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Ethylene Production by Root Nodules and Effect of Ethylene on Nodulation in *Glycine max*

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Nodulated soybean roots produced more ethylene and contained more 1-aminocyclopropane-1-carboxylic acid than uninoculated roots. Nodules produced more ethylene and contained more 1-aminocyclopropane-1-carboxylic acid per gram of material than roots. Almost all of the ethylene produced by the nodules was produced by the plant fractions of the nodules. Ethylene, at physiological concentrations, did not inhibit nodulation in soybeans.

It has been suggested that ethylene, a plant hormone (18), may function as an autoregulatory signal to control nodule formation and development in *Medicago sativa* (19). There is evidence to support this hypothesis. First, exogenous ethylene is a strong inhibitor of nodulation in *Phaseolus vulgaris*, *Pisum sativum*, and *Trifolium repens* (4, 7, 8). Second, some legumes produce more ethylene when they are nodulated than when they are not nodulated (19–21); this production of ethylene may be a plant response to the nodule bacteria (28). Third, endogenous ethylene interferes with nodulation in *Trifolium subterraneum* and *M. sativa* (3, 19, 24).

While several studies have investigated the effect that ethylene has on nodulation and nitrogen fixation in *Rhizobium* associations, none of these studies has looked at the possible role of ethylene as an autoregulatory signal in the control of nodulation in the soybean-*Bradyrhizobium* symbiosis, and none has attempted to determine where the ethylene is produced in any system. The specific objectives of this study were to determine (i) whether soybean roots respond like other legumes and produce more ethylene when they are nodulated; (ii) whether increases in ethylene production are due to the nitrogen nutrition of the plant or to the presence of nodules on the roots; (iii) where, in the nodules or in the roots, ethylene is produced; and (iv) the effect of ethylene on the nodulation of soybeans.

Plant and bacterial material. *Bradyrhizobium japonicum* I-110 ARS (14, 15) and 5MT-7 (10) were used as the plant inocula. Cells were grown in A1E broth at 30°C and shaken at 100 rpm (13). Soybean (*Glycine max* [L.] Merr. cv. Tracy M) seeds were treated, germinated, inoculated, and grown in a greenhouse as previously described (9, 12). In a growth chamber, seedlings were placed in light-proof 1-liter hydroponic vessels, one seedling per vessel. The nutrient solution (12) was changed twice in the second week of growth, three times in the third week of growth, and daily in the fourth week of growth. The vessels were equipped with Swagelok fittings, which facilitated connection of air inlets and ethylene traps (Fig. 1). In order to provide a stable root temperature, the hydroponic vessels were immersed in a large 28°C water bath placed inside the growth chamber. Plants were grown with a 14-h photoperiod and with shoot temperatures of 30°C (day) and 20°C (night). Light was supplied at a photosynthetic photon flux density of about 750 μmol m^{-2} s^{-1}.

Agar tube studies were also conducted in the growth chamber. The tubes (25 by 250 mm) contained 60-ml portions of nutrient media supplemented with 0.5% Noble agar (Difco). The tubes were supported in light-proof boxes, and a 1.5-cm layer of black gravel was placed on the agar surface of each tube to protect the roots from light. Other conditions were as described above.

Growth chamber ethylene measurements. Roots were supplied with ethylene-free air at a rate of 25 ml/min for 18 h before measurements were made. Air was made ethylene free by passage over a heated (600°C) platinum catalyst (6). Ethylene, formed by the roots, was collected for 30 min with 250 mg of freshly activated silica gel in a U-shaped tube fashioned from a 24-cm-long, 2-mm-outside-diameter section of aluminum tubing and immersed in a dry ice-isopropanol bath. For analysis, the cold U tube was transferred to the column inlet of a gas chromatograph, and the ethylene was released from the silica gel by immersing the U tube in an 80°C water bath. An isothermal (35°C) gas chromatograph equipped with a flame ionization detector and a Porapak N column (3.18 mm by 3 m) was used for ethylene analyses. The column flow rate was 30 ml/min, and helium was the carrier gas.

Isolation and fractionation of bacteroids, cytosol, and plant cell debris. Roots were harvested from greenhouse-grown soybean plants and placed on ice. As quickly as possible, 150 g of nodules was harvested and placed in a glove bag with a nitrogen atmosphere. Within the glove bag the nodules were mixed with cold, anaerobic buffer (11), homogenized, and placed into centrifuge tubes. Nodule fractions were then isolated as previously described (11), except that all operations were conducted under a nitrogen atmosphere and 2 ml of buffer was used for each 1 g of nodules.

