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Gabaculine Inhibition of Chlorophyll Biosynthesis and Nodulation in *Phaseolus lunatus* L.[1]

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ABSTRACT

Gabaculine (3-amino-2,3-dihydrobenzoic acid) was an inhibitor of *in vivo* chlorophyll biosynthesis in lima bean (*Phaseolus lunatus* L. cv Henderson). When applied to roots of 9-day-old plants, 10 micromolar gabaculine was sufficient to terminate biosynthesis of new chlorophyll. The trifoliolate leaves which emerged after gabaculine treatment were yellow. Gabaculine-treated plants had slightly lower dry weights; yet, overall plant size showed very little change. Chlorophyll fluorescence induction kinetics and CO_2 exchange measurements were used to monitor both immediate and long-term effects of gabaculine on photosynthesis. A lowered rate of the decline from the maximum level of fluorescence was observed after 10 hours for nitrate-supplemented plants, and all treated plants showed a slightly increased level of original fluorescence after 6 days. No change was observed in the rate of photosynthesis by unifoliolate leaves. The trifoliolate leaves, though not able to photosynthesize, were able to continue respiration. This suggested that heme biosynthesis for mitochondrial cytochromes was not abolished. In untreated lima bean, root nodules were induced by *Rhizobium* sp. 127E15. Following gabaculine treatment, root nodules formed, but were largely ineffective in nitrogen fixation. Nodule dry weight, nitrogen fixation activity, and leghemoglobin content were decreased by gabaculine.

Gabaculine is a potent, mechanism-based inhibitor of certain pyridoxal phosphate-linked enzymes (5, 15, 17, 23). In plants, it inhibits glutamate-1-semialdehyde aminotransferase (5, 17), thereby blocking the pathway of protoporphyrin biosynthesis (17). Because gabaculine is a potent inhibitor of protoporphyrin synthesis, it represents a useful tool in examining the relationship between photosynthesis and Chl availability. However, its use thus far has been limited to (a) examining the enzymes of the pathway of Chl synthesis (5), (b) studying the relationship between Chl availability and the biosynthesis of Chl-binding proteins (15), and (c) evaluating phytochrome as a product of the Chl biosynthetic pathway (9). Few reports have addressed the long-term effects of gabaculine on the photosynthetic apparatus or processes such as nitrogen fixation which depend upon a continued supply of photosynthate.

The aim of this study was to examine the effect of gabaculine on lima bean. We have asked three specific questions. First, does gabaculine, when administered to plant roots, inhibit Chl biosynthesis in lima bean leaf tissue? Second, what are the long-term physiological responses of the lima bean to gabaculine inhibition? Third, what effect does gabaculine have on the ability of lima bean to form root nodules which are competent in nitrogen fixation? Our results suggest that gabaculine is a potent inhibitor of Chl biosynthesis when applied to the lima bean root system. However, it has very little effect on long-term photosynthetic rates in leaf tissue which was green prior to gabaculine treatment. Furthermore, gabaculine appears to inhibit formation of nodules which are able to effectively fix nitrogen.

MATERIALS AND METHODS

Lima beans (*Phaseolus lunatus* L. cv Henderson) were planted in 900 ml mason jars (2 plants/jar) containing a vermiculite/perlite mixture and grown under conditions which have been described previously (20). Half of the plants were grown in a nutrient solution (20) containing KNO_3 (1.22 g/L), and half without nitrate. After 2 d of growth, all plants were inoculated with *Rhizobium* sp. 127E15 which were obtained and cultured as previously described (20). Gabaculine (3-amino-2,3-dihydrobenzoic acid) was obtained from Fluka Chemical Corp. On the morning of d 9, just prior to the onset of the daylight cycle, 150 ml of aqueous gabaculine (0, 10, 25, 50, or 500 μM) was added to the plants in place of their normal watering. Day 9 was chosen for gabaculine addition since nodulation had begun; yet, nodules were not fully established (2). The unifoliolate leaves were greened, but not fully expanded, and the trifoliolate leaves had not yet emerged. Plants were monitored during the next 16 d and were harvested on d 25.

