Selection and Initial Characterization of Partially Nitrate Tolerant Nodulation Mutants of Soybean

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ABSTRACT

Since NO₃⁻ availability in the rooting medium seriously limits symbiotic N₂ fixation by soybean (Glycine max L. [L.] Merr.), studies were initiated to select nodulation mutants which were more tolerant to NO₃⁻ and were adapted to the Midwest area of the United States. Three independent mutants were selected in the M₅ generation from ethyl methanesulfonate or N-nitroso-N-methylurea mutagenized Williams seed. All three mutants (designated NOD1-3, NOD2-4, and NOD3-7) were more extensively nodulated (427 to 770 nodules plant⁻¹) than the Williams parent (187 nodules plant⁻¹) under zero-N growth conditions. This provided evidence that the mutational event(s) affected autoregulatory control of nodulation. Moreover, all three mutants were partially tolerant to NO₃⁻; each retained greater acetylene reduction activity when grown hydroponically with 15 millimolar NO₃⁻ than did Williams at 1.5 millimolar NO₃⁻. The NO₃⁻ tolerance did not appear to be related to an altered ability to take up or metabolize NO₃⁻, based on solution NO₃⁻ depletion and on in vivo nitrate reductase assays. Enhanced nodulation appeared to be controlled by the host plant, being consistent across four Bradyrhizobium japonicum strains tested. In general, the mutant lines produced less dry weight than the control, with root dry weights being more affected than shoot dry weights. The nodulation trait has been stable through the M₅ generation in all three mutants.

Of the numerous environmental factors which have an effect on soybean (Glycine max L. Merr.) nodulation, NO₃⁻ availability plays a key role in Midwest field environments. It is well known that NO₃⁻ limits the early phases of nodule initiation, as well as the subsequent functional (nitrogenase) activity of the nodule (8). Although sensitivity of nodulation to NO₃⁻ has been the subject of numerous studies, no definitive reports have been published which elucidate the mechanisms of inhibition. For soybean it appears that the host plant, rather than the bacterial strain, is in primary control of the level of N₂ fixation (5).

With this in mind several laboratory groups have attempted to induce variability in host nodulation response. Recently, nodulation mutants have been selected from pea (Pisum sativum) (12), soybean (1, 2), and white bean (Phaseolus vulgaris L.) (15). Of specific interest are the extensive studies of Gresshoff's group (6) to isolate and characterize mutants from Bragg soybean which form more nodules than the parent in the presence and absence of nitrate. These nts mutants have been shown to be altered in an autoregulatory signal derived from the shoot (3, 4) which, in normal plants, typically suppresses nodulation on new roots once nodulation has occurred (14, 16). The nts 382 soybean mutant has many more nodules than the Bragg parent under nil nitrogen conditions (1, 2), but these nodules are smaller and less effective. In the presence of nitrate, the nts 382 mutant has higher nitorgenase activity than does the parent (1). Herridge and Bergersen (11) have also identified three Korean lines which also have greater numbers of nodules than control plants. It is therefore evident that the soybean plant is subject to manipulation with respect to nodulation in the presence of nitrate.

Midwest United States soybean production occurs under high levels of residual soil N which limits nodulation (8). Since the nts mutants selected by Gresshoff's group were derived from the cultivar Bragg which is a Maturity Group VII line and cannot be grown to maturity under Midwest growing conditions, the current study was initiated to select nodulation mutants from a background which can be field tested in the major Midwest soybean production area. This paper provides the initial characterization of three soybean lines with enhanced nodulation capability which were selected from mutagenized populations of Williams (Maturity Group III).

MATERIALS AND METHODS

Plant Material

Soybean (Glycine max [L.] Merr., cv Williams) seeds were mutagenized with EMS or NMU as previously described (17). In brief, the four mutagen-treatments (two chemicals and two postwash intervals) consisted of presoaking all seed for 16 h in vigorously aerated water, then treating separate seed lots for 2 h with 50 mM EMS or 5 h with 2.5 mM NMU, followed by either 5- or 9-h postwashes. The mutagenized seed (designated M₁) was planted in the field and, at maturity, the four M₂ seed lots were individually bulk harvested. The M₂ seed lots were then screened for nodulation mutants either in the field or in greenhouse gravel beds.

