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Genetics

Isolation and properties of soybean [*Glycine max* (L.) Merr.] mutants that nodulate in the presence of high nitrate concentrations

(nitrate inhibition/*nts* mutants/nitrate-tolerant symbiosis/ethyl methanesulfonate mutagenesis/nitrogen fixation)

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ABSTRACT **Soybean seeds [*Glycine max* (L.) Merr. cv. Bragg] were mutagenized with ethyl methanesulfonate. The M_2 progeny (i.e., the first generation after mutagenesis) of these seeds were screened for increased nodulation under high nitrate culture conditions. Fifteen independent nitrate-tolerant symbiotic (*nts*) mutants were obtained from 2500 M_2 families. In culture on sand with KNO_3, nodule mass and nodule number in mutant lines were several-fold those of the wild type cultured under the same conditions. Inheritance of the nts character through to subsequent generations was observed in the 10 mutants tested. Mutant *nts*382 also nodulated more than the wild type in the absence of nitrate. Furthermore, nitrate stimulated growth in both the wild type and *nts*382, and these lines had similar nitrate reductase activity. These results indicate that *nts*382 is affected in a nodule-development regulatory gene and not in a gene related to nitrate assimilation.**

Regulation of symbiotic nitrogen fixation in legumes is manifested in a developmental as well as a physiological context. Regulation occurs at all stages of nodule development, affecting nodule initiation, nodule growth, and the onset of nodule senescence (1, 2). Under optimum conditions for plant growth, soil nitrate is a major external factor controlling legume symbioses (3). High nitrate levels suppress the symbiosis, as shown by decreased nodule mass as well as decreased nitrogenase activity per unit of nodule mass. The mechanism of nitrate inhibition is not fully understood (1), but two hypotheses that have received the most support are (*i*) that decreased carbohydrate availability resulting from nitrate reduction and assimilation limits nodule growth and nitrogenase activity (4) and (*ii*) that products of nitrate reduction, particularly nitrite, are responsible. Nitrite, the first intermediate in the nitrate assimilation pathway, is a presumptive candidate for the molecule responsible for nitrate inhibition of nitrogen fixation, since it binds to and inhibits nitrogenase and leghemoglobin (5, 6).

Modern techniques in molecular biology, such as site-directed mutagenesis, have enabled the isolation of many *Rhizobium* mutants that have led to an increased understanding of the heritable attributes of the microsymbiont required for functional symbiosis (7). In contrast, only a few heritable host factors have been reported to influence the extent of symbiosis in legumes (8). The most striking variation has resulted in decreased nodulation or absence of nodulation (8). Nevertheless, variants within existing germ plasms with increased nodulation have been demonstrated in some species (9–15). These promising variants with enhanced nodulation have previously been selected in the absence of nitrate (9, 15).

Consistent with the trend of greater emphasis on bacterial genetics rather than host genetics, attempts to circumvent nitrate inhibition by genetic manipulation of the symbionts have been confined to *Rhizobium* (16–18). Results of these studies have implied that the host genome is responsible for this regulatory phenomenon. However, to date only minor differences in symbiotic tolerance to nitrate have been demonstrated between host species (19) and between cultivars within a species (19, 20).

MATERIALS AND METHODS

Seeds of soybean cv. Bragg were mutagenized with ethyl methanesulfonate ($EtOSO_2Me$), NaN_3, or γ rays. $EtOSO_2Me$ was the most efficient mutagen for generating chlorophyll-deficient mutants (19) and plants from this treatment were used in the subsequent screening. Screening was carried out on the second mutant generation (M_2 progeny).

M_1 seeds were mutagenized with $EtOSO_2Me$ (either at 0.44% for 4 hr or at 0.5% for 6 hr) and planted in the field. A detailed procedure for the mutagenesis can be obtained from the authors. After the crop matured, M_1 plants were harvested separately to give M_2 families.

