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Effect of Postemergence, Supplemental Inoculation on Nodulation and Symbiotic Performance of Soybean (*Glycine max* (L.) Merrill) at Three Levels of Soil Nitrogen

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The influence of a supplementary bradyrhizobial inoculation after an initial seed slurry inoculation with the same strain on nodulation and N_2 fixation in soybeans was examined in the greenhouse. The plants were grown in a Typic Eutrocrepts soil: sand mixture containing 25, 65, or 83 mg of N per kg (i.e., native soil N plus ^{15}N-labeled ammonium sulfate). Harvests were made at early flowering and physiological maturity. The supplementary inoculations which were made 14 or 21 days after planting (DAP) caused formation of substantially more nodules than the single slurry inoculation did. Autoregulation was therefore not completely successful in preventing subsequent infections. For the slurry-inoculated plants, at both harvests the proportion of N derived from fixation was greatest in the soil containing the least N, and only slight increases in N_2 fixation resulted from a second inoculation. The inhibition of N_2 fixation at the higher N levels was significantly reduced by a second inoculation at 21 DAP; this treatment resulted in at least a doubling of both the percentage and total amount of N_2 fixed by the single slurry inoculation at physiological maturity. The N_2 fixation increases resulting from the supplementary inoculation at 14 DAP were less pronounced and not significant. Greater N_2 fixation was frequently not reflected by increased total N or dry matter yield, suggesting that the major benefit of the increased fixation was a decreased dependence of plants on soil N for growth.

Maximum nitrogen fixation in a legume requires that the legume be adequately nodulated. Scanty and poorly distributed nodules on the root system do not usually satisfy the nitrogen needs of the plant (1), resulting in greater reliance on soil N for growth. Supplemental inoculation to correct initial suboptimal nodulation has been recommended by Ciafardini and Barbieri (1), who reported greater nodulation and N_2 fixation in soybeans inoculated by using this approach than in those given a single slurry inoculation. Although Wadisirisuk et al. (18), using a sequential inoculation with an effective strain, reported increased nodulation in soybeans, N_2 fixation was not significantly enhanced. They found, however, that the initial low level of N_2 fixed as a result of an earlier inoculation with an ineffective bradyrhizobial strain was increased by a later inoculation with an effective strain. These results appear to indicate that a second inoculation may be most beneficial under conditions of initial impairment of the N_2 fixation potential of soybeans.

High soil N is one of the factors that commonly inhibit nodulation and N_2 fixation. Although reduced symbiotic efficiency (especially in a soil high in N) may not affect soybean yield (12, 19, 20), the resulting increased soil N uptake results in a depletion of soil N and represents an inefficient use of a legume in a cropping sequence (11). The present study examined the hypothesis that a second inoculation might enhance nodulation on plants grown in soils containing high levels of N.

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MATERIALS AND METHODS

A Typic Eutrocrepts soil, pH 7.4, containing 20 mg of 2 M KCl-extractable (available) native N per kg was used for this greenhouse study. Other soil characteristics have been given by Zapata et al. (21). The sieved (2-mm mesh size) soil was mixed with washed sand in a 3:1 soil-sand ratio, and pots (17 cm in diameter and 18 cm high, with three holes at the bottom) were filled with 4 kg of this mixture.

The three N treatments used were (i) addition of 100 ml of an aqueous ammonium sulfate solution containing 10 atom% ^{15}N excess to soil to give a total of 25 mg of available N per kg (designated N1), (ii) addition of 100 ml of an aqueous ammonium sulfate solution containing 2 atom% ^{15}N excess to the previous mixture, to give the equivalent of 65 mg of N per kg (designated N2), (iii) addition of 100 ml of distilled water to soil containing 83 mg of 2 M KCl-extractable N per kg at the initiation of the present study (designated N3) (this soil was used in a previous experiment with faba bean [*Vicia faba* L.] and barley [*Hordeum vulgare*] and had initially been treated with 150 mg of N per kg of 1 atom% ^{15}N excess ammonium sulfate).