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Abbreviations: nts, NO₃⁻ tolerant symbiont; EMS, ethyl methanesulfonate; NMU, N-nitroso-N-methylurea.
SCREING PROCEDURE

Field Screen

The field nodulation screen consisted of planting mutagenized M₅ seed into a field plot that had been fertilized with 180 kg N ha⁻¹ as NH₄NO₃. The seeds were not inoculated, because the field plots have sufficient endogenous populations of *Bradyrhizobium japonicum* to effectively nodulate soybean. Approximately 3000 plants from each of the four mutagenized seed lots described above were evaluated. The NO₃⁻ treatment delayed nodulation of most plants. Therefore, plants were not evaluated until approximately 2 months after planting when all plants exhibited some nodules. The plants were carefully removed, the soil was shaken from the roots, and plants that had an excessive number of nodules relative to the population average were visually selected. Selected plants were transplanted to an adjacent field area where they were shaded for a period of time to minimize the transplant shock. Two of the most promising mutants selected from the field nodulation screen were designated NOD1 and NOD2 and were used in subsequent studies. The NOD1 line was derived from NMU treated (9-h postwash) seed and the NOD2 line was derived from EMS treated (5-h postwash) seed. At maturity, individual M₅ selected plants were harvested to provide M₄ seed for further evaluation and increase. Ten M₄ seed from each of the NOD1 and NOD2 lines were advanced as single plants to provide M₃ seed. The M₃ seed from individual plants were bulked and advanced to the M₂ generation to provide sufficient seed for detailed testing. Subsequent studies using M₃ generation seed were, however, traceable back to a single M₁ plant, and the NOD1 and NOD2 lines are subsequently referred to as NOD1-3 and NOD2-4, respectively.

Greenhouse Screen

The greenhouse nodulation screen consisted of growing plants hydroponically in gravel beds which were subirrigated from 50-L nutrient barrels, similar to the field system previously described (7). Plants were supplied with a modified Hoagland nutrient solution consisting of 0.94 mM Ca(NO₃)₂, 0.62 mM KNO₃, 0.5 mM MgSO₄, 0.05 mM KH₂PO₄, and 0.10 mM NH₄H₂PO₄ (pH 6.0), 36 μM Fe as Sequestrene 330 chelate⁴ (Ciba-Geigy Corporation, Greensboro, NC), 12.5 μM KCl, 6.25 μM H₂BO₃, 1.25 μM MnSO₄, 0.1 μM ZnSO₄, 0.13 μM CuSO₄, and 0.004 μM (NH₄)₆Mo₇O₂₄. Seeds were coated with a commercial peat-based *B. japonicum* inoculum (Urban Laboratories, Urbana, IL) prior to planting. Nutrient solutions were changed once at 2 weeks after planting and seedlings were evaluated at 3 weeks after planting. The seedlings were carefully removed from the gravel and saved if they possessed a greater number of nodules than the average population. Selected plants were transplanted into a separate hydroponic unit and individual plants were harvested at maturity to provide M₃ seed for further evaluation and increase.

Of the seedlings selected from the greenhouse screening, one of the more striking lines (NOD3) was derived from NMU (5-h postwash) treated seed. Subsequent studies were conducted with M₅ generation seed from a single M₄ plant (hereafter designated NOD3-7). All three mutants (NOD1-3, NOD2-4, and NOD3-7) are independent, since they were selected from different mutagenized seed lots.

