Plant Physiol. 78, 34–40
0032-0889/85/78/0034/07/$01.00/0

A Supernodulation and Nitrate-Tolerant Symbiotic (*nts*) Soybean Mutant[1]

Received for publication August 24, 1984 and in revised form January 4, 1985

BERNARD J. CARROLL, DAVID L. MCNEIL[2], AND PETER M. GRESSHOFF*
Department of Botany, Australian National University, Canberra, ACT 2600, Australia

ABSTRACT

The nodulation characteristics of soybean (*Glycine max*) mutant *nts*382 are described. The mutant nodulated significantly more than the parent cultivar Bragg in the presence and absence of several combined nitrogen sources (KNO_3, urea, NH_4Cl, and NH_4NO_3). The number of nodules on the tap root and on lateral roots was increased in the mutant line. In the presence of KNO_3 and urea, nitrogenase activity was considerably higher in *nts*382 than in Bragg. Mutant plants were generally smaller than wild-type plants. Although *nts*382 is a supernodulator, inoculation with *Rhizobium japonicum* was necessary to induce nodule formation and both trial strains CB1809 (= USDA136) and USDA110 elicited the mutant phenotype. Segregation of M_3 progeny derived from a M_2 wild-type plant indicated that the mutant character is inherited as a Mendelian recessive. The mutant is discussed in the context of regulation of nodulation and of hypotheses that have been proposed to explain nitrate inhibition of nodulation.

Nodule formation in legumes is tightly regulated. Indeed, symbiotic development is subject to both external factors and internal (or 'autoregulation') control mechanisms. Several environmental conditions, such as light intensity (photosynthate- or non-photosynthate mediated), temperature, pH, and soil moisture, influence nodulation (19). However, under optimum conditions for plant growth, exogenous nitrate represents a major environmental factor controlling the extent of symbiosis (5, 13). Small amounts of nitrate have been demonstrated to stimulate nodulation. Above these minute concentrations, however, nodule fresh weight is inversely related to the level of nitrate in the growth medium (20). To a lesser extent, other sources of combined nitrogen have also been shown to inhibit nodulation (7, 8, 10, 24, 27). The extent of this inhibition does vary considerably with the legume species, the form of combined nitrogen administered, and the experimental system. Some sources of combined nitrogen, for example urea or NH_4^+, cause acidification of the growth medium (14), and the inhibitory effects of these nitrogen sources may be mediated indirectly through a reduction in pH rather than through the nitrogen status of the plant. To circumvent this complication, Vigue *et al.* (30) controlled pH fluctuations by the inclusion of a pH-buffering carboxy resin in the pots (17). In this system using soybeans, nitrate but not urea suppressed nodule fresh weight per plant at the range of concentrations tested. This was partially explained by reduced uptake of nitrogen in urea-fed plants. In contrast to the effect of nitrate and urea on nodule mass, rates of acetylene reduction per unit of nodule mass were similar for nitrate and urea treatments (30).

In the absence of externally supplied combined nitrogen, nodulation is tightly regulated with the number of infections greatly exceeding the final number of mature nodules (2). Interruption of invasion is related to the effectiveness of the host-*Rhizobium* association (21) as well as to other internal factors not directly related to the nitrogen status of the plant (23). Generally, ineffective strains of *Rhizobium* form more nodules, especially after the initial stages of nodule formation (21). Nutman also showed that excision of effective (but not ineffective) red clover nodules resulted in a transient increase in the number of nodules subsequently formed (22). In fact, removal of the nodule meristem of effective nodules was sufficient to stimulate subsequent nodule development. The effect of nodule meristem excision on nitrogenase activity was not considered, but the argument that an inhibitory factor emanating from the growing point, and not the bacterial tissue, of the nodule was supported by the finding that excision of the main root tip had the same effect as nodule meristem removal. Clear evidence for internal regulation or autoregulation independent of nitrogen fixation and in the early stages of nodule initiation was reported by Pierce and Bauer (23). Using the spot inoculation technique (26), they showed that inoculation of soybean roots with *R. japonicum* several hours prior to a second inoculation substantially reduced nodulation by the second inoculation. In a split-root system for soybeans, Kosslak and Bohlool (16) showed that autoregulation prior to nodule appearance and nitrogenase activity was not restricted to root tissue immediately adjacent to the inoculated area, since prior inoculation of one side of a split-root suppressed nodulation on the other side (16). Studies by Calvert *et al.* (4) characterized this rapid regulatory phenomenon (23) further and showed that suppression of nodulation due to prior inoculation is mediated through suppression of nodule emergence rather than by inhibition of root hair infection. Clearly, the pending fruition of an infection is subject to internal (or auto-) regulation that exists both prior and subsequent to nitrogen fixation.

Recently, we isolated 15 independent soybean mutants that continued to nodulate in the presence of nitrate (6). These lines were designated *nts* (nitrate-tolerant-symbiosis) mutants. Previously, only four nodulation mutants of soybean had been discovered and all of these naturally occurring mutants are characterized by decreased nitrogenase activity. Soybean plants homozygous for the recessive mutation rj_1 rj_1 are resistant to nodulation; however, the blockage can be partially circumvented by inoculation with high *R. japonicum* cell densities (18). Rj_2, Rj_3, *and* Rj_4 are strain-specific dominant mutations that condition ineffective nodulation (3, 28, 29).

In this paper we report the nodulation characteristics of mutant line *nts*382. The effects of various nitrogen sources on nodulation, nitrogen fixation, and plant growth are described, as well

[1] Supported by an Agrigenetics sponsored research program and an Australian Government Postgraduate Award to B. J. Carroll.

[2] Present address: Western Australia Department of Agriculture, Kununurra Research Station, Kununurra, Western Australia, Australia.

as the influence of two *R. japonicum* strains on the expression of the supernodulation phenotype. Data are presented that indicate that the mutant character is inherited as a Mendelian recessive.