The strain of *Bradyrhizobium japonicum* used was J2, obtained from Nitragin Co., Milwaukee, Wis. This strain was cultured in yeast extract mannitol broth by agitation on a shaker at room temperature. After 10 days of growth, the cells were centrifuged at 10,000 × *g* and suspended in sterile distilled water to give a Petroff-Hausser count of about 10^9 cells per ml. The three bradyrhizobial inoculation treatments used were a peat slurry (SL) on seed and SL plus a bradyrhizobial suspension applied at either 14 days after planting (DAP) ($SLSU_{14}$) or 21 DAP ($SLSU_{21}$). For the slurry inoculation, 10 g of sterile peat was inoculated with 5 ml of the washed bradyrhizobial suspension and immediately applied as a slurry onto surface-sterilized (17) soybean seeds (*Glycine max* L. cv. Chippewa). For the supplemental postemergence inoculations at 14 or 21 DAP, 50 ml of an aqueous suspension containing approximately 10^7 bradyrhizobial cells per ml (determined with a Petroff-Hausser counting chamber) was poured onto the surface of the soil at the base of the stem. Similar amounts of sterile distilled water were applied to the other treatments. Uninoculated soybean plants at each soil N treatment served as controls and as reference crops. The pots were arranged in a completely randomized design. The amount of nitrogen fixed was calculated using the following isotope dilution equations (10, 16):

$$\text{percentage of } N_2 \text{ fixed} = \left(1 - \frac{\text{atom percent } ^{15}\text{N excess in fixing crop}}{\text{atom percent } ^{15}\text{N excess in reference crop}}\right) \times 100$$

$$\text{total } N_2 \text{ fixed} = \frac{\% \text{ } N_2 \text{ fixed}}{100} \times \text{total N in fixing crop}$$

Nitrogen derived from soil (in the cases of the N1 and N2 treatments) was estimated as the difference between total N in plant and the N derived from fertilizer and fixation. Because the initial ^{15}N in the N3 soil was not determined, soil plus fertilizer N uptake (total N − N_2 fixed) rather than soil N uptake was determined for this treatment.

Four seeds were planted on 20 December, and seedlings were thinned to two after emergence. Supplementary 12-h lighting of 100 μmol/m^2 per s photosynthetic active radiation (400 to 700 nm) was provided during the day (high-pressure sodium lamp; Phillips, Eindhoven, The Netherlands), and the mean day and night temperatures were 28 and 20°C, respectively. Triplicate treatments were arranged in a completely randomized design. Plants were watered from below, by capillarity from water contained in saucers. Harvests were made at 35 and 80 DAP, when plants were at the first-flower and physiological maturity growth stages (7), respectively. For the first harvest, all the above-ground plant material was bulked and dried at 70°C for 48 h. The pods present at the second harvest were harvested separately from the shoots. This separation was done to reduce sampling errors (9). Plant samples were dried at 70°C for 2 days, weighed, ground to pass through 0.2-mm-mesh sieves, and analyzed for total N (6) and ^{15}N/^{14}N isotopic ratios (8). In cases in which plants were separated into different parts, a weighted atom percent ^{15}N excess for the whole plant was obtained using the equation described by Fried et al. (9).

Nodule counts were made on the following sections of the root: 0 to 5, 5 to 10, 10 to 15, and >15 cm from the stem base.

Statistical analysis was by two-way analysis of variance, and statistical differences were assessed by the Duncan multiple-range test at a significance level of $P < 0.05$.

RESULTS

Numbers of nodules. No nodules were formed on the uninoculated controls. Nodulation on the SLSU-inoculated plants at the first harvest (35 DAP) was generally greater than for the SL-inoculated plants (Fig. 1) (means, 17, 90, and 46 nodules per plant for SL, $SLSU_{14}$, and $SLSU_{21}$ inoculations, respectively). The soil N treatment effects were generally less pronounced than those of methods of inoculation.

At the second harvest, the greatest and most consistent increases in nodulation had occurred on the $SLSU_{21}$-inoculated plants (Fig. 1), with the lowest number of nodules always occurring on the SL-inoculated plants. However, unlike at the first harvest, the $SLSU_{21}$-inoculated plants from the second harvest formed more nodules in each soil than did the $SLSU_{14}$-inoculated plants. The average increase in nodule numbers was approximately twofold.