During the interval between gabaculine application and plant harvesting, two methods were used to monitor the photosynthetic physiology of the unifoliolate leaves. First, the kinetics of Chl fluorescence were measured with a fluorimeter (14) which was modified for intact leaf tissue with fiber optics. An excitation beam from a 300 W projector was passed through a 450 nm broadband interference filter (Detric Optics) and focused onto the leaf (140 $\mu E\ m^{-2}\ s^{-1}$) with fiber optics (Dolar Jenner Industries). Fluorescence was collected by a second fiber optics bundle and monitored by a photomultiplier tube protected with a 680 nm narrow band interference filter. Plants were dark adapted for 30 min prior to fluorescence measurements. Second, *in vivo* rates of photosynthesis and respiration were estimated by measuring whole-leaf CO_2 exchange rates (Analytical Development Company, Ltd. infrared CO_2 gas analyzer). A chamber was placed over a 4.09 cm^2 area of intact leaf. CO_2 exchange rates were monitored for illuminated plants (380 $\mu E\ m^{-2}\ s^{-1}$). Alternatively, plants were placed in the dark for 1 h prior to determining dark respiration rates. Each measurement represents the average of two independent determinations.

The plants were harvested on d 25 and dry weights of roots, stems, leaves, and nodules were determined. Length of roots and stems, leaf areas, internode lengths, and nodule number were measured. Chl content was determined after extraction with 80% acetone (1). Leghemoglobin content of nodules was determined

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both by Western blotting with antibody against leghemoglobin (18, 25) and by difference spectroscopy of nodule extracts for leghemoglobin-heme (3). Heme was quantified using a difference extinction coefficient of 23.4×10^3 M^{-1} cm^{-1} (3). Antibody against leghemoglobin was prepared as previously described (7). The concentration of leghemoglobin on Western blots was determined by densitometry (Kontes Fiber Optic Scanner model 800) and quantified by comparison with blots of isolated leghemoglobin. All absorption measurements were obtained in an SLM/Aminco DW-2C spectrophotometer. Nitrogen fixation activity was determined by acetylene reduction (20).

RESULTS

This study was initiated to ascertain the effect of gabaculine on photosynthesis and nodulation of lima bean. We made a number of predictions based upon the assumption that gabaculine blocks higher plant protoporphyrin biosynthesis. First, if gabaculine can be absorbed by the roots and translocated to the partially expanded unifoliolate leaves, we expected a long-term decline in photosynthesis owing to a block in Chl turnover. We hoped to use the kinetics of Chl fluorescence and CO_2 exchange measurements to map the time course of this decline. Second, if gabaculine can be absorbed by the roots and translocated to the developing portion of the shoot, we expected an inhibition of further development owing to an inhibition of Chl and heme biosynthesis. Third, we expected an inhibition of nodule development owing to (a) a decreased supply of photosynthate to the developing nodule and (b) an interruption of nodule heme biosynthesis. We had already established that gabaculine does not affect the growth of free-living *Rhizobium* sp. 127E15, even at concentrations as high as 500 μM (data not shown). However, root nodules are rich in leghemoglobin and, thus, have a high heme content. The heme moiety is thought to be synthesized by the bacteria (6, 11, 19, 21, 22). However, there is a possibility that the heme is coordinately synthesized by both plant and bacteria (10, 12, 13) or by the plant alone. Therefore, a major goal was to observe the leghemoglobin content of nodules from gabaculine treated plants.

Figure 1 shows 14-d-old plants which were treated with gabaculine and illustrates two important metabolic consequences of gabaculine inhibition. First, shoot growth and development were not influenced by gabaculine. Plant height and emergence of first and second trifoliolate leaves were nearly identical in all treatments. Second, Chl biosynthesis in the leaf tissue was profoundly affected. Unifoliolate leaves did not lose Chl; rather, there was slightly less Chl per leaf area due to further leaf expansion. Newly emerged trifoliolate leaves were chlorotic. Clearly, the inhibitor was effectively absorbed by the roots and translocated to all parts of the shoot tissue. Results shown in Figure 1 imply that shoot growth in treated plants resulted from photosynthate supplied by the unifoliolate leaves and suggest that gabaculine (or cessation of Chl biosynthesis) has minimal long-term effects on photosynthesis in greened leaves.