For *nts* (nitrate-tolerant symbiotic) mutant selection, 12 seeds from each family were planted at 2-cm depth in a pot of river sand (25-cm diameter, 25-cm height) and inoculated with *Rhizobium japonicum* strain CB1809 [equivalent to United States Department of Agriculture (USDA) strain 136]. The plants were cultured in the presence of nitrate for 5–7 weeks, then carefully removed from the sand and visually screened for the extent of nodulation. Pots were initially watered with nutrient solution three times per week; frequency of watering was increased to daily as the demand for water increased with growth. The nutrient solution was the same as that used by Herridge (21), except that all nutrients other than $CaCl_2$ and KNO_3 were administered at one-quarter strength for the first 2 weeks. The nitrate concentration in the nutrient solution was 5 mM throughout. To minimize fluctuations in nitrate levels, the pots were flushed with nutrient solution (1.4 liters) at each watering. Five millimolar KNO_3 was chosen on the basis of a nitrate concentration vs. nodule dry weight profile obtained with the parent cultivar Bragg (Fig. 1). The plants were grown outside during summer and in glasshouses during winter, spring, and autumn. The plants were grown at a latitude of 37° 17′ S with mean summer minimum and maximum temperatures of 12.4°C and 26.9°C, respectively. Glasshouse temperatures were held between 14°C and 30°C. Subsequent generations of *nts* mutants were tested under the conditions described above. However,

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Abbreviation: $EtOSO_2Me$, ethyl methanesulfonate.

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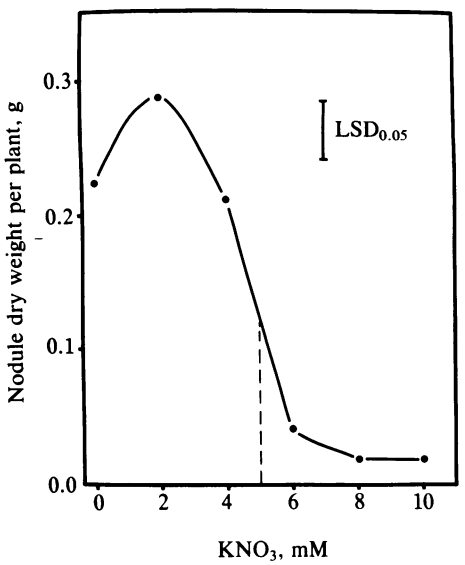

FIG. 1. Effect of nitrate concentration on nodule dry weight in parent cultivar Bragg. Plants were harvested 7 weeks after planting. Inoculant was *R. japonicum* strain CB1809. $LSD_{0.05}$, least significant difference ($P < 0.05$).

smaller pots (20-cm diameter, 20-cm height) were occasionally used instead of the pots described for M_2 screening. As well as verifying the stability of the nts phenotype with *R. japonicum* CB1809 as the inoculant strain, we also tested *R. japonicum* USDA 110 on several of the mutant lines. Purity of *Rhizobium* cultures was verified by strain-specific phage-typing.

The acetylene-reduction assay was used to estimate nitrogen fixation (22). Intact plants were placed in 1040-ml air-tight jars and assayed in 6% acetylene over a 40-min period. Subsequently, seedlings were replanted and grown through to seed. Leaf nitrate reductase activity was measured on nitrate-grown mutant and wild-type plants by use of an *in vivo* assay (minus nitrate) described by Nicholas *et al.* (23).

RESULTS

When 2500 families (≈25,000 M_2 seedlings) were screened for nodulation on nitrate, 15 of these families segregated the nts phenotype. These variants had significantly increased nodulation in the presence of 5 mM KNO_3 compared with wild-type siblings and the parent cultivar (Table 1). Expression of the nts character was not confined to mutant plants inoculated with *R. japonicum* CB1809 (= USDA 136). *R. japonicum* USDA 110 also elicited the nts phenotype in the 6 mutants so far tested (see Table 1). Stable inheritance of the nts character has been demonstrated through to at least the M_3 generation in the 10 mutant families listed in Table 1. Many other selections showed marginally increased nodulation, but these will not be further discussed.

Nodulation of *nts* Mutants. Table 1 shows nodule number for *nts* mutants and wild type cultured on 5 mM KNO_3 for 5–7 weeks. Nodule number per plant was substantially higher in the mutants than in the wild type, as was nodule fresh weight. After 6–9 weeks culture on 5 mM KNO_3, *nts* mutants had 5–20 times the nodule mass of wild-type plants (Table 2).

