International Journal of Agriculture, Environment & Biotechnology
Citation: IJAEB: 7(3): 471-480 September 2014
DOI Number: 10.5958/2230-732X.2014.01351.5

Plant Physiology

Effect of Nitrogen and Sulphur Nutrition on Nitrogen assimilating Enzymes in Soybean Roots and Nodules

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Paper No. 232 Received: April 2, 2014 Accepted: July 12, 2014 Published: September 6, 2014

Abstract

Soybean is an important legume crop with high protein content but deficient in sulphur (S) containing amino acids *viz* methionine and cysteine. Soybean protein quality can be improved by nutrient fertilization. Present studies report the effect of nitrogen (N) and S nutrition on nodulation, activities of ammonia assimilating enzymes and biochemical parameters in soybean roots and nodules. Nitrogen as urea @ 31.25 kg ha^{-1} and sulphur as gypsum @ 20 kg ha^{-1} alone significantly increased nodule number, fresh and dry weight per nodule, root length and nodular leghemoglobin content whereas total soluble proteins, free amino acids, glucose and sucrose content did not vary significantly in nodules and roots. Treatment of N or S significantly increased glutamate dehydrogenase activity in cytosol and bacteroidal fractions from 70 to 90 days after sowing whereas aspartate aminotransferase, glutamate synthase, glutamine synthetase and alanine aminotransferase activities showed non-significant variations in roots at different stages of development under the influence of N or S alone or their combination. Results suggested that N and S could improve nodulation and vegetative growth in soybean but ammonia assimilating enzyme activities in roots/nodules did not vary significantly.

Highlights

- Nitrogen and sulphur alone or in combination increased nodulation and vegetative growth in soybean.

Keywords: Soybean, nitrogen, sulphur, nitrogen metabolizing enzymes, leghemoglobin

The assimilation and metabolism of inorganic nitrogen in plants is a complex process that involves several enzymes. Nitrate (NO_3^-) is reduced to ammonia (NH_4^+) by the action of nitrate reductase (NR; NADH, EC 1.6.6.1 and NADPH, EC 1.6.6.2) and nitrite reductase (NiR, EC 1.7.7.1) which further get incorporated into amino acids via glutamine synthetase (GS, EC 6.3.1.2)/glutamate synthase (GOGAT, EC 1.4.7.1) pathway and by glutamate dehydrogenase (GDH; NADH, EC 1.4.1.2) (Cruz *et al.,* 2004). Ammonia is initially incorporated into amide position of glutamine by glutamine synthetase which in turn is converted with 2-oxoglutarate to glutamate by glutamate synthase. GDH recycles glutamate and catalyzes direct incorporation of NH_4^+ into glutamate by reversible reductive amination of 2-oxoglutarate (Inokuchi and Okada, 2001; Cruz *et al.,* 2004). Both glutamine and glutamate are best utilized for the synthesis of aspartate, catalyzed by aspartate aminotransferase (AspAT, EC 2.6.1.1) (Chopra *et al.,* 2003). Alanine aminotransferase (AlaAT, EC 2.6.1.2) is

Table 1. Effect of nitrogen and sulphur supply on nodule number & weight, leghemoglobin content and root length & weight in soybean at different developmental stages.

