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https://doi.org/10.5109/24005

出版情報：九州大学大学院農学研究院紀要. 37 (2), pp.139-147, 1992-12. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., 37 (2), 139-147 (1992)

Effects of Nitrogen Application on Physiological Characteristics of Nitrate-Tolerant Mutants of Soybean

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(Received July 13, 1992)

Nitrate-tolerant mutants of soybean (*Glycine max* [L.] Merr.), ntsll16 and nts1007 and their parent cultivar Bragg were grown in field. Plants were harvested at flowering, pod elongating and pod filling stages; and analyzed for nitrogenous components and sugar.

The highest total N and ammonium-N concentration were found in nodules followed by leaf, stem and root. There were small differences in the concentrations between N- and non-N-applied plants. Nts1007 showed higher concentrations in almost all plant parts than Bragg and ntsll16. Allantoin-N concentrations was highest in nts1007, especially in nodule. Nitrogen application brought about the remarkable decrease of allantoin-N in stem and root. Slight differences in sugar concentrations were observed between N-applied and non -N-applied plants and among three lines. The concentrations in root and nodule of nts1007 were higher than Bragg and ntsll16, indicating that a heavily nodulating mutant, nts1007 was not recognized to be restricted in energy supply for nitrogen fixation as compared to Bragg or ntslll6.

There were small differences in the characteristics in pods between N- and non-N-applied plants of all lines except for allantoin-N and sugar of nts1007. Allantoin-N showed higher concentration in nts1007 than in other two lines at pod elongating stage. Sugar contents showed a decreasing tendency that was distinct in nts1007 applied with nitrogen. The high allantoin-N and low sugar concentrations in pod shells of nts1007 were considered to be due to higher N fixation by superabundant nodulation.

INTRODUCTION

Legume root nodule, the morphologically defined structure, is responsible for symbiotic nitrogen fixation in legumes. Legume nodule initiation and development are governed by both external and internal signals or factors to the two symbiotic partners (Mathews et al., 1992). External nitrogen sources such as nitrate depress nodule formation (Carroll and Gresshoff, 1983; Herridge, 1982). Carroll ***et al. (1985)*** isolated a number of soybean mutants that produce nodule numbers in excess of the wild-type cultivar and continue to nodulate prolifically in the presence of external nitrogen sources. These mutants have been termed supernodulating and nitrate-tolerant symbiotic (nts) mutants. Nitrate tolerance is a useful trait for soybean production because it enables soybean plants to maintain a nitrogen fixing ability in N-fertile soils, and can be used in breeding programs for high yielding (Haider *et* ***al.,*** 1991). Hussain ***et al.*** (1992) in the field experiment showed that nts1007 was inferior in growth parameters, grain yield and total dry matter production to Bragg and a hypernodulat-

ing mutant, ntslll6. Nodulation was extremely abundant at three sampling times in both N- and non-N-applied plants of nts1007. Applied N depressed grain yield, total dry matter production and harvest index in Bragg and ntslll6, but nts1007 revealed the increased values. They concluded that this might be due to high nitrate tolerance and abundant nodulation in nts1007. To understand the physiological behaviors of a hypernodulating mutant, ntsll16 and a supernodulating mutant, nts1007 in comparison with those of the parent cultivar Bragg in two nitrogen levels at different stages under natural field conditions, the present study was carried on.

EXPERIMENTAL

Sample preparation

Soybean (*Glycine max* (L.) Merr.) cultivar Bragg (wild-type, nod+, fix+) and its mutants ntslll6 (nitrate tolerant, hypernodulating, nod++, fix+) and nts1007 (nitrate tolerant, supernodulating, nod+++, fix+) were grown as shown in the previous paper (Hussain et al., 1992).

Two plants of each replication were harvested separately at flowering, pod elongation and pod filling stages. After the sampling, plants were separated into leaf, stem including petiole, root, nodule and pod to measure various parameters. Pods were separated into large, medium and small pods at pod elongating stage; and into filling pods and young ones at pod filling stage. Filling pods were separated into seeds and shells. At maturity, five plants of each plot were harvested for yield parameters. All the plant parts were lyophilized, weighed and ground with a cyclotec 1093 sample mill (100 - 120 mesh, Tecator AB, Sweden) for determination of organic and inorganic components. The maturity times of the three cultivars were different as shown in the previous paper (Hussain et ***al., 1992).***

Determination of nitrogenous constituents and sugars

Total Nitrogen:

The ground materials were digested and analyzed colorimetrically for ammonia according to the modified method of Cataldo et ***al.*** (1974) with replacement of sulfuric acid by a 30:1 mixture of sulfuric acid and salicylic acid (Eastin, 1978).