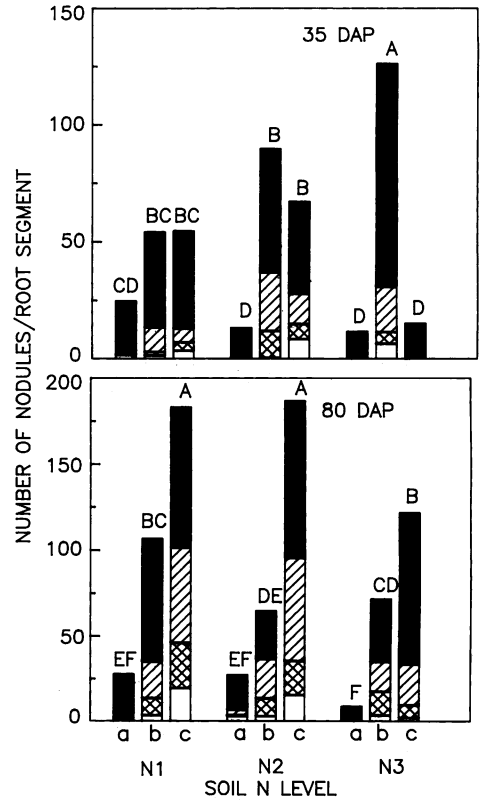

FIG. 1. The effects of SL (a), $SLSU_{14}$ (b), or $SLSU_{21}$ (c) on nodules formed on the 0- to 5- (□), 5- to 10- (▩), 10- to 15- (▨), and >15-cm (■) segments on soybean grown in soil containing 25 (N1), 65 (N2), or 83 (N3) mg of 2 M KCl-extractable N per kg at planting. Bars (total numbers of nodules) topped by the same letter(s) at each harvest are not significantly different ($P < 0.05$), as determined by the Duncan multiple-range test.

Nodule distribution. Without exception, the greatest proportion of nodules was formed on the 0- to 5-cm segment of the root at the first harvest (Fig. 1). The relative magnitudes, however, were influenced by the method of inoculation. On the average, a far greater proportion of the nodules was located on this root portion in the SL-inoculated plants than in either the $SLSU_{14}$- or $SLSU_{21}$-inoculated plants. In terms of absolute numbers, however, the supplemental inoculations resulted in more nodules on the 0- to 5-cm segment than the SL inoculations did. Few nodules were observed beyond the 5-cm portion of the root in any of the SL-inoculated plants at the first harvest. Nodulation below the 0- to 5-cm root zone was enhanced by the supplemental inoculations (Fig. 1).

At the second harvest, the pattern of nodule distribution on the SL-inoculated plants was essentially similar to that of the first harvest, with hardly any nodules being formed below the topmost 5-cm root segment (Fig. 1). The most significant increases in numbers of nodules between the first and second harvest occurred in the $SLSU_{21}$-inoculated plants, with the relative increases being greater below the 5-cm root zone than above it.

Nodule weight. First-harvest nodule weights on the

TABLE 1. Effect of method of inoculation on dry weights of nodules produced by soybeans grown at three soil N levels

Inoculation treatment[a]	Avg dry wt of nodules (mg/plant) on soybeans[b] at:					
	35 DAP			80 DAP		
	N1	N2	N3	N1	N2	N3
SL	97.7 B	42.0 C	70.0 BC	177.5 ABC	153.5 BC	90.0 D
$SLSU_{14}$	86.3 BC	180.5 A	158.8 A	210.5 AB	151.0 C	147.0 CD
$SLSU_{21}$	89.0 BC	74.3 BC	49.3 BC	215.5 A	228.5 A	194.5 ABC

[a] See Materials and Methods for details.
[b] Values at each harvest followed by the same letter(s) are not significantly different ($P < 0.05$), as determined by the Duncan multiple-range test.

$SLSU_{14}$-inoculated plants were higher at the N2 and N3 levels than at the N1 level; they were also higher than those of both the SL- and $SLSU_{21}$-inoculated plants in the N2 and N3 soils (Table 1).

At the second harvest, total nodule weights were similar on the SL- and $SLSU_{14}$-inoculated plants for all soil N treatments. The $SLSU_{21}$ inoculation gave significantly higher total nodule weights per plant than the SL treatment at the N2 and N3 levels but not at the N1 level. They were also greater than nodule weights of the $SLSU_{14}$-inoculated plants, but the differences were significant only in the N2 treatment (Table 1).