Families *nts*382 and *nts*1116 were studied in more detail than any of the other mutant families. These two mutants were compared with Bragg for nodulation in the presence and absence of nitrate. Not only did *nts*1116 and *nts*382 nodulate more than the wild type during culture on nitrate (Table 1), but there was also a striking difference between these mutants and the wild type in the absence of nitrate (Fig. 2, Table 3).

Table 1. Nodule number for *nts* mutants and wild-type plants cultured on 5 mM KNO_3 for 5–8 weeks

Selected family	Nodules per plant (mean ± SD)	
	nts mutants	Wild type
382*	146 ± 71	26 ± 11
1007*	179 ± 39	13 ± 4
1116*†	79 ± 60	19 ± 7‡
246†	115 ± 74	8 ± 2
733*	213 ± 177	18 ± 10
183*	269 ± 70	19 ± 8
97§	120	32 ± 7
501*§	251	19 ± 8
2062	233 ± 45	34 ± 19
2264†	409 ± 148	12 ± 6‡

Unless noted, the data are for M_2 plants and wild type refers to wild-type siblings of respective *nts* mutants. The inoculant strain for these data was *R. japonicum* CB1809 (= USDA 136); however, *R. japonicum* USDA 110 also elicited the nts phenotype in the six mutant lines so far tested (see * below).

*The nts phenotype also was elicited when *R. japonicum* USDA 110 was used as the inoculant strain.

†Data for M_3 plants.

‡Parent cultivar Bragg.

§Data for *nts* mutants from one plant.

After 45 days culture without nitrate, *nts*1116 plants had 5 times as many nodules as parent cultivar Bragg; with nitrate present, there were 9 times as many nodules on *nts*1116 plants (Fig. 2*A*). Two-way analysis of variance of the data in Fig. 2*A* showed that there was a significant genotype effect but no significant effect of nitrate on nodule number per plant for *nts*1116 or for Bragg. Consistent with reports in the literature (2), we found that nodule growth is more sensitive to nitrate inhibition than is nodule initiation in wild-type soybean. Wild-type plants grown on nitrate had only 40% of the nodule mass of N_2-dependent wild-type plants (Fig. 2*B*). Nodule mass was consistently higher in *nts*1116 than in Bragg (Fig. 2*B*). In the absence of nitrate, *nts*1116 plants had 2.5 times the nodule mass of Bragg plants, and culture on nitrate accentuated the difference, with *nts*1116 having 9 times the nodule mass of the wild type (Fig. 2*B*). In contrast to Bragg, supplementing the nutrient solution with 2.5 mM KNO_3 throughout the 45 days of culture did not inhibit nodule growth per plant in *nts*1116 (Fig. 2*B*).

A similar trend was seen for *nts*382 (Table 3). Winter-grown Bragg and *nts*382 plants were harvested at 31 and 64 days after planting. In the early stages of nodule develop-

Table 2. Nodule fresh weight for *nts* mutants and wild-type plants cultured on 5 mM KNO_3 for 6–9 weeks

Selected family	Nodule fresh weight per plant, mg (mean ± SD)	
	nts mutants	Wild type
1007	1196 ± 377	62 ± 25*
246†	1537 ± 647	198 ± 134
733	1086 ± 349	86 ± 53
183	1715 ± 411	187 ± 91*
501	445 ± 252	64 ± 35
2062‡	1929 ± 391	176 ± 118*
2264	941 ± 307	86 ± 53

Unless noted, the data are for M_3 plants and wild type refers to parent cultivar Bragg. The inoculant strain was *R. japonicum* CB1809 (= USDA 136).

*Non-*nts* siblings of respective mutants were used as wild-type controls.

†‡Data from M_4 and M_2 plants, respectively.