Treatment	Nodule number			Fresh weight/Nodule (mg)			Root length (cm)			Root fresh weight (g)			Leghemoglobin (mg/g)		
	Days after sowing (DAS)														
	70	80	90	70	80	90	70	80	90	70	80	90	70	80	90
Control	66.7	105.7	139.0	10.1 (2.79)	8.06 (3.37)	11.45 (2.76)	14.0	16.6	17.5	1.84 (0.69)	3.49 (1.50)	4.4 (2.12)	0.48	0.38	0.55
Nitrogen (Urea @31.25 kg N ha^{-1})	97.7	150.7	137.3	11.4 (3.36)	10.82 (4.37)	10.19 (3.00)	14.33	16.2	16.9	2.25 (0.79)	3.19 (1.41)	4.65 (2.00)	0.61	0.36	0.47
Sulphur (Gypsum (@ 20 kg ha^{-1})	95.3	115.7	129.0	12.3 (3.67)	10.32 (3.95)	9.05 (2.62)	14.07	17.2	20.1	1.75 (0.64)	2.5 (1.15)	6.15 (2.46)	0.59	0.40	0.52
Nitrogen (Urea @31.25 kg ha^{-1}) + Sulphur (Gypsum @ 20 kg S ha^{-1})	91.0	151.0	134.7	9.2 (4.92)	7.94 (5.27)	9.65 (2.9)	17.23	18.0	20.9	2.04 (0.72)	4.74 (1.73)	5.32 (3.59)	0.65	0.50	0.63
Critical Difference (P≤0.05)															
Treatments DAS	15.16 13.12			0.89 (0.35) 0.77 (0.30)			2.11 1.83			NS (0.44) 0.53 (0.38)			0.04 0.03		

Table 2. Effect of nitrogen and sulphur supply on total soluble protein, free amino acids, glucose and sucrose content in soybean nodules and roots at different developmental stages.

Treatment	Total soluble protein (g/100g)			Free amino acids (g/100g)			Glucose (mg/g)			Sucrose (mg/g)		
	Days after sowing											
Nodules	70	80	90	70	80	90	70	80	90	70	80	90
Control	2.15 ± 0.34	3.21 ± 0.56	1.20 ± 0.09	1.05 ± 0.25	0.67 ± 0.12	1.35 ± 0.69	13.50 ± 3.42	9.83 ± 1.35	8.20 ± 1.24	3.47 ± 1.27	0.56 ± 0.28	0.74 ± 0.22
Nitrogen (Urea @31.25 kg ha^{-1})	2.67 ± 0.23	3.42 ± 0.14	1.31 ± 0.16	1.24 ± 0.32	0.94 ± 0.15	1.06 ± 0.36	15.87 ± 1.44	10.56 ± 1.14	9.13 ± 0.12	4.72 ± 0.47	0.38 ± 0.27	0.42 ± 0.24
Sulphur (Gypsum @ 20 kg ha^{-1})	2.57 ± 0.51	3.66 ± 0.81	1.10 ± 0.06	1.24 ± 0.16	1.13 ± 0.10	1.19 ± 0.05	16.76 ± 3.02	11.22 ± 1.55	9.23 ± 0.69	5.14 ± 1.46	0.49 ± 0.42	0.75 ± 0.22
Nitrogen (Urea @ 31.25 kg N ha^{-1}) + Sulphur (Gypsum @ 20 kg S ha^{-1})	2.47 ± 0.22	3.37 ± 0.37	0.85 ± 0.15	1.20 ± 0.11	0.86 ± 0.10	1.14 ± 0.39	16.22 ± 3.31	9.43 ± 0.74	7.90 ± 1.23	4.45 ± 0.47	0.44 ± 0.32	0.87 ± 0.42
Critical Difference (P≤0.05)												
Treatments Days after sowing	NS 0.32			NS 0.25			NS 1.61			0.28 0.54		
Roots												
Control	1.61 ± 0.12	2.56 ± 0.62	0.81 ± 0.16	0.45 ± 0.01	0.45 ± 0.08	0.48 ± 0.10	7.62 ± 1.36	3.59 ± 1.99	5.61 ± 1.03	2.70 ± 0.62	0.02 ± 0.13	0.13 ± 0.04
Nitrogen (Urea @31.25 kg N ha^{-1})	1.79 ± 0.12	2.71 ± 0.31	0.87 ± 0.12	0.49 ± 0.02	0.54 ± 0.04	0.58 ± 0.09	8.24 ± 1.06	5.34 ± 1.05	6.27 ± 1.33	2.36 ± 0.48	0.03 ± 0.01	0.18 ± 0.06
Sulphur (Gypsum @20 kg ha^{-1})	1.77 ± 0.32	2.62 ± 0.44	0.91 ± 0.07	0.47 ± 0.06	0.45 ± 0.09	0.60 ± 0.15	9.03 ± 0.66	4.75 ± 1.24	6.02 ± 0.20	2.57 ± 0.32	0.04 ± 0.01	0.18 ± 0.10
Nitrogen (Urea @ 31.25 kg N ha^{-1}) + Sulphur (Gypsum @ 20 kg S ha^{-1})	1.93 ± 0.16	2.95 ± 0.47	1.08 ± 0.25	0.52 ± 0.09	0.41 ± 0.11	0.53 ± 0.09	9.99 ± 1.05	5.93 ± 0.41	7.37 ± 2.16	3.47 ± 0.47	0.10 ± 0.02	0.23 ± 0.08
Critical Difference (P≤0.05)												
Treatments Days after sowing		NS 0.27			NS NS			NS 1.06			0.28 0.24	