MATERIALS AND METHODS

Soybean (*Glycine max* [L.] Merr.) cv Bragg was used in this study. Mutant line *nts*382 was selected for increased nodulation in the presence of high nitrate concentrations. The isolation procedure has been described in detail (6) and is summarized in Figure 1. M_4 plants of mutant line *nts*382 were used here. Plants were cultured in either pots of river sand or in Leonard jars (9).

In experiments testing the effect of various nitrogen sources on nodulation and N_2 (C_2H_2) fixation, plants were cultured in 20 cm diameter pots of sand. Three Bragg or *nts*382 seeds were planted 1 cm below the surface and inoculated with *Rhizobium japonicum* strain CB1809 (= USDA136). The pots were reinoculated at day 4. The nutrient solution was as used by Herridge (12), except that all nutrients other than $CaCl_2$ and the nitrogen source were administered at quarter strength for the first 2 weeks. KNO_3 (2.75 and 5.5 mM), urea (5.5 mM, *i.e.* 11 mM N), NH_4Cl (5.5 mM), NH_4NO_3 (5.5 mM, *i.e.* 11 mM N) and KCl (as control) were added to the nutrient solution as required. The pots were watered daily with 700 ml of nutrient solution, which was sufficient to flush out residual nutrients from the previous watering.

M_2 family 382 was one of 15 families that segregated for the *nts* phenotype (Fig. 1). Both *nts* variants and wild-type (non-*nts*) siblings were saved to produce M_3 families (*i.e.* families derived from single M_2 plants). Those M_3 families derived from wild-type M_2 plants were screened for segregation of the *nts* character (Fig. 1). Seeds were planted in 25 cm diameter pots of river sand (12 seeds per pot). The pots were inoculated with *R. japonicum* strain USDA110 at day 0 and day 4. Nutrients were administered as described above, except that all pots received 1.4 L of nutrient solution 3 times a week. After 7 weeks of growth, the plants were screened for the *nts* character.

To control access of *R. japonicum* strains to the roots (9), Bragg and *nts*382 were cultured in Leonard jars. Seeds were surface sterilized by rinsing in 95% ethanol followed by immersion of the seeds for 10 min in 3% NaOCl. After several rinses in sterile distilled H_2O, the seeds were transferred to water agar and germinated at 28°C in the dark. When the radical was approximately 0.5 to 1 cm long (2–3 d after sterilization), the seedlings were transferred to Leonard jars (N-free or supplemented with KNO_3 or urea). The jars either remained uninoculated or were inoculated with *R. japonicum* strains CB1809 or USDA110.

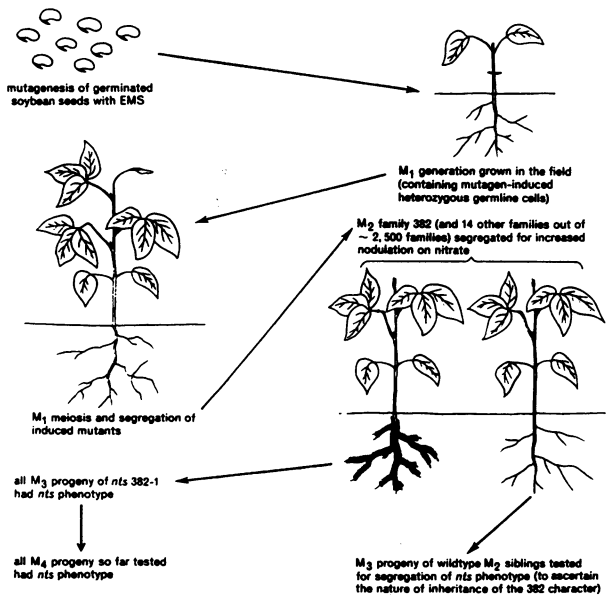

FIG. 1. Schematic representation of the isolation of *nts*382 from mutagenized Bragg soybeans.

The plants were cultured in a temperature-controlled glasshouse (mean maximum temperature = 26.9°C; mean minimum temperature = 14.8°C) at a latitude of 37° 17′S. Incandescent bulbs supplemented natural light such that the photoperiod was 16 h. At harvest, the plants were measured for nodule number, nodule fresh weight, nitrogenase activity, and plant fresh weight.

Acetylene reduction was used to estimate nitrogen fixation on intact plants (11). Plants were incubated in 1040 ml air-tight jars with 2 to 3 ml of distilled H_2O (to prevent desiccation) at 25 to 27°C. The atmosphere in the jars was 6% acetylene in air. Rates were calculated from integrator units obtained from a Hewlett-Packard Integrator-Recorder 3390 coupled to a HP5590A flame ionization gas chromatograph. Samples were taken at 40 min and rates were determined to be linear over that period. Genotype and combined nitrogen effects were statistically tested by analysis of variance using the general statistical program Genstat (1). The LSD was computed when the F statistic was significant (0.05 level of significant). Chi-square analysis was used to statistically test segregation ratios. It was necessary to include Yates correction term in chi-square calculations due to the size of the expected classes (25).

RESULTS

Effect of Combined Nitrogen on Nodulation, Nitrogenase Activity, and Growth. Bragg and *nts*382 were inoculated with *R. japonicum* CB1809 and cultured for 4 weeks in the presence and absence of various combined nitrogen sources. The nitrogen-free treatment received 5.5 mM KCl as a control.