Nitrogen fixation. At the first harvest, although the additional inoculation at 14 DAP had little effect on the percentage of N_2 fixed in plants in the N1 treatment, it resulted in 18- and 2-fold increases in the percentage of N_2 fixed in plants in the N2 and N3 treatments, respectively (Fig. 2). The effects on total N_2 fixed were even greater (36- and 3-fold increases at the N2 and N3 levels, respectively, compared with the small and insignificant increase in total N_2 fixed at the N1

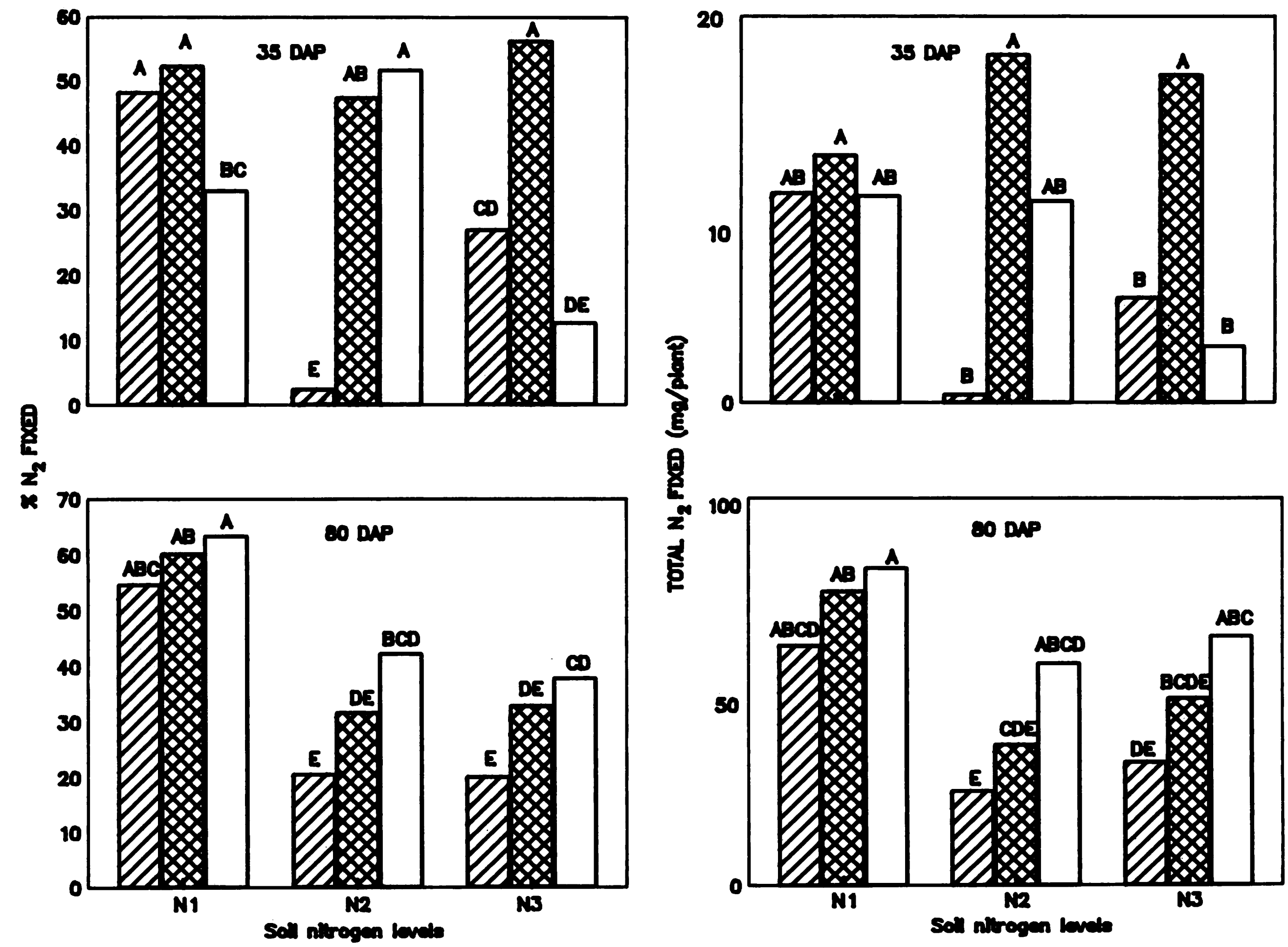

FIG. 2. Nitrogen fixed in soybeans at 35 and 80 DAP in soil containing 25 (N1), 65 (N2), and 83 (N3) mg of 2 M KCl-extractable N per kg, by the following methods of inoculation: SL (▨), $SLSU_{14}$ (▩), or $SLSU_{21}$ (□). Bars at each harvest topped by the same letter(s) are not significantly different ($P < 0.05$), as determined by the Duncan multiple-range test.