We monitored both the kinetics of Chl fluorescence induction and the CO_2 uptake rates of photosynthesis after gabaculine treatment. Figure 2 shows the Chl fluorescence induction kinetics immediately after gabaculine addition (0 h) and at two time-points thereafter (17.5 and 158.5 h). Notice four important features of this figure. First, age dependent changes in fluorescence occurred in the absence of gabaculine treatment. In both nitrate-supplemented and deprived plants, aging of unifoliolate leaves yielded a decline in the variable component of fluorescence. Second, there was no immediate effect of gabaculine. Nearly identical fluorescence tracings were observed during the first 0 to 5 h after gabaculine addition regardless of the inhibitor concentration. Indeed, gabaculine concentrations as high as 500 μM showed similar results (data not shown). Third, nitrate-supplemented lima bean (Fig. 2A), but not nitrate-deprived lima bean (Fig. 2B), showed an interesting fluorescence change upon gabaculine treatment. The rate of decline from F_p[2] was diminished by the inhibitor. This parameter of nitrate-supplemented plants began to decrease at 10, 15, and 36 h for the 50, 25, and 10 μM gabaculine treatments, respectively. The difference between nitrate-supplemented and nitrate-deprived plants suggested that gabaculine inhibition depends in part on nutritional status. It is likely that these changes represent metabolic consequences of gabaculine inhibition, rather than mapping the time course of an inhibition of Chl biosynthesis itself. Fourth, a slight

[2] Abbreviations: F_p, maximum extent of fluorescence emission; F, extent of Chl fluorescence emission, measured as described in "Materials and Methods;" F_o, original level of fluorescence emission.

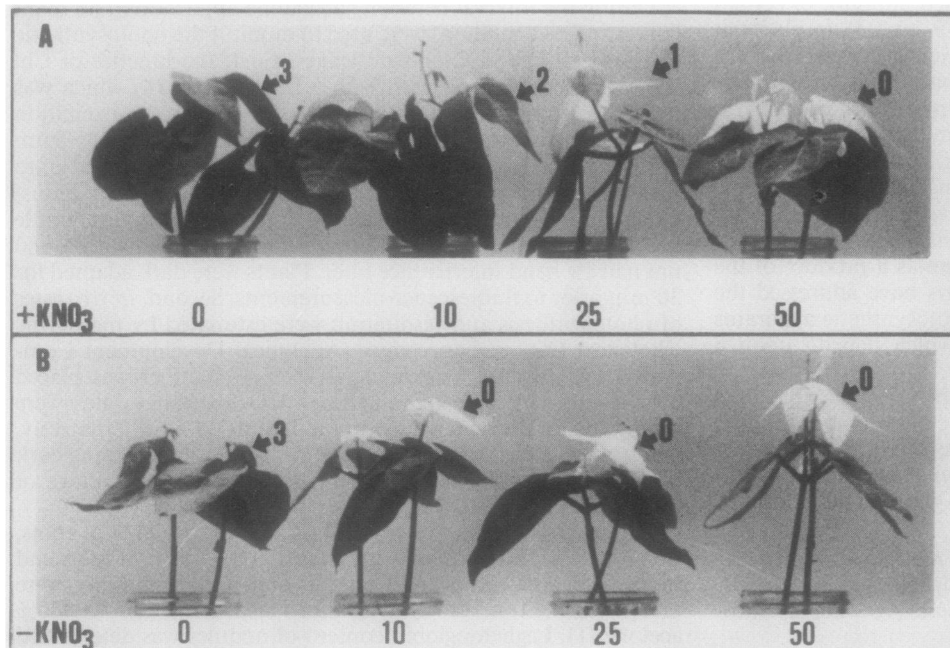

FIG. 1. Lima beans grown in the presence of gabaculine. Gabaculine was added at concentrations of 0, 10, 25, and 50 μM (as indicated below each plant) to nitrate-supplemented plants (A) and to nitrate-deprived plants (B). Trifoliate leaves are denoted by arrows and are rated for relative Chl content on a scale of 0 (yellow) to 3 (green).