Nodule Number. Under all the conditions tested, 4-week-old *nts*382 plants had considerably more nodules than wild-type Bragg plants (Fig. 2). In the parent cultivar, all nitrogen sources reduced nodule number (Figs. 2 and 3). In contrast, mutant line *nts*382 grown on KNO_3 or urea had increased nodule number per plant over that of the KCl controls (Fig. 2). When data for nodule number are expressed per plant fresh weight, nodule number in *nts*382 was unaffected by increasing KNO_3 concentration (Fig. 3). Urea (5.5 mM), on the other hand, caused a small reduction in nodule number per plant biomass in the mutant. The relative degree of inhibition of nodule formation by urea was greater in Bragg than in *nts*382 (Fig. 3). Mutant line *nts*382 formed nodules on 5.5 mM NH_4Cl and NH_4NO_3, which totally prevented nodule formation in Bragg (Figs. 2 and 3). Ammonium chloride inhibited growth to a larger extent than did ammonium nitrate (Table I); however, NH_4NO_3 was more inhibitory than NH_4Cl on nodule formation in *nts*382 (Fig. 3).

Nitrogenase (Acetylene Reduction) Activity per Plant Biomass. In Bragg, supplementing the nutrient media with nitrate or urea caused a substantial reduction in nitrogenase activity measurable 4 weeks after planting (Fig. 4). In contrast, 2.75 mM KNO_3 significantly stimulated acetylene reduction per gram plant fresh weight in *nts*382. Nitrogenase activity for *nts*382 plants cultured on 5.5 mM KNO_3 was not significantly different from the nitrogen-free mutant plants (Fig. 4). For both Bragg and *nts*382, urea was more inhibitory than equimolar KNO_3 concentrations. Regardless of the combined nitrogen supply, *nts*382 had higher nitrogenase activity than Bragg, but the difference under N-free conditions was not significant at the 0.05 level of significance. Nitrogenase activity per plant fresh weight in 4-week-old *nts*382 plants cultured on 2.75 and 5.5 mM KNO_3 was significantly higher than for Bragg plants cultured in the absence of nitrogen

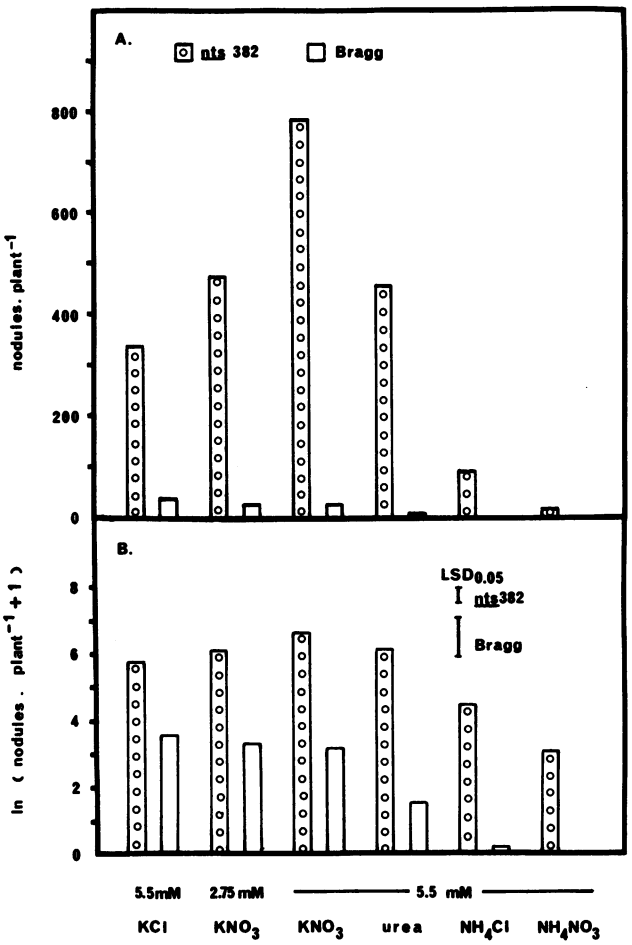

FIG. 2. Effect of nitrogen source on nodule number per plant in Bragg and *nts*382 plants. Plants were cultured and harvested as in Table I. Raw data (panel A) required log$_e$ transformation (panel B) to satisfy assumptions for an analysis of variance. Each column represents the mean of four to eight plants. Genotype, combined nitrogen, and interaction effects were significant.