Assay for ethylene production by nodule components. The plant cell debris fraction and the bacteroid fraction were resuspended in sufficient buffer (11) to give a final volume of 45 ml. A 15-ml portion of the nodule fraction was placed into a 25-ml flask, and the flask was closed with a stopper and incubated in a shaking water bath at 28°C and 250 rpm. The plant fractions were incubated under an N_2 atmosphere and under air. The bacteroid fractions were incubated under N_2. After 30 min, the flasks were removed from the water bath, and the atmosphere above the incubation mixture was immediately pushed through a cold U tube containing activated silica gel (see above) by a stream of nitrogen (25 ml min^{-1} for 5 min). The ethylene content was determined by gas chromatography.

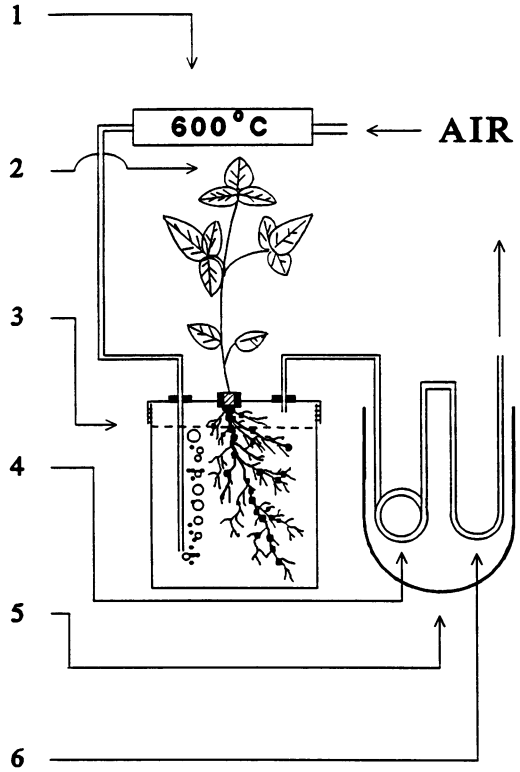

FIG. 1. Chamber design used for the determination of ethylene production by plant roots. 1, air purifier; 2, soybean plant; 3, flowthrough chamber; 4, moisture trap; 5, dry ice-isopropanol cold bath; 6, U tube ethylene trap containing silica gel.

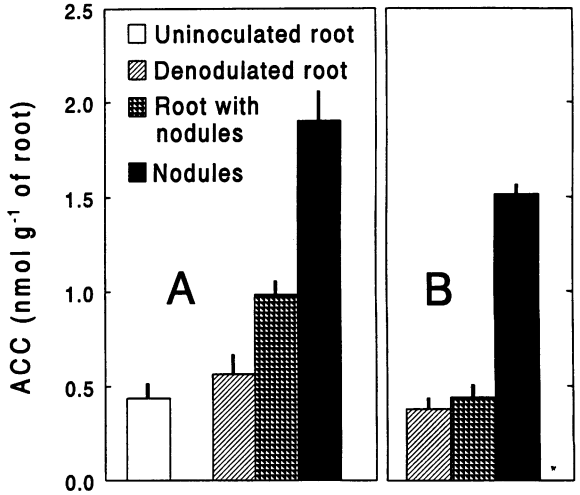

FIG. 2. ACC contents of root and nodule materials. (A) Data for uninoculated plants and for plants that received the highly effective wild-type inoculum. (B) Data for plants that received the ineffective strain 5MT-7 inoculum. Plants were grown for 4 weeks in the growth chamber. The values for roots and denodulated roots are averages ± standard errors of nine measurements. In panel A the value for nodules is the average ± standard error of seven measurements. In panel B, nodule materials from nine plants were pooled to obtain enough mass to make measurements; the value is the average ± standard error of two measurements.

Closed-system ethylene assay. Ethylene production by roots was estimated by incubating freshly harvested material in a sealed Seal-A-Meal (Dazey Corp., Industrial Airport, Kans.) bag containing 50 ml of H_2O and 100 ml of air. Ethylene production by nodules was estimated by incubating freshly harvested material in sealed test tubes. Preparations were incubated in the dark for 30 min at 37°C. Syringe samples were removed, and ethylene content was determined by gas chromatography.

Protein determination. Samples and standards were dissolved in 1 M NaOH at 80°C for 30 min, the base was neutralized with 2.0 M HCl, and protein contents were determined by the Coomassie blue dye-binding method (2, 17, 25).