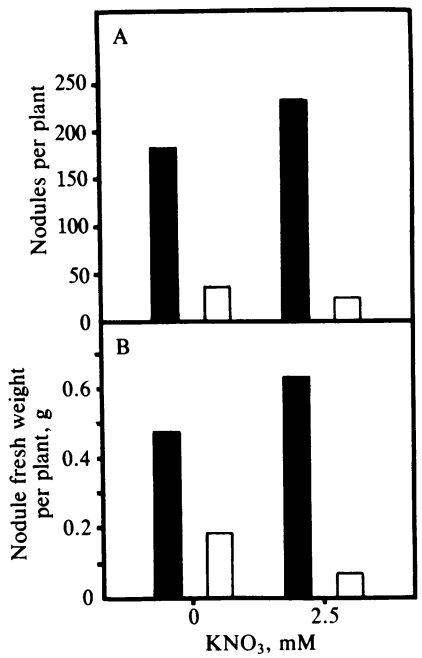

FIG. 2. Nodule development in *nts*1116 (shaded bars) and wild-type (Bragg, unshaded bars) plants grown in the presence or absence of 2.5 mM KNO_3. Nodule number (*A*) and nodule fresh weight (*B*) are expressed per plant. The inoculant strain was *R. japonicum* CB1809 (= USDA 136) and the pots (20-cm diameter, 20-cm height) were watered daily with nutrient solution. Plants were harvested 45 days after planting. Each column represents the mean for 4–14 plants.

ment, *nts*382 plants had a significantly higher nodule number and nodule fresh weight than did wild-type plants. Irrespective of nitrate supplementation, considerable differences in nodulation were also observed 64 days after planting. In the absence of nitrate, *nts*382 had 6 times the nodule number and twice the nodule fresh weight of the wild type and, as was the case for *nts*1116, administering nitrate during growth increased the difference in nodulation between *nts*382 and Bragg. Fig. 3 shows the root systems of *nts*382 and wild-type Bragg plants that had been cultured in the absence (5.5 mM KCl control) or presence of 5.5 mM KNO_3 for 4 weeks. Fig. 3 clearly illustrates the striking enhancement in nodulation observed in this mutant.

Table 3. Plant fresh weight and nodule development in *nts*382 in presence or absence of 5.5 mM nitrate

Days after planting	Line	KNO_3, mM	Plant fresh weight, g	Nodules per plant	Nodule fresh weight per plant, mg
31*	*nts*382	0	2.31†	95‡	215‡
	Bragg	0	2.17	3	8
	*nts*382	5.5	3.64	35	74
	Bragg	5.5	3.12	2	4
64§	*nts*382	0	11.8¶	431‡	1583‡
	Bragg	0	21.1	69	886
	*nts*382	5.5	28.5	414	1886
	Bragg	5.5	40.5	29	174

The inoculant strain was *R. japonicum* USDA 110 and the pots were watered daily with nutrient solution. Plants were harvested at 31 and 64 days after planting. Each entry for 31-day-old plants represents the mean for 4 or 5 plants, whereas for the older plants each entry is the mean for 9–28 plants. The effects of plant genotype and nitrate treatment for the different aged plants were analyzed by using two-way analysis of variance.

*Plants were at the primary leaf/first trifoliolate leaf stage of development.

†Significant nitrate-treatment effect on 31-day-old plants.

‡Nitrate treatment, genotype, and interaction effects were significant.

§N_2-dependent plants had four to five fully open trifoliolate leaves, whereas nitrate-fed plants had six to nine trifoliolate leaves.

¶Significant nitrate-treatment and genotype effects on 64-day-old plants.

Acetylene-Reduction Activity for *nts* Mutants. Four weeks after planting and culture without nitrate, *nts*382 had approximately the same nitrogenase activity per plant as the parent cultivar Bragg (Table 4). In contrast, *nts*382 plants cultured on 2.75 mM KNO_3 had 10 times the nitrogenase activity of wild-type plants cultured under identical conditions. Similar results were obtained for other mutants cultured on 5 mM nitrate. For example, 9-week-old *nts*1116 plants had 7 times the activity of the wild type. In other experiments, *nts*2264 and *nts*1007 plants had 8 times the nitrogenase activity of wild-type plants (Table 4).