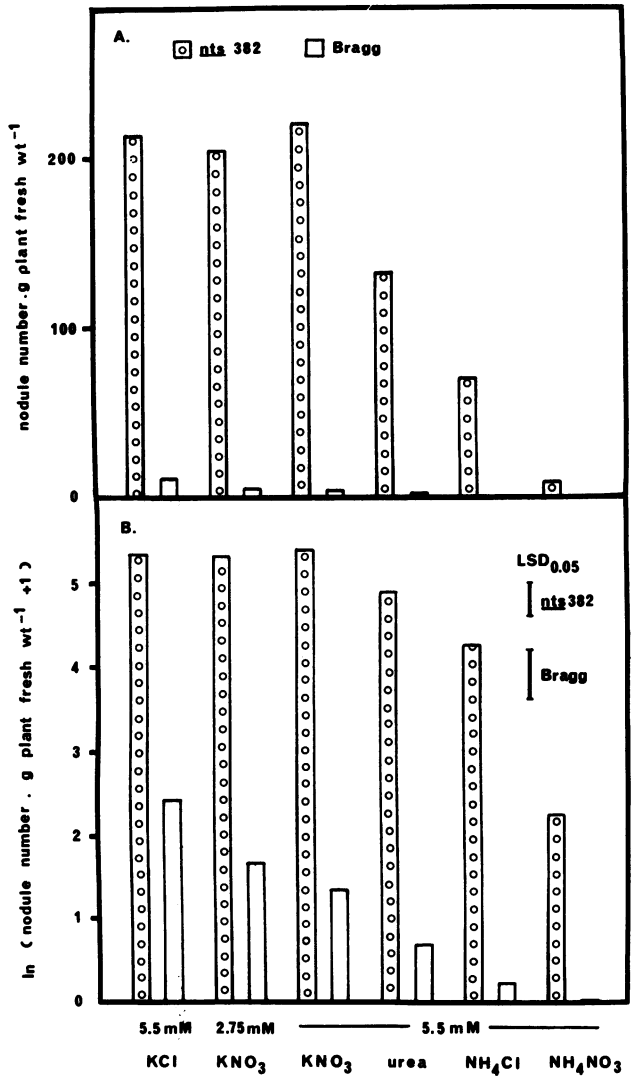

FIG. 3. Effect of nitrogen source on nodule number per plant biomass in Bragg and *nts*382. Plants were cultured and harvested as in Table I. Raw data (panel A) required log$_e$ transformation (panel B) to satisfy assumptions for an analysis of variance. Each column represents the mean of four to eight plants. Genotype, combined nitrogen, and interaction effects were significant.

(Fig. 4).

Visual Characteristics of Nodulation by nts382. Figure 5 illustrates a sample of plants that contributed to the data presented above. The photographs indicated that prolific nodulation in *nts*382 may occur at the expense of root growth. Mutant line *nts*382 continued to nodulate prolifically throughout development. Figure 6 shows the tap root of a *nts*382 plant harvested at the podfill stage of development.

Plant Fresh Weight. Nitrate (both concentrations) and urea treatments produced healthy vigorous plants and stimulated growth of Bragg and *nts*382 over that of the respective KCl controls (Table I). Regardless of genotype, plants cultured on 5.5 mM NH_4Cl and NH_4NO_3 were unhealthy and stunted. Ammonium nitrate produced no effect on plant fresh weight after 4 weeks of culture, whereas NH_4Cl inhibited fresh weight accumulation in both Bragg and *nts*382. Plant fresh weight was consistently higher in Bragg than in *nts*382 (Table I).

Axenic Culture in Leonard Jars. The observations obtained from pots inoculated with *R. japonicum* strain CB1809 and described above were confirmed in Leonard jars supplemented with KNO_3 and urea. Both *R. japonicum* stains tested, namely CB1809 and USDA110, elicited the *nts* phenotype in mutant line 382. Uninoculated controls of *nts*382 and Bragg did not nodulate.

Nodulation Pattern. Table II shows the tap root nodulation pattern of N_2-dependent *nts*382 and Bragg plants. Tap root length was less in nodulated *nts*382 plants than in Bragg plants cultured under identical conditions (Table II). The tap root nodulation interval is defined as the distance between the uppermost and lowermost nodule on the tap root. This parameter was larger in *nts*382, both in absolute terms (2 times that of Bragg) and when expressed as a percentage of tap root length (3 times that of Bragg). Nodule density (nodules/cm) was also increased in *nts*382 (Table II). Nodule density, expressed on the total tap root length was 9 times higher in *nts*382 than in Bragg. Within the nodulation interval, the nodule density was 2.5 times higher in the mutant line (Table II). A similar contrast in nodulation pattern was observed on lateral roots and, furthermore, on *nts*382 plants cultured on various nitrogen sources (Fig. 5).

Inheritance of the *nts*382 Character. Following mutagenesis on M_1 seeds, resultant M_1 plants were grown through to produce M_2 seeds. Seeds from each M_1 plant were grouped together to give M_2 families. Mutant line *nts*382 was selected from a M_2 family that segregated for the mutant phenotype (Fig. 1). From 17 plants in M_2 family 382, two expressed the *nts* phenotype and

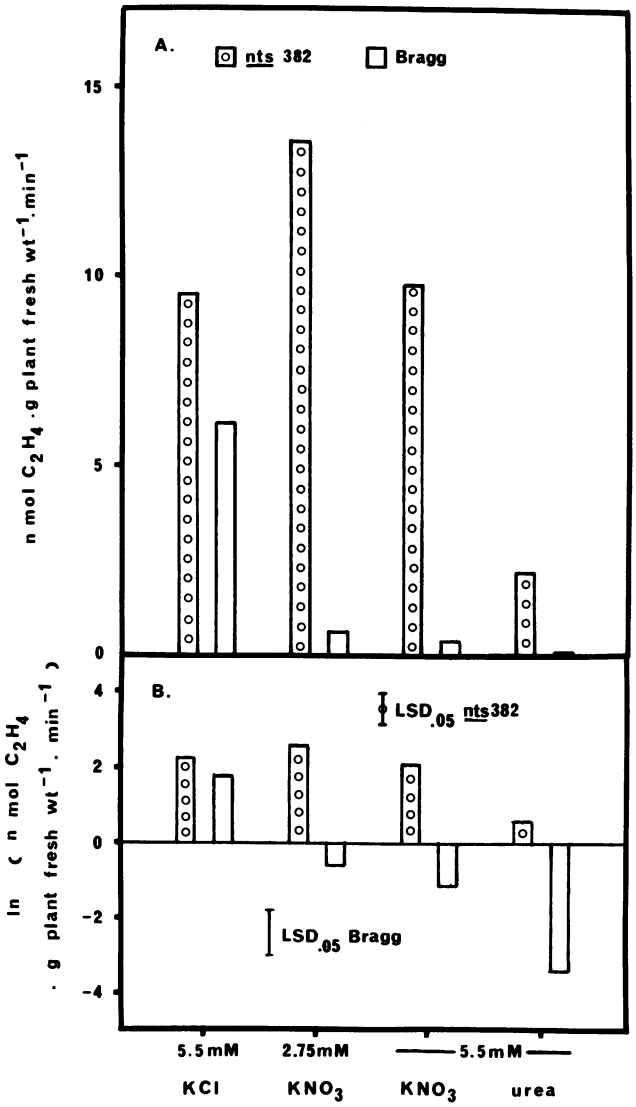

FIG. 4. Effect of potassium nitrate and urea on nitrogenase (acetylene reduction) activity in Bragg and *nts*382 plants. Plants were cultured and harvested as in Table I. Raw data (panel A) required $\log_e$ transformation (panel B) to satisfy assumptions for an analysis of variance. Each column represents the mean of four to eight plants. Genotype, combined nitrogen, and interaction effects were significant.