Root and nodule ACC content. Root and nodule material from greenhouse-grown plants was assayed for 1-aminocyclopropane-1-carboxylic acid (ACC) content by the method of Lizada and Yang (22), with the following modifications: (i) a 1:1 mixture of bleach (Clorox) and saturated NaOH was used, and (ii) ground samples were neither filtered nor concentrated.

Ethylene production by nodulated roots. Plants were grown for 4 weeks in the growth chamber. Root ethylene production was measured with the aid of a flowthrough system that allowed the ethylene produced by the root system to be trapped without disturbing the plant (Fig. 1). The presence of nodules increased the production of ethylene. In three separate experiments uninoculated plants produced 5.3 ± 0.8, 5.6 ± 1.6, and 17.2 ± 3.0 pmol of ethylene h^{-1} g (fresh weight) of plant $material^{-1}$, and nodulated plants produced 48.4 ± 10.6, 14.1 ± 3.6, and 29.4 ± 9.8 pmol of ethylene h^{-1} g^{-1}, respectively. Thus, in each experiment, the nodulated treatment group produced more ethylene than the uninoculated group, and, on average, nodulated plants produced 3.4 times more ethylene than uninoculated plants. Part of this greater ethylene production may have been due to the improved nitrogen nutrition of nitrogen-fixing plants. Alfalfa, corn, and pea plants supplied with nitrogen produce more ethylene than nitrogen-stressed plants do (5, 16, 21), and this study shows that soybean plants behave in a similar manner. In this study uninoculated soybean plants that received no nitrate produced less ethylene than uninoculated plants that received 2.5 mM nitrate (6.4 ± 0.8 versus 19.4 ± 4.2 pmol g of $roots^{-1}$ h^{-1}, respectively). Thus, some of the ethylene produced by nodulated plants is a consequence of improved nitrogen nutrition.

Location of ethylene production. Two studies were conducted to determine the location of ethylene production. One involved the use of a closed system for the estimation of ethylene production. For this study, roots and nodules were separated and placed into separate sealed containers, and the amount of ethylene produced by each preparation was measured. The results indicated that nodules accumulated almost twice as much ethylene per gram (fresh weight) as roots did (198 ± 33 versus 95 ± 27 pmol g of nodule or root $material^{-1}$, respectively). These results must be considered with caution as high levels of ethylene production occur when plants are wounded. For this reason, in a second study I examined the amounts of ACC present in both roots and nodules. In plant systems ACC is a precursor of ethylene (1). Thus, plant materials that contain more ACC are likely to produce more ethylene. Studies with root and nodule materials showed that nodules contain considerably more ACC (and thus would be expected to produce more ethylene) than roots do (Fig. 2A).

TABLE 1. Ethylene production and ACC content of each nodule component[a]

Nodule component	Protein content (mg g of nodules^{-1})[b]	Ethylene production (pmol h^{-1} g of nodules^{-1})[c]	ACC content (pmol g of nodules^{-1})[d]
Bacteroids	4.9 ± 0.1	1.6 ± 0.2	5 ± 2
Cytosol	2.4 ± 0.3	24.8 ± 6.2	528 ± 39
Cell debris	9.8 ± 0.5	27.2 ± 1.6	573 ± 24

[a] The plant material was 6-week-old *G. max* cv. Tracy M plants that were inoculated with the wild-type bacteria. The values for bacteroids are averages ± standard errors of two measurements. All other values are averages ± standard errors of three measurements.

[b] Milligrams of protein in the nodule component/grams of nodule material.

[c] (Picomoles of ethylene produced per hour per milliliter of component × milliliters of component)/grams of nodule material.

[d] (Picomoles of ACC per milliliter of nodule component × milliliters of component)/grams of nodule material.

High levels of ACC were also detected in nodules from plants inoculated with ineffective strain 5MT-7 (Fig. 2B). The increase in ACC observed with nodules from plants inoculated with strain 5MT-7 was not due to improved plant nitrogen nutrition. Strain 5MT-7 nodulates, but the nodules formed fix almost no nitrogen (10). Because of the poor nitrogen fixation abilities of strain 5MT-7, plants inoculated with this strain resemble uninoculated plants. Shoot weights and the ratios of shoots to roots are similar in uninoculated plants and plants that receive strain 5MT-7. Plants that received the wild-type inoculum had significantly higher shoot weights and higher ratios of shoots to roots (data not shown). It is not known whether all ineffective bacteria form nodules that release ethylene. Strain 5MT-7 forms nodules that contain large amounts of 3-indoleacetic acid (10, 11). 3-Indoleacetic acid stimulates ethylene biosynthesis (23).