Other components than total-N:

The ground materials were extracted with hot 80% ethanol. The extract was dried in a rotary evaporator at 40°C, and then dissolved in each 20 ml of both water and dichloromethane. The aqueous layer was separated by centrifugation at 3000rpm for 10 minutes.

Ammonium- and nitrate-N in the supernatant were determined spectrophotometrically by the methods of Cataldo et al. (1974) and Cataldo et ***al.*** (1975), respectively. Amino-N was determined colorimetrically by using the ninhydrin reagent according to Moore and Stein (1948) except the reagent solution was renewed when used. To determine amide-N, the amide of the supernatant was hydrolyzed by the method of Boddey et al. (1987). The released ammonia was determined by the method of Cataldo et al. (1974). Allantoin-N was measured by the method of Vogels and Van der Drift (1970), modified by Ishizuka (1985).

Total sugar content was estimated using Dreywood's anthrone reagent according

to Morris (1948).

RESULTS AND DISCUSSION

Total nitrogen

The highest nitrogen concentration among the plant parts was found in nodule followed by leaf, stem and root (Table 1). There were small differences in the concentration among plant parts of N- and non-N-applied plants. In both N- and non-N-applied plants, the concentration in all plant parts decreased with the growth except for root where it remained almost same. In the N-applied plants of Bragg at all sampling times, N concentrations were slightly higher than those in non-N-applied plants, since the contribution of symbiotic nitrogen fixation in N-accumulation was considered to be small compared to nts mutants. Although distinct tendency was not observed, ntsll16 and nts1007 showed the opposite tendency that N concentrations in non-N-applied plants were higher than those in N-applied plants. These differences might be caused by a high contribution of nitrogen fixation in nts mutants. Among the three lines, nts1007 showed higher N concentrations in all parts except for leaf and nodule than Bragg and ntsll16.

Due to the increased growth of vegetative parts and pod formation with the advance of growth stage (Hussain et ***al.*** 1992), the accumulation of N increased (Fig. 1). Total N accumulation was the lowest in nts1007 due to smaller vegetative growth and shorter growing period (Hussain et al. 1992). The differences in N accumulation between the plants with and without applied-N were not clear.

Table 1. Total nitrogen concentration in plant parts of nts mutants of soybean and the parent cultivar Bragg (mg/g).

Plant part	Soybean line	Flowering stage		Pod elongating stage		Pod filling stage	
		- N	+N	- N	+ N	- N	+N
Leaf	Bragg	43.5f0.8	43.0 f0.5	38.6±0.6	39.6f0.8	37.8f0.4	39.3±0.2
	ntsll16	47.8f0.2	41.0±0.0	37.1 ±0.7	34.2±1.1	35.6f1.6	33.5±0.6
	nts1007	45.4±1.5	45.6f2.1	39.8 ± 1.3	40.0±0.6	40.0f1.7	37.6 ± 1.8
Stem	Bragg	15.1±0.0	16.0±0.5	14.8±0.8	16.7f0.4	12.9±0.0	12.2 ±0.6
	ntsll16	15.5±0.0	13.0f0.6	14.2k0.7	12.6f0.4	11.6f0.7	10.7 ±0.2
	nts1007	17.7±0.3	18.5f0.4	18.8 ±0.7	21.2±0.4	18.2f0.6	14.4±0.4
Root	Bragg	6.3 ±0.0	8.4±0.4	7.2±0.1	7.6k0.5	7.2±0.6	7.3f0.7
	ntsll16	8.9 ±0.2	7.8±0.7	8.3 f0.8	7.8 ± 0.6	8.1±0.1	5.8f0.6
	nts1007	10.6±0.8	12.4±0.2	10.6±0.1	10.0±0.3	10.6±1.5	9.2f0.3
Nodule	Bragg	46.8±2.1	47.0±0.0	45.6±0.4	49.6 f0.9	34.9f4.1	41.7±0.9
	ntsll16	51.5f1.9	52.7f2.1	48.2 ±0.7	46.9±0.1	47.1-t0.6	42.8±5.7
	nts1007	49.0 ±3.1	45.7f2.1	51.4 ±3.7	49.1 k2.4	47.1f0.6	45.8±1.7

+ N, -N: Nitrogen- and non-N-applied, respectively.
Data: Mean value ± standard deviation.