FIG. 5. Effect of potassium nitrate and urea on nodulation in Bragg (a) and *nts*382 (b) plants. Plants were cultured and harvested as in Table I.

the remaining 15 plants expressed the wild-type phenotype and were indistinguishable from the parent cultivar Bragg for nodulation in the presence of nitrate. In the M_2 screen, plants were harvested 6 weeks after planting and culture on 5 mM KNO_3. The nodule per plant for M_2 *nts* segregants was 146 ± 71 (± SD) and for M_2 wild-type (non-*nts*) segregants it was 26 ± 11. Bragg plants cultured under identical conditions had 19 ± 7 nodules per plant.

M_3 progeny derived from M_2 *nts* plants all expressed the mutant character indicating that these M_2 *nts* plants were homozygous for the mutation. Similarly, all M_4 progeny (used in experiments described above) had the *nts* phenotype.

M_2 wild-type (non-*nts*) segregants were also repotted and grown through to seed. The seeds derived from each M_2 plant were grouped together to give M_3 families. One of six such M_3 families segregated 14 *nts* plants:40 wild-type plants. This ratio closely approximates 1:3, the expected segregation ratio for a recessive character in progeny derived from a self-fertilized heterozygous wild-type plant (chi-square was equal to 0.00 and was not significant).

Mutant *nts* plants in the segregating M_3 family qualitatively had a similar phenotype to the original M_2 *nts* selections, and to the M_3 and M_4 progeny derived therefrom. Nodule number per plant for these nitrate-grown *nts* segregants was 789 ± 181 (± SD), whereas wild-type segregants had 32 ± 19 nodules per plant. Furthermore, plant fresh weight was lower in *nts* segregants; 8.23 ± 2.33 (± SD) g per plant compared to 15.19 ± 3.20 g per plant for wild-type segregants.

After 7 weeks culture on 5 mM KNO_3, *nts* segregants had 23 times the nodule fresh weight per plant biomass of wild-type segregants (Table III). Increased nodulation in *nts* plants was observed on the tap root as well as on the lateral roots (Table III); this trend was consistent with the nodulation pattern shown in Figure 5 for *nts*382 grown under a variety of combined nitrogen regimes. As a result of the increased nodule mass, nitrate-grown *nts* segregants had 7 times the nitrogenase (acetylene reduction) activity per plant biomass of nitrate-grown wild-type segregants (Table III).

DISCUSSION

Mutant line *nts*382 was one of several *nts* (nitrate-tolerant-symbiosis) mutants selected from an M_2 population of parent cultivar Bragg for increased nodulation in the presence of nitrate (6). Individual M_2 families (resulting from a single mutagenized M_1 seed) were originally screened. M_2 family 382 segregated for the *nts* character (Fig. 1), as did all of the M_2 *nts* mutant families.

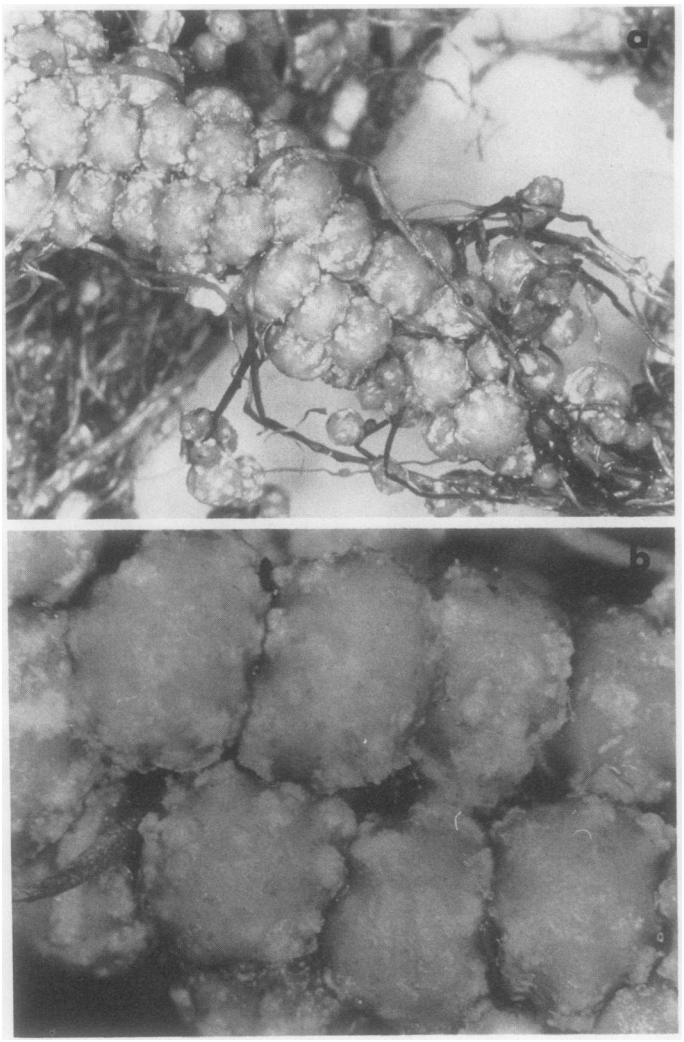

FIG. 6. Nodulation of *nts*382 at the podfill stage of development. a, The tap root of a *nts*382 plant that had been cultured in a 25-cm pot of vermiculite. This plant had received slow-release nitrogen fertilizer throughout growth. b, Close-up of Figure 6a.

