermore, no effect or significant stimulation (by day 10) was obtained with 20 μM AVG compared with controls (0 μM AVG). Finally, by days 14 and 19 all AVG treatments significantly increased nodule number per plant at all NO_3^- concentrations assayed.

The following points were noted: (a) The enhancement of nodulation by AVG increased with NO_3^- supply; (b) 10 μM AVG was the most effective treatment in 2.5 and 5 mM NO_3^--treated plants, whereas 20 μM AVG was best for plants receiving 10 or 20 mM NO_3^-; and (c) the AVG effect was concentration and time dependent, because high AVG concentrations caused both initial inhibition and final stimulation of nodule formation.

Effect of AVG on Ethylene Evolution in Alfalfa

The inhibition of ethylene biosynthesis by exogenously supplied AVG was studied in plants grown in 10 mM NO_3^- and treated with four AVG concentrations. Ethylene evolution was measured 48 and 96 h after inoculation (Fig. 2). At this concentration of NO_3^-, we previously observed an induction of high rates of ethylene evolution and a strong inhibition of nodulation in alfalfa roots (19). Comparable rates of ethylene biosynthesis (about 50 pmol $plant^{-1}$ h^{-1}, measured as ethylene accumulation) were also induced in the present study by 10 mM NO_3^- in AVG-untreated plants. The concentration of AVG that induced a twofold higher number of nodules per plant (Fig. 1) also inhibited ethylene biosynthesis by 80 to 90% (with respect to controls) 48 h after inoculation and inhibitor addition (Fig. 2). Four days after inoculation, the rates of ethylene biosynthesis were still very high in control plants, but AVG continued to inhibit them to the same extent. The effect of AVG was concentration dependent, and highly significant decreases were observed up to 10 μM. However, a further increase to 20 μM AVG resulted in no significant effect compared with 10 μM AVG. Ethylene biosynthesis was very sensitive to AVG, because 1 μM was sufficient to maintain an inhibition of about 66% throughout the period of the experiment (Fig. 2).

Acetylene Reduction and Growth of Plants

Nitrate at 5 mM significantly ($P = 0.05$) inhibited ARA in root nodules of controls and AVG-treated plants (Table I). However, regardless of the NO_3^- treatment, AVG-treated plants showed higher ($P = 0.05$) rates of ARA. Although 1 μM AVG produced substantially enhanced nodulation (average 37%), total ARA was not significantly affected (Table I). At 10 μM, however, an increase of total ARA was observed, likely due to the very high stimulation of nodulation achieved (48 and 129% for 2.5 and 5 mM NO_3^--fed plants, respectively). The effect of AVG on plant growth was minimal at all AVG concentrations, although 20 μM may have slightly retarded growth (data not shown).

DISCUSSION

Nitrate has many complex inhibitory effects on the overall process of nodulation of a legume root by *Rhizobium*. Recently, it was reported that major effects are exerted within a period as short as 18 h after inoculation by preventing both infection thread formation and initial cortical cell divisions (21). The mechanisms, however, remain obscure. In the present study, we found that under some conditions the inhibitory effect of NO_3^- on nodulation of alfalfa could be eliminated