EVALUATION OF MUTANTS

Plant Culture

Soybean seeds were surface sterilized with ethanol for 10 s, then 1% (v/v) sodium hypochlorite (diluted commercial bleach) for 3 min, followed by deionized distilled water. Seeds were planted in autoclaved sand in trays which were watered from the bottom with distilled water as needed. Germination was in a growth chamber maintained at day/night temperatures of 29°C/20°C ± 1°C and a 14-h photoperiod at 400 to 600 μmol photons m⁻² s⁻¹ (measured at plant tops with a LI-185A quantum sensor, Lambda Instruments Co., Lincoln, NE). At 7 d, seedlings were removed from the sand, inoculated by dipping the roots for 10 min in a suspension (10⁶ cells/mL) of *B. japonicum* dispersed in nutrient solution, and transplanted to pots containing nutrient solution. Seedlings were suspended through holes in lids of 7-L black-plastic-lined polyethylene pots, or 2-L black polyethylene pots, as noted with specific experiments. A modified Hoagland nutrient solution was used consisting of 2.5 mM CaCl₂, 1.25 mM K₂SO₄, 1 mM MgSO₄, 0.1 mM K-phosphate (pH 6.5), 12 μM Fe as Sequestrene 330 chelate, 25 μM KCl, 12.5 μM H₂BO₃, 0.25 μM CuSO₄, 2.5 μM MnSO₄, 1 μM ZnSO₄, and 0.007 μM (NH₄)₆Mo₇O₂₄. Nitrate treatments were as noted below with specific experiments. The solution pH was maintained at pH 6.5 ± 0.5 by the use of ion exchange resin columns as previously described (10).

Comparison of Three Nodulation Mutants and Williams

The experiment involved two NO₃⁻ levels (0 and 5.0 mM), four soybean lines (NOD1-3, NOD2-4, NOD3-7, and Williams), two *B. japonicum* strains (USDA 26 and tan 4b), and three replications. (The tan 4b strain was previously selected as a mutant from USDA 26 and had been reported to have greater nodulation capacity [13].) Each 7-L pot contained one plant of each line. A replication involved pooling three plants of a given line, one each from three pots. Nitrate levels were monitored as previously described (9) and replenished daily to maintain the original NO₃⁻ levels. At the end of the experiment (28 d after planting), plants were harvested and separated into shoots and roots at the cotyledonary node. Analyses were conducted for nodule number, acetylene reduction activity, and dry matter of nodules, shoots, and roots.

Determination of NO₃⁻ Uptake Rate

The experimental procedure, with exceptions noted below, was as indicated under “Plant Culture” using 2-L black polyethylene pots and one NO₃⁻ level (5.0 mM) with four pots per treatment. The seedlings were not inoculated in order to evaluate uptake and reduction of NO₃⁻ in the absence of confounding N₂ fixation. Four seedlings of a given line

⁴ Mention of a trademark, vendor, or proprietary product does not constitute a guarantee or warranty of the vendor or product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other vendors or products that may also be suitable.
SOYBEAN NODULATION MUTANTS

(NOD1-3, NOD2-4, NOD3-7, and Williams) were transplanted to individual pots. Nitrate depletion was monitored every 24 h, as noted earlier, and a complete nutrient change was made on d 9 following transplant and initiation of the NO₃⁻ uptake study. Nutrient NO₃⁻ levels on d 9 had been depleted to 15 to 35% of the initial 5 mM level with the four genotypes. The experiment was terminated at 2 weeks post-transplant, and plants were assayed for root and shoot dry matter, total N, and NO₃⁻-N contents. At termination, nutrient NO₃⁻ levels ranged from 23 to 46% of the initial 5.0 mM level with the four genotypes.

Acetylene Reduction Assay

Plant roots were severed from the shoot and individually placed in 500 mL jars. The jars were sealed and 50 mL of acetylene was injected through a septum fitted into the lid. The nodulated roots were incubated for 30 min at 30°C and 0.5 mL subsamples were analyzed for ethylene production by flame ionization gas chromatography (Hewlett Packard 5890A Gas Chromatograph). Acetylene and ethylene peaks were quantified with a Nelson analytical 3000 series gas chromatography system (Nelson Analytical Inc., Cupertino, CA) interfaced to an IBM personal computer for data reduction. After the assay, the nodules were removed from the roots and counted. Plant roots and nodules were then dried for dry matter determinations.