Table I. *Plant Fresh Weight of Bragg and nts382 Plants Cultured for 4 Weeks on Various Nitrogen Sources*

The plants were grown in 20-cm sand pots as described in "Materials and Methods."

Nitrogen source	Plant Fresh Wt[a]	
	Bragg	*nts*382
	g	
0 mM (5.5 mM KCl)	3.24 (100)[b]	1.96 (100)[b]
2.75 mM KNO_3	5.61 (173)	2.37 (121)
5.5 mM KNO_3	8.05 (248)	3.68 (188)
5.5 mM urea	4.80 (148)	3.51 (179)
5.5 mM NH_4Cl	2.12 (65)	1.27 (65)
5.5 mM NH_4NO_3	3.04 (94)	2.18 (111)

[a] $LSD_{0.05}$ = 1.56 for Bragg and 1.08 for *nts*382. [b] Plant fresh weight as per cent of 5.5 mM KCl control.

This observation indicated that each of the mutant families arose from independent mutation events and that the mutants were a result of the mutagenesis program (6). M_2 *nts*382 selections were homozygous for the mutation, since all M_3 progeny derived thereof had the *nts* phenotype, as did all M_4 progeny, including the plants used in some experiments described here. M_3 progeny

Table II. *Tap Root Nodulation Pattern of N_2-Dependent nts382 and Bragg Plants*

Plants were inoculated with *R. japonicum* USDA110 and harvested 9 weeks after planting. The pots were watered daily with N-free nutrient solution throughout the experiment.

Tap Root Parameter	*nts*382[a]	Bragg[b]	$LSD_{0.05}$
Root length (cm)	16.6	28.1	3.1
Nodulation interval (cm)	14.4	7.6	2.1
Nodulation interval (% of root length)	86.5	27.1	6.7
Nodule density on root length (nodules·cm^{-1})	4.32 (2.0)[c]	0.47 (0.67)	—(0.15)
Nodule density on nodulation interval (nodules·cm^{-1})	5.07	2.10	0.99

[a] Each entry in the table for *nts*382 represents the mean of seven plants. [b] Each entry in the table for Bragg represents the mean of 28 plants. [c] Raw data required square-root transformation to satisfy assumptions for an analysis of variance; means and LSD of transformed data are shown in parentheses.

Table III. *Nodulation and Nitrogenase (Acetylene Reduction) Activity in KNO_3-Grown nts and Wild-Type Segregants from a nts382 M_3 Family*

This family was derived from a wild-type M_2 plant. The segregation ratio was 14 *nts*:40 wild type, which approximates 1:3 (chi-square = 0.00 and was not significant). Plants were inoculated with *R. japonicum* USDA110 and harvested after 7 weeks growth on 5 mM KNO_3. Data are expressed per g plant fresh weight.

Symbiotic Parameter	*nts*382[a]	Wild-type[b]	$LSD_{0.05}$
Nodule number·g plant fresh wt^{-1}			
on tap root	9.6 (2.22)[c]	0.7 (−0.45)	(0.20)
on lateral roots	89.2 (9.4)[d]	1.5 (1.1)	(0.4)
mg nodule fresh wt·g plant fresh wt^{-1}	145.3 (12.0)[d]	6.2 (2.5)	(0.5)
nmol C_2H_4·g plant fresh $wt^{-1}\cdot min^{-1}$	8.0 (2.05)[c]	1.1 (0.00)	(0.76)

[a,b] Each entry in the table is the mean of 14 and 18 plants, for *nts* and wild-type, respectively, except that acetylene reduction data are the means of seven *nts* and nine wild-type plants. [c,d] Raw data required either $\log_e$ (c) or square-root (d) transformation to satisfy assumptions for an analysis of variance; means and LSD of transformed data are shown in parentheses.

of a wild-type M_2 plant segregated 3 wild type:1 mutant, indicating that the *nts*382 character is inherited as a Mendelian recessive.

Regardless of the presence or absence of combined nitrogen, *nts*382 nodulated more than the parent cultivar Bragg. This trend was consistent over a range of combined nitrogen sources that caused varying degrees of inhibition of nodulation in Bragg. For example, under conducive conditions for nodulation, 4-week-old N_2-dependent *nts*382 plants had 9 times the nodule number of N_2-dependent Bragg plants. Similarly, under conditions that totally prevented nodulation in Bragg (5.5 mM NH_4Cl or NH_4NO_3), *nts*382 plants were still nodulated. The mutant line also had increased nodulation in Leonard jars, in deep soil pots (soil obtained from soybean field at Breeza, NSW, Australia) and in Georgia (USA) fieldplots. Nodule initiation and nodule growth are coordinated in *nts*382, and *nts*382 plants have a considerably larger nodule mass than wild-type plants (6; Table III). We use the term supernodulator to describe *nts*382, since this soybean

genotype has an increased nodule mass under a wide range of environmental conditions.

Although *nts*382 is a supernodulator, it is not a constitutive nodulator, since it still requires the inducer (*i.e. R. japonicum*) to be present. The two strains of *R. japonicum* used, namely CB1809 and USDA110, elicited the *nts* phenotype in the mutant line. Both these strains form an effective symbiosis with the parent cultivar. A wider spectrum of fast- and slow-growing *R. japonicum* strains, that vary in their ability to nodulate the parent cultivar, were tested on *nts*382 to ascertain whether the *nts* character confers a change in the promiscuity of the host plant. Those strains that nodulated Bragg elicited the mutant phenotype on *nts*382, and strains that were nod$^-$ on the parent cultivar were also unable to induce nodule formation on *nts*382.