a pyridoxal phosphate-dependent enzyme usually found in all plant parts including roots. It catalyzes the reversible reaction between pyruvate and glutamate into alanine and 2-oxoglutarate and hence, tightly links primary carbon metabolism with the synthesis of various amino acids (Rocha *et al.,* 2010).

A large portion of total plant nitrogen in legumes is derived from atmospheric N_2 fixation and is differentially metabolized in bacteroids and cytosolic fraction of the nodule (Ohyama and Kumazawa, 1978). The initial product of nitrogen fixation generated in bacteroid fraction of soybean is ammonia, most of which is exported into surrounding nodule plant cell cytoplasm (O'gara and Shanmugan, 1976), assimilated into organic compounds and transported to the shoots as either ureides or amides (Rawsthorne *et al.,* 1980).

Supply of adequate amounts of nutrients enhances nitrogen fixing capability of legumes (Cheema and Ahmad, 2000; Das *et al.,* 2012). Sulphur application in legumes has been reported to influence nodulation and N metabolism (Lange, 1998) whereas symbiotic N fixation and nitrate utilization appear essential for maximum yield (Pedersen, 2004). Soybean is considered as an alternative to rice in rice–wheat cropping system in Punjab due to its ability to fix atmospheric N and high nutritional quality. Knowing and enhancing the activity of N assimilation and metabolism enzymes could help in the selection of high yielding and protein-rich genotypes. Earlier work from our laboratory has indicated that gypsum @ 20 kg S ha^{-1} showed maximum yield in soybean. In the present studies, effect of gypsum @ 20kg S ha^{-1}, recommended dose of N @ 31.25 kg N ha^{-1} and interactive effect of these two on nodulation, ammonia assimilating enzymes alongwith total soluble proteins, free amino acids, glucose and sucrose contents in soybean roots and nodules were studied under the agroclimatic conditions of Punjab.

Materials and Methods

The field experiment was conducted on soybean cultivar SL 525 during *kharif* season in 2013 in the fields of Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. The soil of experimental field was sandy loam with pH 8.0, organic carbon (0.285%), salts (0.295%) and available P, K, Zn, Fe, Mn and Cu contents were 10.05, 50.0, 1.25, 1.51, 1.84 and 0.32 kg $acre^{-1}$, respectively. The experiment was laid out in randomized block design having a control and three treatments, each replicated four times. The three treatments involved were the application of N and S alone or in combination to the soil at the time of sowing. Nitrogen and sulphur were applied through urea @ 31.25 kg ha^{-1} and Gypsum @ 20 kg ha^{-1}, respectively. The seeds were sown at the rate of 30 kg $acre^{-1}$. Uniformly growing plants were uprooted from the wet fields at 10 d interval from 70 to 90 days after sowing (DAS). Roots with intact nodules were washed with tap water followed by deionized water and the nodules were then carefully detached from the roots with the help of forcep. Uniformly developed nodules and roots were collected and physical parameters including nodule number, dry weight and fresh weight of both roots and nodules and root length were determined and thereafter, enzyme assays were carried out. For extracting glutamine synthetase, glutamate synthase, glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase, fresh nodules were homogenized in cold 100 mM Tris-HCl buffer (pH 7.6) containing 20 mM β-mercaptoethanol, 2 mM $MgCl_2$, 2 mM EDTA and 10 mM cysteine. The homogenate was fractionated into cytosolic and bacteroidal fractions and used for various enzymatic assays. The root cytosolic fraction was obtained by centrifugation of homogenate at 25,000 g and the supernatant was then used for assaying enzymic activities.