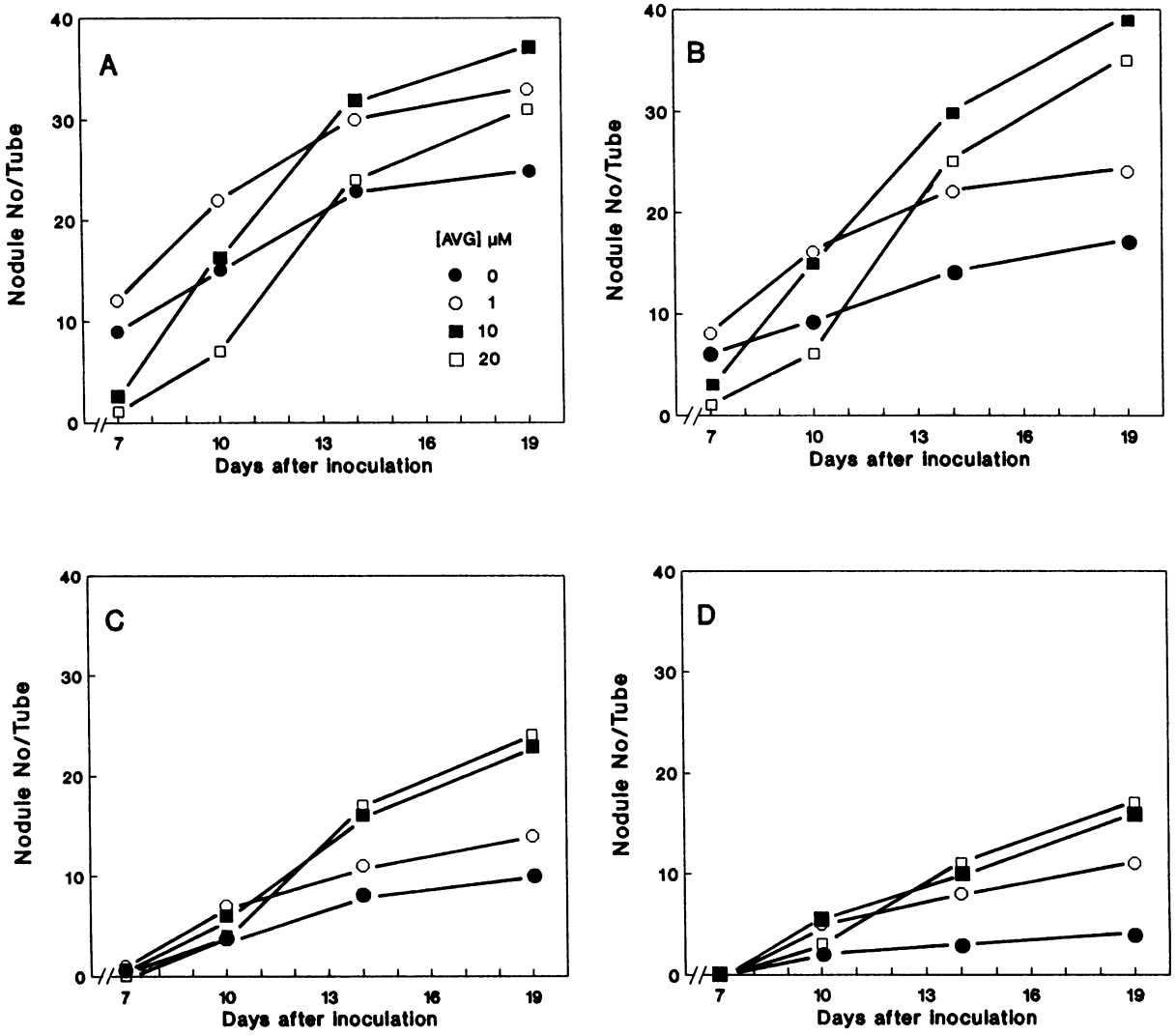

Figure 1. Stimulation of nodule formation by AVG in roots of alfalfa grown in different concentrations of nitrate: A, 2.5 mM; B, 5 mM; C, 10 mM; D, 20 mM. Five plants were grown and inoculated as described in "Materials and Methods." Nodule number per tube was recorded at several times after inoculation. Each tube contained five plants. Values represent the means of at least 12 test tubes. Data were subjected to multivariant analysis of variance, and means were compared by Duncan's new multiple range test. $LSD_{0.01}$, equal to 1.55, was smaller than the symbols.

by the ethylene inhibitor AVG (Fig. 1), which strongly inhibited ethylene biosynthesis in these plants (Fig. 2). Induction of ethylene biosynthesis by NO_3^- and a negative correlation between ethylene evolution and nodulation in alfalfa were previously reported (19). The present results support our hypothesis that the NO_3^- effect could be mediated through the phytohormone ethylene.

Ethylene production by plant tissues is extensively documented in the literature and includes N_2-fixing legumes, particularly *M. sativa* (18, 23), *Vicia sativa* (29), and *Pisum sativum* (20). Ethylene biosynthesis and release is also a normal response of plants to many different stresses, such as wounds (12), toxic chemicals (4), and pathogenic microorganisms (4, 12) as well as *Rhizobium* (18). Surprisingly, we showed that nitrate, although a nutrient and not a stress, induced ethylene biosynthesis in uninoculated and inoculated plants (19). Additional evidence that NO_3^- may induce ethylene biosynthesis has been suggested for other plants (9, 10). Whether NO_3^--induced ethylene production by alfalfa roots is caused directly or indirectly through a complex metabolic process involving hormone imbalance (26) is open to question. The severe inhibition of nodulation in legumes by ethylene has been known for several years (15). Levels of ethylene able to inhibit nodulation have been shown to be induced during infection of roots by specific rhizobia in alfalfa (23; this work, Figs. 1 and 2) and in *V. sativa* (29). All of these studies involving AVG concluded that the main effect of AVG was to inhibit ethylene biosynthesis (as can be seen in Fig. 2). However, other effects of AVG, such as the delayed nodulation at highest levels and the precise mechanism by

which inhibitory effect of NO_3^- is overcome, remain to be understood.