In Bragg, all nitrogen sources caused a significant reduction in the symbiotic parameters that were measured (Figs. 2–5). In contrast, enriching the nutrient solution with KNO_3 did not inhibit any symbiotic parameters in *nts*382 and was in some cases stimulatory (Figs. 2 and 4). Urea marginally stimulated nodule number per plant but significantly inhibited nodule number and nitrogenase activity per gram plant fresh weight in the mutant line. Consistent with the comparative effects of KNO_3 and urea on *nts*382, urea was also more severe on symbiotic development in Bragg (Figs. 2–4). This is different to what Vigue *et al.* (30) found with soybean cultivar Steele. Using a carboxy resin to buffer the culture media (17), they demonstrated that nitrate was more inhibitory on nodulation than was urea. To minimize the drop in pH associated with urea and ammonium utilization (14), the pots in our experiments were flushed daily with 700 ml of nutrient solution. This procedure, without a specific buffering agent, produced healthy vigorous urea-fed plants that had significantly stimulated fresh weights in Bragg and *nts*382 (Table I). Nevertheless, it is unclear whether pH fluctuations played a role in inhibition of the symbiosis by urea, NH_4Cl, and NH_4NO_3. This, however, is not a contentious issue, since the salient feature of *nts*382 illustrated by these experiments is that it consistently nodulated prolifically in comparison to parent cultivar Bragg, regardless of the environmental conditions imposed by the provision of various nitrogen sources.

Since prolific nodulation by *nts*382 was not confined to culture on nitrate, it is unlikely that increased nodulation on nitrate resulted from an inability to utilize nitrate. KNO_3 enhanced the growth of *nts*382 plants (Table I) and, furthermore, *nts*382 has the same nitrate reductase activity as Bragg (6). Indeed, *nts*382 is a mutant in the regulation of nodule initiation and nodule development, and the autoregulation mechanism normally limiting nodulation in wild-type soybeans (23) is anomolous. The nodulation interval is extended and nodule density is increased in *nts*382 (Table II; Fig. 5). Although nodule emergence and subsequent nodule growth are coordinately controlled in *nts*382, specific nitrogenase activity (activity/g nodule fresh weight) is not coordinately regulated with these two nodule development parameters in the mutant (6). The fact that *nts*382 plants have increased nitrogenase activity per plant biomass under adverse conditions for symbiotic development is a result of increased nodule mass.

In *nts*382 mutant plants, regulation of nodulation by environmental conditions (such as by nitrate supply) is coordinated with internal (or auto-) regulation. The supernodulation observed in *nts*382 under N-free conditions persisted when combined nitrogen was added to the nutrient media. The increased tolerance of *nts*382 to nitrate and other combined nitrogen sources was seen in an accentuation in the magnitude of difference between *nts*382 and Bragg as nitrogen sources were added to the nutrient solution (Table IV). For example, in the absence of combined nitrogen *nts*382 plants had 9 times as many nodules as Bragg plants (Table IV). As combined nitrogen was added to the nutrient solution, the degree of inhibition in Bragg was not seen in *nts*382 and for plants cultured on 5.5 mM urea, for example, *nts*382 had 65 times as many nodules per plant as did Bragg. A similar trend was evident for all the other symbiotic parameters measured (Table IV). Clearly, *nts*382 is a mutant in the autoregulation pathway and is also less sensitive to regulation by external conditions. Recently, a pea mutant was described to have similar nodulation characteristics (15). Pea mutant *nod*$_3$ nodulated more than the parent cultivar in the absence of nitrate and also nodulation was less affected by nitrate in the mutant (15).

In addition to being useful in studying regulation of nodule development in general, *nts*382 is of immense value for critically analyzing and refining some hypotheses that have been put forward to explain nitrate inhibition of nodule development. The carbohydrate deprivation hypothesis argues that nitrate reduction and assimilation deprives the nodule of carbohydrate necessary for growth and development. The alternative hypothesis that has also received support is that products on nitrate reduction, particularly nitrite, are responsible for nitrate inhibition (6). The nodulation characterstics of *nts*382 and Bragg indicate that mass flow carbohydrate deprivation or products of nitrate reduction cannot directly cause inhibition of nodule development. Growth of *nts*382 was stimulated by potassium nitrate (Table I) and *nts*382 had normal nitrate reductase activity (6) indicating the mutant did utilize nitrate and yet nodule development was not inhibited early in plant development (0–4 weeks after planting). Carbohydrate deprivation or products of nitrate reduction may inhibit nodule development in wild type, but it must be mediated through the control mechanism that is anomolous in *nts*382. Further detailed studies on nodulation and nitrate metabolism of *nts*382 and related mutants are continuing.

Table IV. *Symbiotic Parameters of nts382 Expressed as a Proportion of Bragg*

Each datum in the table was computed by dividing the value of the symbiotic parameter for *nts*382 by the value for Bragg cultured under identical conditions (*i.e. nts*382 ÷ Bragg). Nitrogen sources are listed in order of increasing severity on nodulation in Bragg. Plants were harvested 4 weeks after planting. The inoculant strain was *R. japonicum* USDA110.

Nitrogen Source	Symbiotic Parameter[a]		
	Nodules /plant[b]	Nodules/g plant fresh wt[c]	Nitrogenase activity[d]
0 mM (5.5 mM KCl)	9	21	1.6
2.75 mM KNO_3	18	51	22.6
5.5 mM KNO_3	31	73	29.5
5.5 mM urea	65	132	36.0
5.5 mM NH_4Cl[e]	∞	∞	—
5.5 mM NH_4NO_3[e]	∞	∞	—

[a] *nts*382 ÷ Bragg. [b,c,d] Data from Figures 2, 3, and 4, respectively. [e] NH_4Cl and NH_4NO_3 totally prevented nodule formation in Bragg.