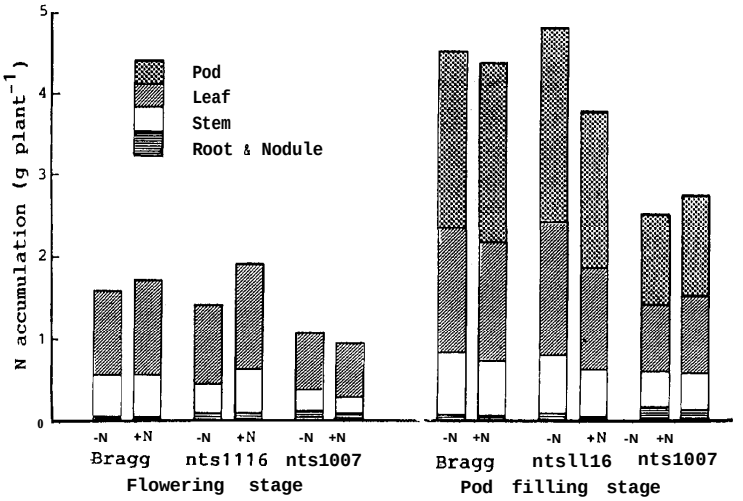

Fig. 1. Nitrogen accumulation of nts mutants and the parent cultivar Bragg.

Table 2. Amino-N concentration in plant parts of nts mutants of soybean and the parent cultivar Bragg (mg/g).

Plant part	Soybean line	Flowering stage		Pod elongating stage		Pod filling stage	
		- N	+ N	- N	+ N	- N	+ N
Leaf	Bragg	1.3±0.0	1.1±0.0	1.0±0.2	1.1±0.0	0.9±0.0	0.8±0.0
	ntsll16	1.1±0.0	0.9±0.0	1.0±0.0	0.9±0.0	0.8±0.0	0.8±0.0
	nts1007	1.oio.o	1.2±0.0	1.1±0.0	1.1±0.0	1.0±0.0	0.9±0.0
Stem	Bragg	1.5±0.0	2.0±0.1	2.4f0.4	3.1±0.1	1.0±0.0	1.1 ±0.1
	ntsll16	1.5±0.1	1.7io.o	2.5f0.3	2.3f0.2	1.0 ±0.0	1.0±0.0
	nts1007	1.7±0.3	2.3±0.0	3.2f0.3	3.3±0.1	1.5±0.0	1.4±0.1
Root	Bragg	0.3±0.0	0.6±0.0	0.6±0.1	0.8±0.1	0.3±0.0	0.3±0.0
	ntsll16	0.6±0.0	0.9f0.3	1.1±0.0	1.0±0.0	0.3±0.0	0.3±0.0
	nts1007	0.7±0.0	1.1±0.3	1.2±0.1	1.2 ±0.4	0.5±0.0	0.4±0.0
Nodule	Bragg	2.5±0.2	3.8±0.0	1.6±0.1		1.2±0.1	2.0±0.2
	ntsll16	2.8±0.1	2.7±0.5	2.7f0.4	2.2±0.0	1.6±0.1	1.2±0.3
	nts1007	2.6±0.0	3.2 ±0.0	3.1±0.0	3.3±0.4	1.9±0.2	1.8±0.0

Ammonium-N

The ammonium-N concentrations among different plant parts of the three lines were in the order of nodule > leaf > stem > root (data not shown). There were small differences among the lines and also between N- and non-N-applied plants. A small amount of ammonium-N was found in root in comparison with other plant parts. This showed immediate assimilation of ammonium-N in roots.

Nitrate-N

There were significant differences in nitrate-N concentration among different parts of the three lines at all sampling times (data not shown). Stem contained the highest nitrate followed by root. In the stem, the concentration decreased with the growth, whereas this tendency was opposite in root. The contents of nitrate-N in leaf and nodule were almost negligible at all sampling times except for nodule at pod filling stage where N-applied plants of the three lines showed higher values than that of non-N-applied plants. The reason why nitrate was detected in nodule remains to be elucidated.

Amino-N

The highest concentration of amino-N was found in nodule. In all the sampling times among the lines, the second highest concentration of amino-N was found in stem followed by leaf and root in both N - and non-N -applied plants (Table 2). The amino-N concentrations in leaf decreased with the growth in both N- and non-N-applied plants. The concentrations in both stem and root first increased moderately until pod elongating stage and then dropped sharply at pod filling stage. In case of nodule, the concentration in Bragg and nts1116 decreased gradually but it showed the same tendency in nts1007 as stems and root. Ishizuka (1977) reported that amino-N concentrations in soybean stem correlated closely with relative growth rates (RGR) and the acceleration of vegetative growth was assumed to be attributable to the increase in concentration of amino acids which seemed to be used directly as block materials for protein synthesis in the vegetative organs. But he could not detect the relationship between doses of N fertilizer and concentrations of amino-N in stem and leaf at reproductive stage. Herridge and Peoples (1990) stated that amino-N contents were essentially unaffected by nitrate supply. In this experiment, there were no remarkable differences in concentration of amino-N of different parts between N- and non-N-applied plants.