TABLE 2. Influence of method of inoculation on uptake of soil N or soil-plus-fertilizer N by soybeans grown at three soil N levels

Inoculation treatment[a]	N uptake (mg/plant)[b]			% of total N derived from soil[b]		
	N1[c]	N2[c]	N3[d]	N1	N2	N3
SL	45.1 A	90.6 C	120.7 E	39.2 CD	78.8 D	77.3 A
$SLSU_{14}$	43.1 A	73.3 B	94.1 CD	34.5 D	63.7 AB	64.9 AB
$SLSU_{21}$	41.1 A	73.3 B	102.0 CD	31.6 D	54.3 BC	60.0 B

[a] See Materials and Methods for details.
[b] Values followed by the same letter(s) are not significantly different ($P < 0.05$), as determined by the Duncan multiple-range test.
[c] Soil N.
[d] Soil-plus-fertilizer N.

level). Averaged values of N_2 fixed indicated that the $SLSU_{14}$ inoculation resulted in the largest amount of N_2 fixed at 35 DAP, i.e., 56% (17 mg of N per plan.), compared with 26% (6 mg of N per plant) and 34% (8 mg of N per plant) by the SL and $SLSU_{21}$ inoculations, respectively.

The values of N_2 fixed at the second harvest are also shown in Fig. 2. For the SL-inoculated plants, those grown in the N1-treated soil derived the highest percentage of N_2 fixed as well as total N from fixation, with similar amounts of N_2 being fixed at the N2 and N3 levels. Compared with N_2 fixation at the first harvest, increases in N_2 fixed at the second harvest attributable to the second inoculation at 14 DAP were smaller, and the amount of N_2 fixed in the $SLSU_{14}$-inoculated plants was not significantly different from that of the SL inoculation under any soil N treatment. The $SLSU_{21}$ inoculation in all cases gave the highest percentage of and total amount of N_2 fixed and differed significantly from the SL-inoculated treatment at the N2 and N3 but not the N1 level.

Nitrogen derived from soil. Data for N absorbed from soil or from soil plus fertilizer by plants at the second harvest are presented in Table 2. Because the initial ^{15}N enrichment in the N3 soil was not determined, only the total N absorbed from soil plus fertilizer could be estimated. The method of inoculation had no effect on the proportion or amount of native soil N accumulated at the N1 level. At the two higher N levels, however, the SL-inoculated plants accumulated more soil N than the $SLSU_{14}$- and $SLSU_{21}$-inoculated plants did. The $SLSU_{21}$ inoculation also resulted in the lowest proportion of soybean N being derived from soil. The lower proportions of N derived from soil in the $SLSU_{14}$-inoculated plants were not significantly different from those of the SL-inoculated plants.

Total N. Large increases in plant N (up to 10-fold) occurred between the first and second harvests (Table 3). The data indicate that at the second harvest, plant N in the N1 soil was not influenced by method of inoculation. At the N2 level, however, $SLSU_{21}$-inoculated plants accumulated significantly more N than the SL- and $SLSU_{14}$-inoculated plants. With plants grown in the N3 soil, all plants accumulated similar amounts of N at the second harvest, except for the larger amounts of N in the $SLSU_{21}$-inoculated plants than in the $SLSU_{14}$-treated plants. Plants which received identical inoculation treatments in the N1- and N2-treated soils accumulated similar amounts of N, which in all cases were smaller than amounts in similarly inoculated plants in the N3-treated soil.

Dry matter. The aboveground dry matter yields are presented in Table 4. Except for the $SLSU_{21}$-inoculated plants grown at the N1 level and the $SLSU_{14}$-inoculated plants grown at the N2 level, which at the first harvest had significantly higher dry matter yields than the plants receiving other inoculation treatments did, inoculation had little effect on dry matter yield at the first harvest. Also, with few exceptions, similarly inoculated plants had similar dry matter yields. At the second harvest, the uninoculated control plants generally gave only slightly lower dry matter yields than the inoculated treatments did, except in the N3 soil, in which the $SLSU_{21}$ inoculation resulted in higher dry matter yield than all the other inoculation methods did. This was the only instance in which a supplementary inoculation significantly enhanced dry matter yield.