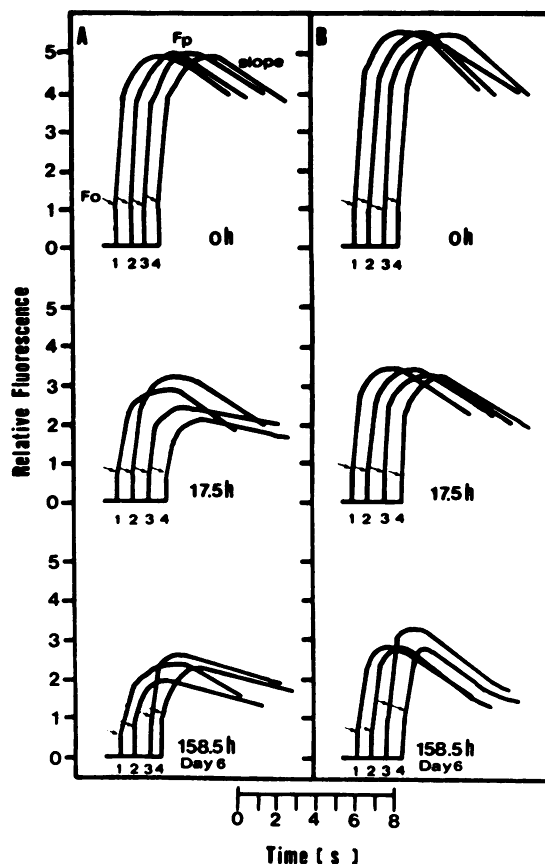

FIG. 2. Chl fluorescence induction kinetics of lima bean unifoliolate leaves. At 0 h, gabaculine was administered to roots of nitrate-supplemented plants (A) and nitrate-deprived plants (B). Additions of 0, 10, 25, or 50 μM gabaculine are indicated by 1, 2, 3, and 4, respectively. Immediately after gabaculine treatment (0 h) and at two subsequent times (17.5 and 158.5 h), plants were dark-adapted for 30 min and the fluorescence induction kinetics were monitored. F_o and F_p denote the level of original fluorescence and the maximum extent of fluorescence, respectively. 'Slope' denotes the decline from F_p.

rise in F_o was observed after 6 d for both nitrate-supplemented and nitrate-deprived lima beans.

Table I shows the CO_2 exchange rates in leaf tissue 2.5 d (unifoliolate leaves) and 18 d (trifoliolate leaves) after gabaculine treatment. The rate of photosynthesis in the unifoliolate leaves was not significantly altered by gabaculine. Equivalent rates were observed from the time of gabaculine treatment until the plants were harvested. However, trifoliolate leaf photosynthesis was not observed in gabaculine-treated plants. These trifoliolate leaves showed a negative CO_2 exchange rate, suggesting that respiration was not abolished by gabaculine treatment. We dark-adapted both untreated and gabaculine treated plants to further examine respiration rates and found no difference between treatments (Table 1).

The plants were harvested at 25 d and various parameters of roots, leaves, and stems were examined. These parameters are quantified in Figure 3. In nitrate-supplemented plants, the dry weights of the root (Fig. 3A), leaf (Fig. 3B), stem (Fig. 3C), and shoot (Fig. 3D) decreased sharply upon addition of 10 μM gabaculine. The Chl content of the unifoliolate leaves was diminished by gabaculine (Fig. 3E). As also shown in Figure 1, Chl was almost completely absent in the trifoliolate leaves (Fig. 3F). The Chl *a*/*b* ratios of unifoliolate leaves was unchanged by the inhibitor (data not shown). We observed similar trends in plants which were nitrate-deprived. In these plants, the dry weights (Fig. 3, A–D) decreased to a lesser extent than the nitrate-supplemented plants. The trifoliolate leaves again showed a large decrease in Chl content (Fig. 3F). The other growth parameters, such as internode number and length, were not significantly different between treatments (data not shown).