As stated above, N_2-dependent *nts*382 plants had a greater nodule mass than did the wild-type controls. This implies that the equality of N_2-dependent *nts*382 and wild-type plants in reducing acetylene (Table 4) resulted from decreased specific activity of nitrogenase (activity per unit of nodule mass) in the mutant. Indeed, in 64-day-old plants grown without nitrate, nitrogenase specific activity for *nts*382 plants was 81.9 ± 16.3 (±SD) nmol of C_2H_4 produced per g of nodule fresh weight per min, compared with 338.9 ± 48.4 for wild-type Bragg

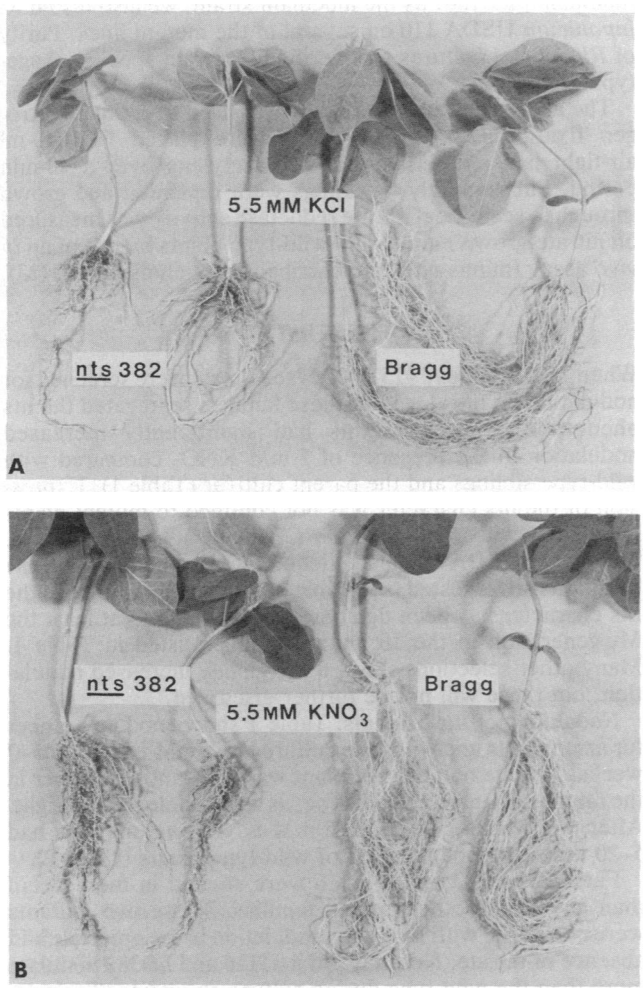

FIG. 3. Bragg and *nts*382 plants after 4 weeks of culture without KNO_3 (5.5 mM KCl control) (*A*) or with 5.5 mM KNO_3 (*B*). Inoculant was *R. japonicum* strain CB1809.

Table 4. Nitrogenase activity of mutant and wild-type plants

nts mutant	KNO_3, mM	Nitrogenase activity, nmol C_2H_4 per plant per min		LSD* ($P < 0.05$)
		Mutant	Wild type	
382†	0	17.0	19.2	NS‡
	2.75	34.2 (3.5)	3.4 (1.2)	−(0.8)§
1116¶	5	143.7	21.4	39.0
2264¶	5	171.9	20.5	71.6
1007¶	5	90.1	10.9‖	21.3

Mutant (M_3 or M_4 generation) and wild-type plants were compared after culture in the presence of nitrate; for *nts*382, a comparison also was made in the absence of nitrate. Nitrogenase (acetylene reduction) activity is expressed per plant. Unless otherwise noted, wild type refers to parent cultivar Bragg. The inoculant strain was *R. japonicum* CB1809 (= USDA 136). Each entry for nitrogenase activity in the table is the mean for 4–9 plants.

*Least significant difference test.

†Harvested after 4-wk culture in sand pots watered daily with nutrient solution.

‡Mutant and wild-type plants were not significantly different.

§Raw data required $\log_e$ transformation to satisfy assumptions for an analysis of variance; means and LSD of transformed data are shown in parentheses.

¶Harvested after culture for 9 wk (for 1116 experiment), 8 wk (for 2264 experiment), or 7 wk (for 1007 experiment) in sand pots watered three times a week with 5 mM KNO_3.

‖Non-nts 1007 siblings (wild-type phenotype) were used for comparison.