Acknowledgments—Dr. David Herridge is thanked for discussion. Kirsten Cowley, Jan Bateman, Angela Higgins, Andrew Carter, and George Serbov are acknowledged for technical help. Josephine Roper is thanked for her skillful typing.

LITERATURE CITED

1. ALVEY N, C BANFIELD, R BAXTER, J GOWER, W KRZANOWSKI, P LANE, P LEECH, J NELDER, R PAYNE, K PHELPS, C ROGERS, G ROSS, H SIMPSON, A TODD, R WEDDERBURN, G WILKINSON 1977 Genstat. A general statistical program. Lawes Agricultural Trust, Rothamsted Experimental Station
2. BAUER WD 1981 Infection of legumes by rhizobia. Annu Rev Plant Physiol 32: 407–449
3. CALDWELL BE 1966 Inheritance of a strain-specific ineffective nodulation in soybeans. Crop Sci 6: 427–428
4. CALVERT HE, MK PENCE, M PIERCE, NSA MALIK, WD BAUER 1984 Anatomical analysis of the development and distribution of *Rhizobium* infections in soybeans roots. Can J Bot 62: 2375–2384

5. CARROLL BJ, PM GRESSHOFF 1983 Nitrate inhibition of nodulation and nitrogen fixation in white clover. Z Pflanzenphysiol 110: 77–88
6. CARROLL BJ, DL MCNEIL, PM GRESSHOFF 1985 Isolation and properties of novel soybean (*Glycine max* (L). Merr) mutants that nodulate in the presence of high nitrate concentrations. Proc Natl Acad Sci USA. In press
7. DARBYSHIRE JF 1966 Studies on the physiology of nodule formation. IX. The influence of combined nitrogen, glucose, light intensity and day length on root hair infection in clover. Ann Bot 30: 623–638
8. DART PJ, DC WILDON 1970 Nodulation and nitrogen fixation by *Vigna sinensis* and *Vicia atropurpurea*: the influence of concentration, form, and site of application of combined nitrogen. Aust J Agric Res 21: 45–46
9. GIBSON AH 1980 Methods for legumes in glasshouses and controlled environment cabinets. *In* FJ Bergersen, ed, Methods for Evaluating Biological Nitrogen Fixation. J Wiley and Sons, Brisbane, pp 139–184
10. GIBSON AH, PS NUMAN 1960 Studies on the physiology of nodule formation. VII. A reappraisal of the effect of preplanting. Ann Bot 24: 420–433
11. HARDY RWF, RD HOLSTEN, EK JACKSON, RC BURNS 1968 The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol 43: 1185–1207
12. HERRIDGE DF 1977 Carbon and nitrogen nutrition of two annual legumes. PhD thesis. University of Western Australia, Perth, Australia
13. HERRIDGE DF 1982 Use of the ureide technique to describe the nitrogen economy of field-grown soybeans. Plant Physiol 70: 7–11
14. ISRAEL DW, WA JACKSON 1982 Ion balance, uptake, and transport processes in N_2-fixing and nitrate- and urea-dependent soybean plants. Plant Physiol 69: 171–178
15. JACOBSEN E, WJ FEENSTRA 1984 A new pea mutant with efficient nodulation in the presence of nitrate. Plant Sci Lett 33: 337–344
16. KOSSLAK RM, BB BOHLOOL 1984 Suppression of nodule development of one side of a split-root system of soybeans caused by prior inoculation of the other side. Plant Physiol 75: 125–130
17. LAHAV E, JE HARPER, RH HAGEMAN 1976 Improved soybean growth in urea with pH buffered by a carboxy resin. Crop Sci 16: 325–328
18. LA FAVRE JS, ARJ EAGLESHAM 1984 Increased nodulation of "non-nodulating' (rj_1 rj_1) soybeans by high dose inoculation. Plant Soil 80: 297–300
19. LIE TA 1974 Environmental effects on nodulation and symbiotic nitrogen fixation. *In* A Quispel, ed, The Biology of Nitrogen Fixation. North-Holland Publishing Company, Amsterdam, pp 555–582
20. MCNEIL DL, TA LARUE 1984 Effect of nitrogen source on ureides in soybeans. Plant Physiol 74: 227–232
21. NUTMAN PS 1949 Physiological studies on nodule formation. II. The influence of delayed inoculation on the rate of nodulation in red clover. Ann Bot 13: 261–283
22. NUTMAN PS 1952 Studies on the physiology of nodule formation. III. Experiments on the excision of root-tips and nodules. Ann Bot 16: 79–101
23. PIERCE M, WD BAUER 1983 A rapid regulatory response governing nodulation in soybean. Plant Physiol 73: 286–290
25. RICHARDSON DA, DC JORDAN, EH GARRARD 1957 The influence of combined nitrogen on nodulation and nitrogen fixation by *Rhizobium meliloti* Dangeard. Can J Plant Sci 37: 205–214
25. STRICKBERGER MW 1976 Genetics, Ed 2. Macmillan Publishing Co, New York, p 146
26. TURGEON BG, WD BAUER 1983 Spot inoculation of soybean roots with *Rhizobium japonicum*. Protoplasma 115: 122–128
27. VAN SCHREVEN DA 1959 Effects of added sugars and nitrogen on nodulation of legumes. Plant Soil 11: 93–112
28. VEST G 1970 Rj_2—a gene conditioning ineffective nodulation in soybean. Crop Sci 10: 34–35
29. VEST G, BE CALDWELL 1972 Rj_4—a gene conditioning ineffective nodulation in soybean. Crop Sci 12: 692–693
30. VIGUE JT, JE HARPER, RH HAGEMAN, DB PETERS 1977 Nodulation of soybeans grown hydroponically on urea. Crop Sci 17: 169–172