All plants in this study were inoculated with *Rhizobium* sp. 127E15. As expected, only the nitrate-deprived plants showed extensive root nodule formation. We were particularly interested in the effects of gabaculine on nodule development. Addition of 10 μM gabaculine to lima bean yielded nodules which were normal sized but had less red pigmentation than untreated plants. These nodules appeared to lack leghemoglobin. Plants treated with 25 μM gabaculine had small nodules which were comparatively whiter. The nodules from 50 μM gabaculine treated lima beans were also small but were more brownish-red in appearance. Figure 4 shows that the nodule dry weight (Fig. 4A) decreased with increasing gabaculine concentration. Nitrogen fixation activity (Fig. 4C) also decreased. The amount of leghemoglobin, measured by Western blots using antibody against the protein moiety (Fig. 4D), was reduced for gabaculine treated plants. The amount of the heme moiety (Fig. 4E) also showed a decrease.

DISCUSSION

Gabaculine is one of a class of potent inhibitors which target pyridoxal phosphate-linked enzymes (23). Many of these inhibitors are mechanism-based in that they require the catalytic environment of a specific active site to irreversibly modify the target enzymes. In the case of gabaculine, a β-proton elimination of the inhibitor yields an aromatic gabaculine derivative (24), which then binds pyridoxal phosphate to form an *m*-carboxyphenylpyridoxamine (23). This product binds so tightly to the active site that it can only be liberated by denaturation. Thus, gabaculine is a highly specific, 'suicide' inhibitor. It has been shown to inhibit γ-aminobutyrate aminotransferase (23, 24), D-amino acid aminotransferase (24), and aspartate aminotransferase (24) from animal and bacterial sources. In plants, gabaculine blocks glutamate-1-semialdehyde aminotransferase, which is crucial in the protoporphyrin biosynthetic pathway (17). However, the ability of gabaculine to block plant mitochondrial heme biosynthesis has been questioned (8, 9). Our aim was to determine if gabaculine, when administered to the root system, would cause inhibition in intact plants. We wanted to quantify the consequences of inhibition both on the photosynthetic parameters of the developing shoot and on the nitrogen fixation capacity of developing root nodules. Our results document a number of effects which gabaculine treatment has on lima bean.

First, extremely low concentrations of gabaculine, administered to roots, are effective in blocking Chl biosynthesis. In nitrate-deprived plants, 10 μM gabaculine induced nearly complete chlorosis of trifoliolate leaves (Fig. 3K). Nitrate-supplemented plants required 25 μM gabaculine for similar results (Fig. 3K). These concentrations are identical to those needed to block Chl accumulation in cyanobacteria (15). Furthermore, Guikema *et al.* (15) also observed that nutrient deficiency caused an increased sensitivity to gabaculine. The cause of this increased sensitivity is not clear. It is possible, for example, that nutrient deficiency enhances the uptake of the inhibitor. It is equally plausible that sensitivity to the inhibitor depends in part on the intracellular pools of metabolites such as glutamate-1-semialdehyde, and that pool sizes are responsive to plant nutrition. If glutamate-1-semialdehyde and gabaculine were to compete for residence at the aminotransferase active site, a nutrition-dependent increase in glutamate-1-semialdehyde would diminish the sensitivity to gabaculine. Nevertheless, our results suggest that gabaculine is a useful chemical in examining the relationship between Chl availability and chloroplast development in whole plants. Leaf emergence and expansion is independent of Chl

Table I. *Photosynthetic and Respiration Measurements for Gabaculine-Treated Lima Bean Plants*

Treatment with Gabaculine	Photosynthetic Rate[a]		Respiration Rate[b]
	Unifoliolate[c]	Trifoliolate[d]	Trifoliolate[d]
μM		*μmolCO$_2$/m^{-2}·s^{-1}*	
+KNO_3			
0 μM	+18.1	+10.1	−3.9
10 μM	+15.0		
25 μM	+15.2		
50 μM	+15.4	−3.5	−3.1
−KNO_3			
0 μM	+17.2	+12.8	−3.6
10 μM	+16.4		
25 μM	+14.7		
50 μM	+18.2	−3.0	−5.3

[a] Positive number represents net photosynthesis. Negative number represents net respiration. [b] Respiration rates were determined after dark adapting plants for 1 h. [c] 2.5 d (52.5 h) after gabaculine addition. [d] 18 d (444 h) after gabaculine addition.