(Fig. 4). Nitrogenase specific activity of *nts*382 plants that received 5.5 mM KNO_3 throughout growth was 105.7 ± 39.6 compared with 154.7 ± 43.6 for Bragg. Clearly, the addition of 5.5 mM KNO_3 to the nutrient solution did not further decrease nitrogenase specific activity in the mutant (Fig. 4).

Growth of *nts* Mutants. Fig. 3 illustrates that prolific nodulation in *nts*382 may have occurred to the detriment of root growth. Indeed, plants displaying the *nts* phenotype were generally smaller than wild-type non-*nts* siblings and parent cultivar Bragg. The composite data of selected M_2 *nts* mutants showed that at the time of screening (≈6 weeks after planting) the mutants were on average 84% as tall as wild-type siblings. This trend was also prominent in subsequent generations. Reduced growth of the mutants was reflected in plant fresh weight, and a more detailed description of this effect is presented in Table 3 for *nts*382. Under glasshouse conditions, plant genotype had no effect on plant fresh-weight accumulation after 31 days of growth. In contrast to the younger plants, however, a significant genotype effect on plant fresh weight had become apparent after 64 days of growth; *nts*382 plants had significantly smaller plant fresh weights than did wild-type Bragg plants. Therefore, *nts*382 grew as well as the parent cultivar during early plant development, but as plant growth continued, prolific nodulation was correlated with decreased plant fresh-weight accumulation. This trend may not hold true for mutant *nts*2264.

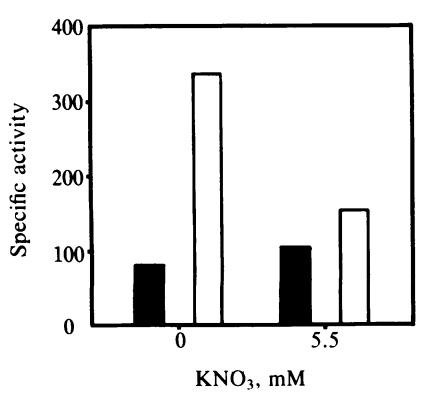

FIG. 4. Nitrogenase specific activity (nmol of C_2H_4 produced per g of nodule fresh weight per min) of Bragg (unshaded bars) and *nts*382 (shaded bars) plants. Plants were cultured without KNO_3 or with 5.5 mM KNO_3 for 64 days prior to harvest. Plants were inoculated with *R. japonicum* strain USDA 110. Each column represents the mean of 5–14 plants. Two-way analysis of variance indicated that genotype, nitrate treatment, and interaction effects were highly significant.

Evidence for Nitrate Assimilation in *nts*382. *In vivo* leaf nitrate reductase activity was the same in nitrate-grown *nts*382 and Bragg plants 18 and 64 days after planting. Nitrate reductase activity in the primary leaf of 18-day-old plants was 14.3 ± 3.0 (±SD) and 16.6 ± 7.2 μmol of nitrite per g of plant fresh weight per hr for *nts*382 and Bragg, respectively.

When the nutrient solution was supplemented with nitrate, plant fresh-weight accumulation was stimulated in *nts*382 as well as in Bragg (Table 3). This stimulation was observed at both 31 and 64 days after planting. Clearly, *nts*382 plants can take up, reduce, and assimilate nitrate.

DISCUSSION

Despite the importance of the root system of plants, the literature is almost devoid of reports detailing mutations that affect root development (24). Our study has demonstrated that *nts* mutants can be isolated from mutagenized soybean populations. The frequency of *nts* mutants correlated well with the frequency of chlorophyll-deficient mutants in the two $EtOSO_2Me$-mutagenized populations used here.

Inheritance of the nts character from one soybean generation to the next has been demonstrated in the 10 selected lines so far tested. All selected lines came from M_2 families that segregated the nts phenotype, indicating that the mutations were a result of the mutagenesis program. Indeed, each of the 15 selections came from independent mutation events, since M_2 families were maintained separately. Preliminary phenotypic grouping indicates that there are three or perhaps four different types of mutants.