Amide-N

Higher amide-N concentrations in stem, root and nodule were found in both N- and non-N-applied nts1007 plants at all sampling times (data not shown). At flowering stage, the highest values were observed in nodule of nts1007. These results could be considered to be caused by higher nitrogen fixation and repressed plant growth in nts1007 due to heavily abundant nodulation. There were almost no differences in leaf.

Allantoin-N

Allantoin-N contents of leaf, stem, root and nodule varied remarkably among lines, nts1007 being much higher than nts1116 and Bragg (Table 3). Among different parts, allantoin-N concentrations were in the following order: nodule > stem > root > leaf for both N-applied and non-N-applied plants and also for different sampling times. There was almost no difference in the concentration in leaf between N- and non-N-applied plants at the reproductive period. However, nitrogen application brought about a remarkable decrease in allantoin-N concentrations of stem and root of the three lines at three sampling times. In stem and root, the concentrations increased gradually with the advance of growth. It was reported that the nodule mass

of soybean was correlated with the ureide content of the leaf, stem and xylem sap (Kushizaki et al., 1964) and that allantoin-N concentrations in field grown soybean decreased with increase of nitrogen application and increased rapidly after the flowering stage and decreased gradually from pod filling stage (Ishizuka, 1972, 1977). Although allantoin formation of soybean was suppressed by basal application of

Table 3. Allantoin-N concentration in plant parts of nts mutants of soybean and the parent cultivar Bragg (mg/g).

Plant part	Soybean line	Flowering stage		Pod elongating stage		Pod filling stage	
		- N	+N	- N	+N	- N	+N
Leaf	Bragg	0.1±0.0	O.lfO.O	O.lfO.O	0.1±0.0	0.1±0.0	0.1±0.0
	ntsl116	0.2±0.0	0.1 ±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
	nts1007	0.3±0.0	0.3±0.0	0.1±0.0	O.lfO.O	0.1 ±0.0	0.1±0.0
Stem	Bragg	1.2 ±0.0	0.7 ±0.0	1.3±0.0	0.8±0.2	2.6k0.2	1.9±0.0
	ntsll16	1.1 ±0.1	0.7-t 0.0	l.o-to.o	0.9±0.0	1.6±0.1	1.1 ±0.0
	nts1007	2.0±0.0	1.4±0.3	2.2±0.5	1.8f0.4	4.2f0.4	2.1±0.0
Root	Bragg	0.1 ±0.0	0.1 ±0.0	0.5±0.0	0.4±0.0	0.6±0.0	0.6±0.0
	ntsll16	0.3±0.0	0.2±0.0	0.5±0.0	0.1 ±0.0	0.6±0.0	0.3±0.0
	nts1007	0.5±0.2	0.4±0.0	0.8±0.4	0.5±0.0	1.1 ±0.3	0.6±0.0
Nodule	Bragg	2.5f0.4	1.6±0.0	3.9k0.8	---	1.8±0.0	2.5k1.3
	ntsll16	2.4f0.4	2.8±0.0	3.6k0.0	8.0+- 1.3	2.0 ±0.6	2.0±0.0
	nts1007	5.5f1.1	7.5rfrO.6	7.7±0.5	6.1±0.2	3.1±0.8	3.7f0.8

Table 4. Total sugar concentration in plant parts of nts mutants of soybean and the parent cultivar Bragg (mg/g).

Plant part	Soybean line	Flowering stage		Pod elongating stage		Pod filling stage	
		- N	+N	-N	+N	- N	+N
Leaf	Bragg	52± 0	54± 1	47± 0	47± 0	54± 4	56± 0
	ntsl116	53± 3	54± 4	53± 0	49± 0	60± 3	66± 0
	nts1007	49± 0	51± 1	47± 1	46± 1	45± 3	42± 3
Stem	Bragg	39± 6	40± 0	29± 0	33± 0	26± 0	31± 3
	ntsll16	42± 4	52± 0	31± 1	30± 3	36± 0	36± 3
	nts1007	39± 3	40± 0	28-t 0	28± 1	27± 1	29± 2
Root	Bragg	26± 0	33± 3	40± 5	38± 0	14± 0	15± 0
	ntsl116	25-t 1	33± 0	39± 1	37± 3	17± 4	15-t 1
	nts1007	32± 3	281 6	33± 1	30± 0	18± 0	16± 0
Nodule	Bragg	79± 6	74± 1	62±13		58fll	76± 1
	ntsll16	68± 2	66± 2	82± 1	67± 1	55± 6	45± 0
	nts1007	65± 7	64± 3	65± 4	63± 3	43± 0	44± 0

Table 5. Allantoin-N and total sugar concentration in pods of nts mutants of soybean and the parent cultivar Bragg (mg/g).