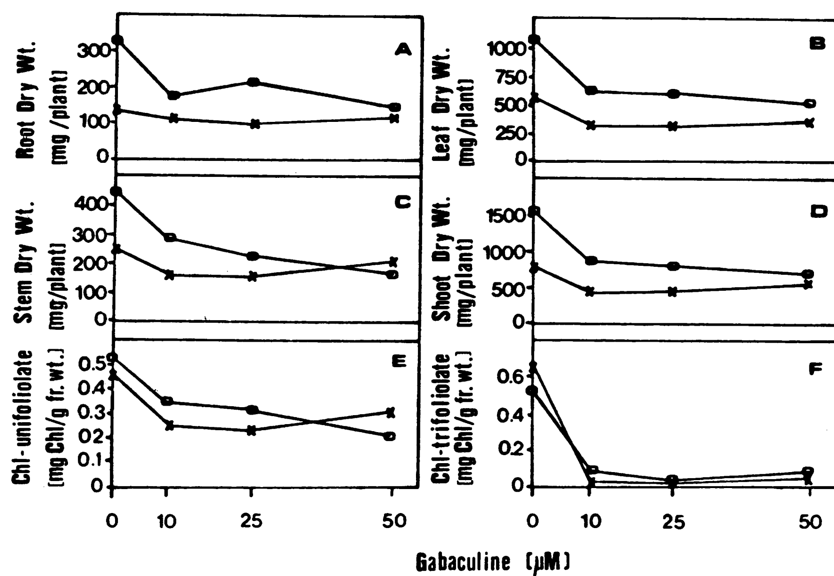

FIG. 3. Effects of gabaculine on the biomass and leaf Chl content of 25-d-old lima bean plants. Root (A), leaf (B), stem (C), and shoot (D) dry weights are expressed in mg per plant. The Chl contents of unifoliolate (E) and the first trifoliolate (F) leaves were examined by spectrophotometric analysis after acetone extraction, and are expressed as mg total Chl per g leaf fresh weight. In all experiments, both nitrate-supplemented (O) and nodulated, nitrate-deprived (×) plants were examined.

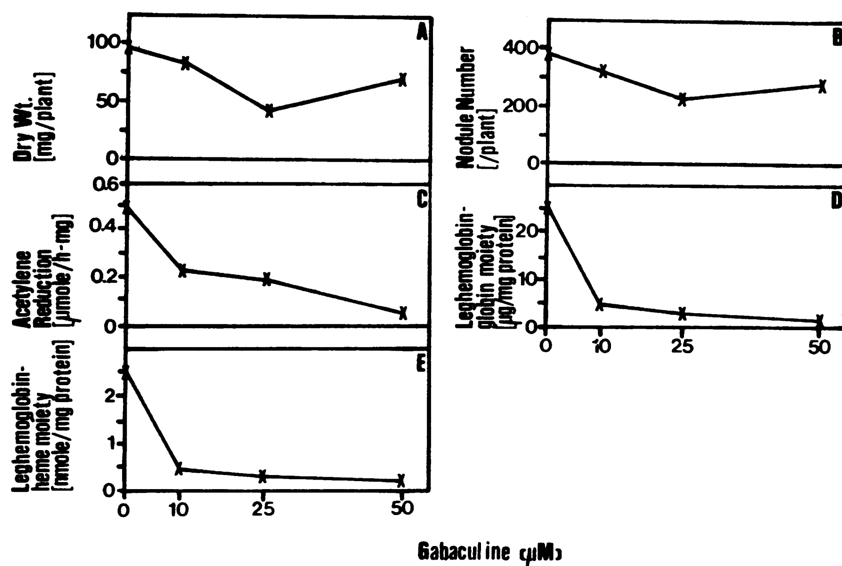

FIG. 4. Effects of gabaculine on the nodulation of lima bean by *Rhizobium* sp. 127E15. Nodule dry weight (A) and the number of nodules (B) are expressed per plant. The acetylene reduction activity supported by these nodules (C) is expressed per mg nodule dry weight. The leghemoglobin content of nodule extracts was determined in two ways: by antibodies specific for the protein moiety (D) and by spectrophotometric assessment of the heme component (E).