DISCUSSION

The age, numbers, spatial distribution, and weight of nodules are factors that influence N_2 fixation by legumes. These nodulation parameters are also affected by the method of rhizobial inoculation (4, 14). Even though it is unlikely that the 50-ml suspension of bradyrhizobia that we used resulted in uniform distribution of cells in the whole soil, the nodulation data suggest that it must have resulted in better distribution than the SL treatment. Seed inoculation gave rise to fewer nodules which were more restricted to the crown roots (within 0 to 5 cm of the stem base) than a supplemental inoculation with bradyrhizobia suspended in water did. The data support earlier observations made with soybean plants grown in the same soil (2, 12). These nodu-

TABLE 3. Effect of method of inoculation on total N yields of soybeans grown at three soil N levels

Inoculation treatment[a]	Total N yield (mg/plant) of soybeans[b] at:					
	35 DAP			80 DAP		
	N1	N2	N3	N1	N2	N3
SL	21.5 CD	15.5 D	21.5 CD	115.0 F	115.0 F	160.0 AB
$SLSU_{14}$	22.0 CD	36.5 A	30.0 ABC	125.0 EF	115.0 F	145.0 BCD
$SLSU_{21}$	28.5 CD	20.0 CD	21.5 BCD	130.0 DEF	135.0 CDE	170.0 A
None	20.0 CD	19.0 CD	15.0 D	115.0 F	120.0 EF	150.0 BC

[a] See Materials and Methods for details.
[b] Values at each harvest followed by the same letter(s) are not significantly different ($P < 0.05$), as determined by the Duncan multiple-range test.

TABLE 4. Effect of method of inoculation on shoot dry matter produced by soybeans grown at three soil N levels

Inoculation treatment[a]	Shoot dry matter (g/plant) produced by soybeans[b] at:					
	35 DAP			80 DAP		
	N1	N2	N3	N1	N2	N3
SL	1.77 BC	1.22 C	1.84 ABC	5.85 BCD	6.15 BCD	6.35 B
$SLSU_{14}$	1.51 C	2.47 AB	1.87 ABC	6.60 AB	5.30 CD	6.30 BC
$SLSU_{21}$	2.07 A	1.39 C	1.65 C	6.30 BC	6.50 AB	7.40 A
None	1.49 C	1.62 C	1.48 C	5.18 D	5.65 BCD	6.25 BC

[a] See Materials and Methods for details.

[b] Values at each harvest followed by the same letter(s) are not significantly different ($P < 0.05$), as determined by the Duncan multiple-range test.

lation differences have been attributed to the fact that there is an increased chance of root-bradyrhizobium contact when bradyrhizobial cells are dispersed and fluid transported in the soil as opposed to when the inoculated cells are localized on seeds (2, 18). The restriction of nodulation to the crown root may be also due to a suppression-of-nodulation (autoregulatory) mechanism by which earlier-formed crown nodules suppress nodulation on other parts of the root (15). The data of Danso and Bowen (2) and Wadisirisuk et al. (18), however, which include data on plants inoculated by suspension only, suggest that the restricted ability of bradyrhizobia to migrate into the rhizosphere may exert a more pronounced effect on the numbers and vertical distribution of nodules than the autoregulatory mechanism does. The present results showed that the second inoculation (given as a suspension) not only led to the formation of greater numbers of nodules on the lower segments of the roots but also increased nodulation severalfold on the topmost 0 to 5 cm of the root. This suggests that the autoregulatory mechanism was not capable of fully suppressing subsequent nodulation by the second inoculation at either 14 or 21 DAP. The $SLSU_{21}$ inoculation gave higher nodulation at physiological maturity than the $SLSU_{14}$ inoculation did, possibly because more infectible sites were available on the roots at 21 than 14 DAP. It is also plausible that the longer delay in inoculation might have further reduced any impact of autoregulation.

The significantly increased nodulation resulting from the $SLSU_{21}$ inoculation was also reflected in greater nodule weights in the N2 and N3 soils but not in the N1 soil.

Decreases in the proportion of plant N derived from N_2 fixation commonly occur as available soil N increases (3, 13). However, since the inhibitory effect of N added to soil decreases with time (5), it was considered possible that nodulation and N_2 fixation from a supplementary postemergence inoculation would be inhibited less than they would in an SL inoculation made at planting. On the basis solely of data obtained by the traditional SL inoculation method, the fact that smaller amounts of N_2 were fixed by plants at the higher N levels (N2 and N3) than at the N1 level suggests an inhibition of N_2 fixation that is due to soil N levels, i.e., increasing soil N resulted in suboptimal levels of N_2 fixation. It was therefore expected that the greatest increase in N_2 fixation (e.g., through management practices) would be in the N2 and N3 soils, in which the SL inoculation resulted in only 21 and 20%, respectively, of soybean N being derived from N_2 fixation, compared with 55% at the N1 level.