Many processes are affected by ethylene, *e.g.* those related to defense mechanisms (12), cell division and DNA synthesis (2), root extension, and lateral root initiation (14). All of these could satisfactorily explain the effects on nodule formation. Nodulation appears to be under the control of the plant host by means of an autoregulatory process (5, 11, 16, 25). Recently, developing as well as mature nodules were reported to elicit feedback regulation of nodule number in alfalfa (6) and soybean (7), which is consistent with the high rates of ethylene evolution by nodulated alfalfa roots (18).

Evidence from Gresshoff's group, ourselves, and others suggests that a common factor might be involved in autoregulation and in nitrate inhibition. This factor may accelerate the maturation of the root tissue and thus shorten the transient susceptibility of the root cells to infection (3), resulting in a decreased probability that root cells will be infected or that fewer infections will develop into nodules. Endogenous ethylene induced in response to infection and/or NO_3^- might be a good candidate for this factor. Unfortunately, the patterns of ethylene synthesis in supernodulation mutants and their wild-type parents have not been characterized. It is also interesting that some *Bradyrhizobium* strains in the rhizosphere produce rhizobitoxine which acts as an ethylene biosynthesis inhibitor in the plant (1, 22). Thus, alleviation of NO_3^- inhibition by high doses of inoculum in wild-type soybean (17) is consistent with such an effect of *Bradyrhizobium*. *Rhizobium* infection induces ethylene biosynthesis by legume root (18, 23, 29). By eliciting ethylene evolution, NO_3^- may significantly reinforce such a plant response so as to preclude infection and/or nodule formation.

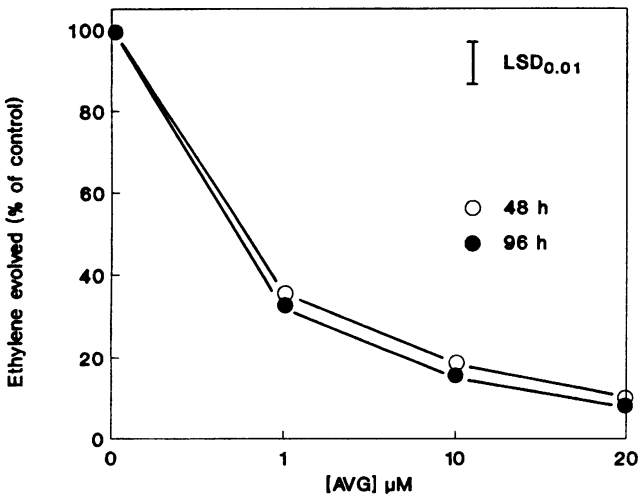

Figure 2. Inhibition of ethylene evolution, following addition of AVG, in inoculated alfalfa roots growing on nitrate. Seedlings were grown in test tubes on 10 mM nitrate, inoculated, and supplied with different concentrations of AVG as described in Figure 1 and "Materials and Methods," and ethylene evolution was measured 48 and 96 h after inoculation. Each point, mean of four replicates with 15 plants per replicate. Data were subjected to analysis of variance, and means were compared by Duncan's new multiple range test.

Table I. *ARA of Alfalfa Nodules Formed in the Presence of Nitrate and AVG*

A set of plants from each treatment was obtained as indicated in Figure 1 and used at day 20 after inoculation for the acetylene reduction assay (see "Materials and Methods").

Nitrate Concentration	AVG Concentration (μM)		
	0	1	10
mM	nmol C_2H_4 $tube^{-1}$ h^{-1}[a]		
2.5	430	420	483
5	387	Not determined	412
$LSD_{0.05}$		20	

[a] Data are means of seven replicates with five plants per replicate. Statistics as in Figure 2.

In our experimental system, the AVG-increased nodulation also resulted in a slight but significant ($P = 0.05$) increase in acetylene reduction (Table I). This discrepancy with a previous report (23) may be due to the fact that different cultivars of alfalfa and completely N_2-dependent plants were used in the previous study instead of plants fed low NO_3^- concentrations. The latter point has been thoroughly discussed, and the use of low nitrate-grown plants has been recommended (8, 28).

ACKNOWLEDGMENTS

We want to thank Prof. Peter M. Gresshoff for critical reading and discussion during the preparation of the manuscript.

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