It is the plant rather than the bacterial portion of the nodule that is responsible for the production of ethylene within the nodule. Nodules were fractionated into their component parts (bacteroids, cytosol, and cell debris) by using differential centrifugation, and a portion of each preparation was incubated in a sealed flask. The results showed that about 97% of the ethylene produced by the nodule components came from the two plant fractions (the cytosol and cell debris fractions). Only very small amounts of ethylene were associated with the bacteroid fractions (Table 1). The highest specific activity for ethylene production (amount of ethylene produced per milligram of protein) was associated with the cytosol fraction. The ACC contents of the nodule components followed the same pattern as ethylene production.

Effect of ethylene on nodulation. Soybean plants were inoculated with *B. japonicum* and grown for 3 weeks in the growth chamber in agar tubes containing 0, 1, 10, 100, or 1,000 μM ethephon (2-chloroethylphosphonic acid), an ethylene-releasing compound that inhibits nodulation in *Pisum sativum* at concentrations of 2 ppm (14 μM) and above (4). The treatment had a dramatic effect on several indicators of plant growth. Shoot length was significantly reduced by 10 to 1,000 μM ethephon (Fig. 3A). Also, internode length and plant wet and dry weights were similarly affected by ethephon (data not shown). The highest ethephon level had a distinct inhibitory effect on root length (Fig. 3A) and lateral root formation (Fig. 3B). The trends observed with ethephon were not greatly influenced by the amount of nitrate N (0.8 and 2.5 mM) supplied to the plants (data not shown). In contrast to what has been observed with other legume

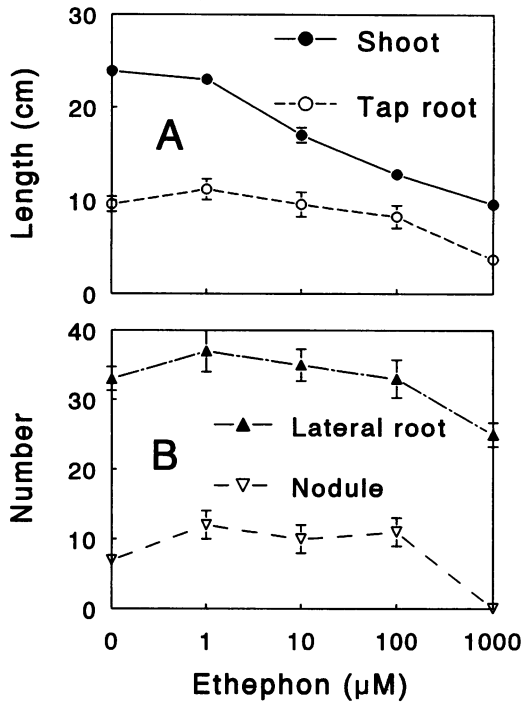

FIG. 3. Effect of ethephon on shoot and root lengths, nodulation, and lateral root number. Plants were grown for 3 weeks in agar tubes in the growth chamber. The values are averages ± standard errors of 20 measurements.

systems, nodule number was not decreased by 1 to 100 μM ethephon. Only the highest concentration (1,000 μM) inhibited nodulation (Fig. 3B). At this level ethephon also severely interfered with normal root and shoot elongation. Aminoethoxyvinylglycine (1 and 10 μM), an inhibitor of ethylene biosynthesis (26, 27) that has been shown to stimulate nodulation in *M. sativa* (19, 24), did not stimulate nodulation in soybeans grown with 0.8 and 2.5 mM nitrate (data not shown).

Conclusions. Previous workers have shown that roots containing *Rhizobium*-induced nodules produce more ethylene than roots without nodules. In this study I extended this observation to include the *Bradyrhizobium*-soybean symbiotic association. This greater production is due in part to the improved nitrogen nutrition of the nodulated plants, but there is also the following evidence that ethylene production by the root nodules is a source of ethylene in nodulated roots: (i) nodules, when removed from the roots and incubated in a closed container, produced more ethylene than the remaining root material did; (ii) nodules contain much more ACC, the precursor of ethylene, than roots do; and (iii) nodulation of the roots with strain 5MT-7 resulted in increased ethylene production (this was true even though this strain is a poor nitrogen fixer). This study also showed that it is the plant portion of the nodules that is responsible for ethylene production by the nodules and that nodulation in the soybean-*B. japonicum* system is not as sensitive to the effects of ethylene as the *Rhizobium* systems that have been investigated in the past are; low and intermediate levels of ethylene do not interfere with nodulation in soybeans.

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