At physiological maturity, supplemental inoculation increased N_2 fixation in all cases. However, the magnitude of the increase differed with the different soil N levels. In the N2 and N3 treatments, the $SLSU_{21}$ inoculation doubled the percentage and total amount of N_2 fixed by the SL treatment, while the corresponding increase at the lowest N level was far less (from 55 to 64%, or 0.07 to 0.09 mg per plant). These observations support the hypothesis that N_2 fixation in soils with high N levels might be less inhibited in plants receiving supplemental inoculation than in those inoculated only at planting. It may also be inferred that supplemental inoculation might have a greater impact in soils of very high N content than in low N soils. The availability of N to plants from added N fertilizer decreases rapidly with time (9). It is therefore most likely that enhanced N_2 fixation in soils with high N levels was due to the formation of extra nodules from the 21-DAP inoculation when the available N in soil had been substantially reduced. Nitrogen fixation in these later-formed nodules would consequently be expected to be less inhibited by inorganic N than in the earliest-formed nodules (from the SL inoculation). Besides, many of the nodules of the $SLSU_{21}$ inoculations are likely to be younger and probably are more active in N_2 fixation for a greater part of the reproductive stages of soybeans than the earlier-formed nodules of the SL treatment are. This delayed-nodulation phenomenon would also account for the greater N_2 fixation at physiological maturity by the $SLSU_{21}$ than by the $SLSU_{14}$ inoculation.

The lower N_2 fixation rate resulting from the $SLSU_{21}$ inoculation than from the $SLSU_{14}$ inoculation at 35 DAP could be attributed to insufficient time between the 21-DAP inoculation and the 35-DAP harvest period, i.e., the infection and effective functioning of nodules required more time than was available before harvest. None of the increases in N_2 fixation from the supplemental inoculations, however, could completely protect soybeans from the inhibitory effect of N on N_2 fixation, since the highest percentage of N_2 fixed at physiological maturity in either the N2 or N3 soil (42 and 38%, respectively, in $SLSU_{21}$-inoculated plants) was lower than the lowest percentage of N_2 fixed by the traditional SL method at the N1 level.

It has been shown in several experiments that soil N is often not a limiting factor in soybean yield (9, 20), a fact which is supported by the results obtained in our study. For each soil N level, the higher level of N_2 fixation in plants was not generally reflected in higher plant N level or dry matter yield. This implies that N did not limit growth and that the plants accumulated different amounts of N from the different sources available. The lower-N_2-fixing plants in our study relied more on soil N for growth than the higher-N_2-fixing plants did. For example, the $SLSU_{21}$-inoculated plants fixed more of their nitrogen at the N2 level but assimilated a lower amount of soil N (about 24% less) than the SL-inoculated plants did (Table 2). Higher symbiotic effectiveness, even when unaccompanied by higher yield, has important implications for the eventual fertility of the soil; by utilizing less soil N, subsequent rotational crops will have more soil N available for growth.

The early advantage in N_2 fixation gained by the 14-DAP over the 21-DAP supplementary inoculation disappeared by the physiological-maturity harvest. The consistent trend found at this later harvest of higher N_2 fixation corresponding with the longer delay in applying the supplemental inoculation suggests the possibility that longer delays in applying the supplemental inoculation, i.e., beyond the 21 DAP used in this study, were necessary for still higher increases in N_2 fixation. This would then agree with the suggestion of Ciafardini and Barbieri (1) that supplemental inoculation may be beneficial only if the reinoculation occurs 20 to 30 days following the appearance of nodules on the primary roots (i.e., at least 30 to 40 days after the first SL inoculation, allowing a minimum of 10 days for the appearance of the first nodules). However, this hypothesis is not supported by the data of Danso and Bowen (2), who observed that supplemental inoculation 42 days after the first inoculation with an effective strain was not superior in N_2 fixation to slurry inoculation at planting or supplemental inoculations at 14 and 28 days after the first inoculation.