Mutant *nts* plants were generally smaller than wild-type siblings or parent cultivar Bragg. However, we observed considerable variation between mutants, both in the degree of supernodulation and in total plant growth. There appears to be an inverse relation between these two broad phenotypes. Some marginal *nts* mutants (not described here) showed up to 40% increased leaf area compared to wild type, while still exhibiting a marginal nts phenotype. This genetic diversity or even altered genetic constitutions (i.e., double mutants or heterozygotes) may modulate the correlation between reduced growth and supernodulation and aid the agronomic application of this mutant phenotype.

Theoretically, enhanced nodulation in the presence of nitrate can result from the plant's inability to either (*i*) utilize nitrate or (*ii*) regulate nodule initiation and/or nodule growth in the normal manner. Mutants *nts*382 and *nts*1116 fall into the latter category. In addition to supernodulation in the presence of nitrate, both of these mutants showed enhanced nodulation in the absence of nitrate. For example, under nitrate-free conditions, 64-day-old *nts*382 plants and 45-day-old *nts*1116 plants had 6 and 5 times as many nodules as the respective Bragg controls. Furthermore, there is convincing evidence that *nts*382 can utilize nitrate. Nitrate stimulated plant fresh-weight accumulation in *nts*382 to a similar extent as it did in Bragg (Table 3), and *nts*382 and Bragg had similar leaf nitrate reductase activities. These results showed that *nts*382 and *nts*1116 are mutants in a nodule-development regulatory gene(s) and not in a gene directly associated with nitrate metabolism.

Nitrate represents a major environmental factor that controls nodulation in legumes. Besides such external regulatory factors, nodulation is internally regulated by a process that has been termed autoregulation. Nodules that are effectively fixing nitrogen "signal" younger root tissues to restrict further nodule development. Removal of nodules or infection by a nonfixing *Rhizobium* removes this nodulation inhibition (25–27). Autoregulation is also prevalent before the establishment of nitrogen-fixing nodules. Pierce and Bauer (28) showed that an initial inoculation of soybeans, several hours prior to a second inoculation, reduced the capacity of the latter to induce nodule formation. Similarly, nodule formation on one half of a soybean root system is inhibited by prior inoculation of the other half prior to the onset of nitrogen fixation (29). The results reported here suggest that *nts*382 and *nts*1116 may be mutants in the autoregulation pathway.

As a consequence of a larger nodule mass, nitrate-grown *nts* mutants had higher nitrogenase activity per plant than did wild-type controls. Depending on the selected line and the age at harvest, *nts* mutants had 8–9 times the acetylene reduction activity of the wild type (Table 4). In the absence of nitrate, *nts*382 had the same activity as wild-type plants. This result reflected decreased specific activity (activity per unit of nodule mass) of nitrogenase in the mutant (Fig. 4). The control of nodule initiation and nodule growth is separate from nodule functioning in *nts*382, and these prolifically nodulating plants cannot support optimum levels of specific nitrogenase activity.

Specific nitrogenase activity by wild-type soybeans was lower in nitrate-grown plants than in N_2-dependent plants (Fig. 4). This was not the case in *nts*382 plants. In this mutant, nitrate treatment did not further depress nitrogenase specific activity. The specific activity of nitrogenase in *nts*382 plants from both growth conditions approximated the specific activity in nitrate-inhibited wild-type plants. However, it has not yet been established whether the factor(s) limiting nodule function is the same in *nts*382 nodules and nitrate-inhibited wild-type nodules.

The *nts* mutants reported here are of immense academic interest and will be useful in ascertaining the mechanisms of nitrate inhibition and of regulation of nodulation in general. They (in conjunction with other symbiotically altered soybean mutants that we obtained) indicate that an induced mutagenesis and large scale screening program can yield valuable material. Mutants *nts*382 and *nts*1116 are very similar to an $EtOSO_2Me$-induced pea mutant designated nod_3 that has enhanced nodulation in the presence of nitrate (30, 31). From an agronomic viewpoint, the potential value of nitrate-tolerant-symbioses in legumes has been described recently (32). These include increased residual nitrogen and improved soil structure following the soybean crop, enhanced establishment of introduced inoculant strains, decreased need for nitrate fertilization, and improved nitrogen status of the soybean crop. This large set of independent *nts* mutants of soybean should help to confirm or deny these possible benefits.

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