The activity of glutamate dehydrogenase was assayed following the method of Bulen (1956) according to which, the decrease in absorbance was recorded at 340 nm for 3 min at 15 sec interval on UV-visible spectrophotometer. Glutamine synthetase was assayed by the method of Kanamori and Matsumoto (1974). The activity of glutamate synthase was determined according to Misra and Oaks (1981). The activities of aspartate aminotransferase and alanine aminotransferase were determined by following the method of Tonhazy (1960a, b), respectively. The soluble protein content was also determined in the enzyme extracts according to the method of Lowry *et al.,* (1951) for the estimation of specific activity of enzymes.

Roots as well as nodules were used for the extraction and estimation of total soluble proteins (Lowry *et al.,*

1951), free amino acids (Lee and Takahashi, 1966), glucose (Dubois *et al.,* 1956) and sucrose (Roe, 1934) content. Nodules were also used for the extraction of leghemoglobin in Drabkin's solution and optical density was recorded with spectrophotometer at 540 nm (Wilson and Reisenauer, 1963).

The data was analysed by factorial CRD using CPCS1 software to determine the critical differences in various biochemical parameters and enzymatic activities in control and various treatments *viz* N and S alone or in combination, if any at $P \leq 0.05$.

Results and Discussion

Nodule number increased significantly with application of fertilizers either alone or in combination during plant growth at 70 and 80 days after sowing, maximum response being given by the nitrogen treatment at both the stages (Table 1). Significant increase in the nodule number with plant growth was observed in control and gypsum applied @ 20 Kg S ha^{-1} whereas N alone or in combination with S resulted in increased nodule number up to 80 DAS. Fresh and dry weight per nodule increased significantly with application of fertilizers at 70 and 80 DAS and maximum increase in fresh weight per nodule was observed at 70 and 80 DAS with gypsum and urea, respectively. However, nodule fresh weight did not change significantly with combined treatment of N and S. During the crop development, fresh weight per nodule decreased from 70 to 90 DAS with gypsum and urea when applied alone and decreased up to 80 DAS in control and with combined treatment of S and N and increased thereafter at 90 DAS. Root length, fresh weight and dry weight increased significantly with crop progression from 70 to 90 DAS in all the treatments. Nitrogen or sulphur alone did not show significant increase in these parameters at different developmental stages but combined application of these fertilizers significantly increased root length and dry weight. Leghemoglobin content decreased from 70 to 80 DAS and then, increased thereafter at 90 DAS in all the treatments with maximum increase in combined treatment of N and S. Total soluble proteins in soybean nodules increased over the growth period, reaching a peak at 80 DAS and then, decreased at 90 DAS whereas free amino acids followed an inverse pattern (Table 2). Nodule soluble protein and free amino acid content did not change in response to applied N, S or by combination of two. Glucose and sucrose content in nodules decreased from 70 to 90 DAS and varied non-significantly among different treatments. Slight increase was observed by S application at 70 DAS and by the combined application of S with N at 90 DAS.