Component	Soybean line	Treatment	Pod elongating stage			Pod filling stage		
			Large	Medium	Small	Filling pod		Young pod
						Seed	Shell	
Allantoin-N	Bragg	−N		2.4±0.8	2.2±0.0	0.3±0.0	4.7±0.4	4.6±0.0
		+N		2.0±0.0	1.9±0.0	1.4±0.2	***4.8k0.8***	***5.2f0.4***
	nts1116	- N		2.0±0.0	1.7±0.0	1.8f0.4	4.8±0.3	4.9±0.4
		+N		2.1±0.0	2.1 ±0.0	1.8f0.5	4.1±1.1	4.7±0.5
	nts1007	- N	4.4f1.5	4.9f0.8	4.1 ±0.9	4.0f0.4	7.5±0.5	7.4f0.7
		+N	5.2t1.4	4.6±1.6	4.5±0.9	2.3±0.0	4.4±0.4	2.8-t0.9
Total sugar	Bragg	−N		92± 4	56-t 3	71± 4	19± 0	44± 4
		+N		90±19	65± 0	69± 1	18± 1	43± 0
	ntsll16	- N		99± 2	80± 2	64-t 0	17± 0	41± 0
		+N		11± 8	69± 5	66± 1	17± 1	41± 5
	nts1007	- N	83±18	109±20	45± 0	63± 3	16± 0	37± 1
		+N	61±13	80±23	59± 6	50± 3	17± 4	27± 6

nitrogen fertilizer through inhibition of nodule formation, it was increased by top-dressing at latter stage than flowering stage (Hoshi et al. 1977). In the present experiments, the concentrations in nodule of nts1007 increased until pod elongating stage and then decreased at pod filling stage. In ntsll16 and Bragg, the concentrations decreased gradually from flowering to filling stage, but nts1007 did not show such descending tendency. The higher levels and late descending of allantoin-N in nts1007 apparently were attributed to large production of allantoin due to super-abundant nodulation. Day *et al.* (1987) showed that ureide contents of nodules and xylem sap of a supernodulating mutant, nts382 were higher than those of Bragg. The high allantoin-N concentrations in nts1007 applied with nitrogen were considered to be caused by its sustaining high nitrogen fixing ability until later growth stage due to the highly nitrate tolerance in contrast to ntsll16 and Bragg.

Total sugar

Slight differences in the sugar concentration of leaf, stem, root and nodules were observed among the three lines (Table 4). There were also small differences between N- and non-N-applied plants in reproductive growth periods for each line. Day *et al.* (1987) mentioned that there was little difference in total carbohydrate content between nts382 and Bragg, indicating that heavily nodulated mutants might not be inferior in energy supply for nitrogen fixation to the parent cultivar Bragg. The concentrations in root and nodule of nts1007 were higher rather than Bragg and ntsll16. Therefore, low specific nitrogen fixing activity of supernodulating mutants could be considered to be originated from the other cause than the shortage of energy supply (Day *et al.* 1987).

Among the plant parts, the values were highest in nodules followed by leaf, stem

and root. The sugar concentrations of leaf and stem were more or less same throughout reproductive period in the three lines but the concentrations decreased greatly in root and gradually in nodules after pod elongating stage for the three lines.

Physiological state of pods

There were small differences in all characteristics between N- and non-N-applied plants for all lines except for allantoin-N and sugar of nts1007 (Table 5, the data except for allantoin and sugar were not shown). Nts1007 showed higher concentration of allantoin than the other lines at pod elongating stage, and allantoin concentrations thereafter increased in the three lines. However, allantoin-N in nts1007 decreased consistently in young pods of N-applied plant.

Sugar content of nts1007 at both stages showed a decreasing tendency with N-treatment. The high allantoin-N and low sugar concentrations in pod shells of nts1007 were considered to be due to higher nitrogen fixation and energy consumption of microsymbionts caused by super-abundant nodulation, although the sugar concentrations in vegetative organs did not differ from those in the other lines.

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