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LITERATURE CITED

1. **Ciafardini, G., and C. Barbieri.** 1987. Effects of cover inoculation of soybean on nodulation, nitrogen fixation, and yield. Agron. J. **79:**645–648.
2. **Danso, S. K. A., and G. D. Bowen.** 1989. Methods of inoculation and how they influence nodulation pattern and nitrogen fixation using two contrasting strains of *Bradyrhizobium japonicum*. Soil Biol. Biochem. **21:**1053–1058.
3. **Danso, S. K. A., C. Hera, and C. Douka.** 1987. Nitrogen fixation in soybean as influenced by cultivar and *Rhizobium* strain. Plant Soil **99:**163–174.
4. **Danso, S. K. A., and J. D. Owiredu.** 1988. Competitiveness of introduced and indigenous cowpea *Bradyrhizobium* strains for nodule formation on cowpeas [*Vigna unguiculata* (L.) Walp.] in three soils. Soil Biol. Biochem. **20:**305–310.
5. **Deibert, E. J., M. Bijeriego, and R. A. Olson.** 1979. Utilization of ^{15}N fertilizer by nodulating and non-nodulating soybean isolines. Agron. J. **71:**717–723.
6. **Eastin, E. F.** 1978. Total nitrogen determination for plant material. Anal. Biochem. **85:**591–594.
7. **Fehr, W. H., C. E. Caviness, D. T. Burmood, and J. S. Pennington.** 1971. Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. Crop Sci. **11:**929–931.
8. **Fiedler, R., and G. Proksch.** 1975. The determination of ^{15}N by emission and mass spectrometry in biochemical analysis: a review. Anal. Chim. Acta **78:**1–62.
9. **Fried, M., S. K. A. Danso, and F. Zapata.** 1983. The methodology of measurement of N_2 fixation by non-legumes as inferred from field experiments with legumes. Can. J. Microbiol. **29:**1053–1062.
10. **Fried, M., and V. Middelboe.** 1977. Measurement of amount of nitrogen fixed by a legume crop. Plant Soil **47:**713–715.
11. **Hansen, A. P., M. B. Peoples, P. M. Greshoff, C. A. Atkins, J. S. Pate, and B. J. Carroll.** 1989. Symbiotic performance of supernodulating soybean (*Glycine max* (L.) Merrill) mutants during development on different nitrogen regimes. J. Exp. Bot. **40:**715–724.
12. **Hardarson, G., M. Golbs, and S. K. A. Danso.** 1989. Effect of nodulation patterns on nitrogen fixation by soybean (*Glycine max* [L.] Merrill). Soil Biol. Biochem. **21:**783–787.
13. **Hardarson, G., F. Zapata, and S. K. A. Danso.** 1984. Effect of plant genotypes and nitrogen fertilizer on symbiotic nitrogen fixation by soybean cultivars. Plant Soil **82:**397–405.
14. **Kamicker, B. J., and W. J. Brill.** 1987. Methods to alter the recovery and nodule location of *Bradyrhizobium japonicum* inoculant strains on field-grown soybeans. Appl. Environ. Microbiol. **53:**1737–1742.
15. **Kosslak, R. M., and B. B. Bohlool.** 1984. Suppression of nodule development on one side of a split-root system of soybean caused by prior inoculation of the other side. Plant Physiol. **75:**125–130.
16. **McAuliffe, C., D. S. Chamblee, H. Uribe Arago, and W. W. Woodhouse, Jr.** 1958. Influence of inorganic nitrogen on nitrogen fixation by legumes as revealed by ^{15}N. Agron. J. **55:**334–337.
17. **Vincent, J. M.** 1970. A manual for the practical study of the root-nodule bacteria. International biological programme handbook no. 15. Blackwell Scientific Publications, Ltd., Oxford.
18. **Wadisirisuk, P., S. K. A. Danso, G. Hardarson, and G. D. Bowen.** 1989. Influence of *Bradyrhizobium japonicum* location and movement on nodulation and nitrogen fixation in soybeans. Appl. Environ. Microbiol. **55:**1711–1716.
19. **Weber, C. R.** 1966. Nodulating and non-nodulating soybean isolines: response to applied nitrogen and modified soil conditions. Agron. J. **58:**46–49.
20. **Welch, L. F., V. Boome, C. G. Chamblis, A. T. Christiansen, D. L. Mulvaney, M. G. Oldham, and J. W. Pendleton.** 1973. Soybean yields with direct and residual nitrogen fertilization. Agron. J. **65:**547–550.
21. **Zapata, F., S. K. A. Danso, G. Hardarson, and M. Fried.** 1987. Time course of nitrogen fixation in field-grown soybean using nitrogen 15 methodology. Agron. J. **79:**172–176.