RESULTS AND DISCUSSION

Nodulation Characteristics

Nodule numbers, dry weights, and acetylene reduction activities of the three mutants and of Williams were markedly higher at 0 than at 5 mM NO₃⁻ (Table I). The results are averaged over two B. japonicum strains since there was no statistical strain effect. The inhibitory effect of NO₃⁻ on nodule number, dry weight, and acetylene reduction activity was more marked in Williams than in any of the three mutant lines. When grown on 0 mM NO₃⁻ and compared with the Williams parent, nodule number for the mutants was increased 2.3- to 4.1-fold, while nodule weight and C₂H₂ reduction activities were increased 1.4 to 2.2- and 1.8 to 3.0-fold, respectively. Averaged over both NO₃⁻ treatments, the mutants had significantly greater nodule numbers, nodule dry weights, and acetylene reduction activities than did the Williams control (statistics not shown). The NOD3-7 line had significantly greater numbers of nodules than the other lines at 0 mM NO₃⁻. NOD1-3 and NOD2-4 lines had similar numbers of nodules, both being significantly greater than for Williams. The greater number of nodules for NOD3-7 did not, however, result in greater nodule mass, due to the very small nodule size. The general phenomenon of greater nodule numbers on the selected mutants, when cultured on either 0 or 5 mM NO₃⁻, supports the conclusion that these selected mutants are altered in the autoregulatory pathway, a conclusion also drawn by Carroll et al. (1) for their mutants. Auto-regulation of nodule number has been previously documented for soybean (14, 16). Preliminary grafting studies between the Williams parent and the NOD3-7 mutant (data not shown) supported the previous conclusion (3, 4) that the nodulation phenotype is shoot controlled.

Acetylene reduction activity of all three mutants was significantly higher than for Williams at both 0 and 5 mM NO₃⁻ levels, respectively (Table I). In a separate experiment, all three mutants retained greater acetylene reduction activity at 15 mM NO₃⁻ than did the Williams parent at 1.5 mM NO₃⁻ (data not shown). Thus, nodulation traits were inhibited by NO₃⁻ for the selected mutants, but the effect was much less pronounced relative to the Williams parent. Although our mutants, as well as those selected by Carroll et al. (1), were visually selected following growth under NO₃⁻ levels which partially inhibited nodulation of the parent, subsequent studies indicated that the same lines would likely have been selected in the absence of any NO₃⁻. The screen in the presence of NO₃⁻ did, however, accentuate the difference, since the nodulation pattern for the Williams parent was more sensitive to NO₃⁻ than for the selected mutant lines.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Growth Condition</th>
<th>Soybean Line</th>
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<tbody>
<tr>
<td></td>
<td>mm NO₃⁻</td>
<td>NOD1-3</td>
<td>NOD2-4</td>
</tr>
<tr>
<td>Nodule number (No. plant⁻¹)</td>
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<td>427</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>122</td>
<td>119</td>
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<tr>
<td>Nodule weight (mg plant⁻¹)</td>
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</tr>
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<td></td>
<td>5.0</td>
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<td>91</td>
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<td>C₂H₂ reduction (μmol C₂H₂ plant⁻¹ h⁻¹)</td>
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<td>14.4</td>
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<td></td>
<td>5.0</td>
<td>2.0</td>
<td>2.4</td>
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<tr>
<td>Root dry weight (g plant⁻¹)</td>
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<tr>
<td></td>
<td>5.0</td>
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<td>1.30</td>
</tr>
<tr>
<td>Shoot dry weight (g plant⁻¹)</td>
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<td>0.32</td>
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<tr>
<td></td>
<td>5.0</td>
<td>3.42</td>
<td>3.98</td>
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Table I. Effect of NO₃⁻ on Nodulation and Growth of Selected Nodulation Mutants and the Williams Parent

Seeds were germinated in sand and transplanted to nutrient solution on d 7. Roots were inoculated immediately prior to transplanting. Plants were harvested after 21 d growth in nutrient solution with either 0 or 5 mM NO₃⁻. Data were pooled over two B. japonicum strains since statistical analyses indicated no significant differences between strains. Each value is the mean of six samples.
accumulated less lower than the statistically significant for Williams (Fig. 1). Whether both tive that added Nitrate (1, 2) from collected growth shoot nodulation all parent, Root replicate lines. Nitrate soybean 1.

Plant

CUMULATIVE NO₃⁻ UPTAKE (mmol NO₃⁻/PLANT)

DAYS AFTER NITRATE ADDITION

[Graph showing cumulative NO₃⁻ uptake from nutrient solution by four soybean lines. Nitrate uptake was determined by measuring depletion from the nutrient solution daily. Values represent means of four replicate pots for each soybean line.]