content (Fig. 1). It will be of interest to examine chloroplast ultrastructure and membrane protein composition in order to determine the extent to which Chl availability (or presence of Chl precursors) influences these parameters.

Second, during our 25-d observation period, shoot development was not affected by gabaculine (Fig. 1). All of the plants grew to a similar height. Trifoliolate leaves, which had not emerged at the time of gabaculine addition, fully expanded. Furthermore, the unifoliolate leaves remained green after gabaculine treatment. Our results indicated that photosynthetic rates of the trifoliolate leaves, but not unifoliolate leaves, were inhibited by gabaculine (Table 1). Respiration rates of trifoliolate leaves were not altered by gabaculine (Table 1), Although gabaculine inhibits protoporphyrin biosynthesis, these results suggest that synthesis of mitochondrial heme was not completely blocked. Other studies have also suggested that the mitochondria are insensitive to gabaculine (8, 9, 16). Our results cannot distinguish between two possibilities. First, mitochondrial heme may represent such a small percentage of leaf protoporphyrin, that even a minor leak through the gabaculine block may be sufficient to support respiration. Second, the mitochondrial inner membrane may more effectively exclude gabaculine than does the chloroplast envelope. Further experimentation will be required to clarify this point.

Third, gabaculine appears to exert no significant long-term effect (16 d) on photosynthesis. Our results showed that steady state rates of CO_2 gas exchange were not altered in unifoliolate leaves (Table I). There was also no drastic change in Chl fluorescence induction kinetics. We did observe two effects of gabaculine on fluorescence transients. (a) A lower rate of decline from F_p occurred after 10 h. Interestingly, only the nitrate-supplemented lima beans exhibited this change. These plants would have the more active sink demands due to faster growth. Therefore, it is possible that the decline in this kinetic parameter resulted from a change in the sink demand as a result of alterations in carbon fixation (4). (b) Gabaculine induced a rise in F_o after 6 d. This could result from minor changes in the light harvesting apparatus owing to the lack of Chl turnover. This rise also occurs for gabaculine treated cyanobacteria (RL Henry, JA Guikema, unpublished data).

Fourth, gabaculine inhibited the formation of effective root nodules. Nearly equivalent numbers of root nodules were observed in both the untreated and gabaculine inhibited plants (Fig. 4B). However, the nodules of treated plants lost the capacity to fix nitrogen and, therefore, were ineffective (Fig. 4C). Furthermore, leghemoglobin, an important nodule-specific polypeptide, was found in greatly diminished amounts in treated nodules (Fig. 4, D and E). These results suggest that gabaculine interferes with normal nodule development, inhibits the expression of at least one nodule-specific polypeptide, and yields nearly ineffective root nodules.

Two events could account for the synthesis of ineffective nodules. First, gabaculine blocks Chl synthesis in newly developing leaves. This inhibition must certainly result in a lowered photosynthetic potential and, therefore, must yield a diminished photosynthate supply to the developing nodule. It is possible that nodule development is sensitive to the photosynthate supply. A second possibility is more likely, however; gabaculine may target an enzyme within the nodule and thereby yield a nodule which is ineffective in nitrogen fixation. Aspartate aminotransferase and glutamate decarboxylase may possibly be targets of gabaculine. However, the depressed leghemoglobin content in treated plants is especially intriguing and suggests that the ineffective nodules may stem from a block in protoporphyrin biosynthesis. If this were true, then gabaculine would be a useful chemical for examining whether the heme moiety is synthesized solely by the bacteria (6, 11, 19, 21, 22) or co-ordinately by the plant and bacteria (10, 12, 13).

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