Figure 1. Cumulative NO₃⁻ uptake from nutrient solution by four soybean lines. Nitrate uptake was determined by measuring depletion from the nutrient solution daily. Values represent means of four replicate pots for each soybean line.

Root and Shoot Dry Weight

Averaged over NO₃⁻ treatments, root and shoot growth of all nodulation mutant lines was decreased, relative to the Williams parent, with root growth being affected more than shoot growth (Table I). Nodulation mutants previously selected (1, 2) from the Bragg background also showed tendencies for restricted root growth, leading that group to conclude that nodulation effectively competed with roots for available carbohydrate. Whether these differences in root growth, relative to respective parent lines, are linked to the nodulation phenotype remains to be established. All lines responded to added NO₃⁻ with increased root and shoot growth, although the Williams parent was more responsive. This accounted for the statistically significant line × NO₃⁻ interaction noted for both root and shoot dry weights (statistics not shown).

Nitrate Uptake and Reduction

The cumulative rate of NO₃⁻ uptake per plant was similar for Williams and the NOD2-4 line over the 14-d uptake period (Fig. 1). The NOD1-3 and NOD3-7 lines had similar cumulative NO₃⁻ uptake rates per plant, both being significantly lower than the Williams control and the NOD2-4 line.

Consistent with NO₃⁻ uptake results, the NOD3-7 line accumulated less shoot and root reduced N, and the NOD1-3 line accumulated less shoot reduced N, compared to the Williams control (Table II). Nitrate-N contents of roots were consistently higher than for shoots, but within root or shoot fractions, NO₃⁻ content was not significantly different among the lines tested. Likewise, leaf and root nitrate reductase activities were not different among the three mutants and Williams (data not shown). Similarly, the nts 382 mutant had the same nitrate reductase activity as did the Bragg parent (2). The observation that nodulation of our selected mutants was, however, partially inhibited by NO₃⁻ (Table I) does not allow one to totally rule out involvement of uptake and metabolism of NO₃⁻ in the nodulation response. The observation that root growth of the mutants was less than that of Williams in this study (Table II), when plants were not nodulated (not inoculated), indicated that root growth restriction of the mutants was not totally due to competition for carbohydrate when plants were nodulated (Table I).

Nodulation Response to B. japonicum Strains

There was no evidence of a bacterial strain × host line interaction in nodulation response (data not shown), although only four strains were evaluated (USDA 110, CB 1809, USDA 26, and a mutant strain of USDA 26, a strain designated as tan 4b and which had been reported to have enhanced nodulation capacity [13]). From these limited data, however, it was concluded that the host plant was responsible for the observed phenotypic nodulation expression. A similar preliminary conclusion was drawn with respect to six of the nts mutants which nodulated equally well with two B. japonicum strains (1).

Conclusions

The overall similarity in phenotype of the nodulation mutants described in this paper and those nts mutants previously reported on by Carroll et al. (1, 2) is striking, considering that different parent lines and mutagenesis treatments were involved. Our mutagenesis studies involved a Maturity Group III, indeterminate soybean, Williams, while those of Carroll et al. (1, 2) involved a Maturity Group VII, determinate soybean, Bragg, as parent materials. The observation that two different mutagens (EMS and NMU) were capable of inducing similar mutations in our study indicates that the gene control of this trait is not especially stable. Selection of nodulation mutants in a background adaptable to the Midwest will facilitate testing of the potential agronomic importance of this

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Plant Part</th>
<th>NOD1-3</th>
<th>NOD2-4</th>
<th>NOD3-7</th>
<th>Williams</th>
<th>LSD (0.05)</th>
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</thead>
<tbody>
<tr>
<td>Reduced N (mmol plant⁻¹)</td>
<td>Shoot</td>
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<td>2.67</td>
<td>1.99</td>
<td>2.61</td>
<td>0.24</td>
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<tr>
<td></td>
<td>Root</td>
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<td>NO₃⁻ N (mmol plant⁻¹)</td>
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<td>NS</td>
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<td>889</td>
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<td>434</td>
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trait in the major soybean production area of the United States.

LITERATURE CITED