Total soluble proteins, free amino acids and glucose content of roots did not vary significantly among various treatments (Table 2). Total soluble proteins increased significantly from 70 to 80 DAS and then, decreased up to 90 DAS whereas glucose followed the reverse trend. Sucrose content decreased significantly with the crop development from 70 to 80 DAS but increased thereafter at 90 DAS although the contents were still lower than those at 70 DAS. Significant increase in the sucrose content compared to control roots was observed by the combined application of N and S at all the stages of crop development.

Total GDH and GOGAT activity in soybean nodule bacteroidal fraction increased from 70 to 80 DAS (Fig 1). N or S alone or in combination significantly increased GDH activity compared to control at 80 and 90 DAS, but it varied non-significantly among these treatments. Combined application of N and S increased both total and specific activities of GOGAT in bacteroidal fraction of nodules compared to control and other treatments at all the developmental stages of crop. Total activity of GS increased significantly from 80 to 90 DAS. Specific activity of nodule GS, GOGAT and GDH did not change or remain constant in response to N or S treatments. Total as well as specific activities of aminotransferases in bacteroidal fraction of nodules were maximum at 70 DAS and decreased significantly at 80 and 90 DAS. Total aspartate aminotransferase increased significantly in all the treatments at 70 DAS and maximum activity was observed with sulphur application. Total as well as specific activity of alanine aminotransferase did not change significantly by the fertilizer treatments at different developmental stages.

Total GDH activity in nodule cytosolic fraction increased significantly from 70 to 90 DAS in control and 70 to 80 DAS in other treatments (Fig 1). Although GDH activity increased in all the treatments at different development

stages but it increased significantly at 80 DAS in all the treatments and with S alone at 90 DAS. Total GOGAT activity in nodule cytosolic fraction increased significantly from 70 to 80 DAS and then declined further at 90 DAS whereas GS activity showed the opposite trend to GOGAT i.e. GS decreased from 70 to 80 DAS and then, increased significantly thereafter attaining a peak at 90 DAS. Aspartate aminotransferase activity in cytosolic fraction of nodules increased significantly from 70 to 90 DAS whereas AlaAT decreased from 70 to 80 DAS and showed significant increase with growth period. However, GOGAT, GS activities and aminotransferases were not affected in response to different treatments.

GDH and GOGAT activities in roots increased from 70 to 80 DAS and then decreased upto 90 DAS whereas GS and aminotransferases decreased from 70 to 80 DAS and increased thereafter. However, N or S alone or in combination did not significantly changed the activities of N metabolism enzymes except for AspAT which showed significant increase in its activity at 70 and 90 DAS in all the treatments (Fig 2).

In the present studies, recommended N doses showed maximum nodulation followed by the combined treatment of N and S. Nutrient requirement of the crops depends upon many factors and also on the synergistic or antagnostic effects of one nutrient on the other. It has been reported that starter N stimulates early seedling growth and nodulation (Daramola *et al.,* 1982). Sulphur improves nodulation activity (Olivera *et al.,* 2004; Scherer *et al.,* 2008) and affects growth of leguminous plants through its effects on N fixation (Varin *et al.,* 2009). It increases nitrogenase activity due to higher ferredoxin and ATP concentration in bacteroids of root nodules of legumes (Scherer *et al.,* 2008). Ferredoxin has a significant role in nitrate and sulphate reduction and in N assimilation in root nodule bacteria (Havlin *et al.,* 2007). Root length, fresh and dry weight increased in the treatments where S has been applied alone or in combination with N. Sulphur fertilizers promoted roots growth and nodules development on legume roots thus modifying root architecture by altering primary and lateral root growth (Scherer *et al.,* 2008; Kutz *et al.,* 2002). In soybean, fixed N is assimilated in different ways between bacteroid and cytosolic fractions. Legume nodules rhizobial bacteroids reduce N to ammonia and then export it to the surrounding plant cytosol rather than metabolizing it further due to insufficient GS activities in bacteroids (Ohyama and Kumazawa, 1980). In soybean, most of GDH and AspAT activities were located in cytosol whereas AlaAT and GOGAT are in bacteroid fraction of nodules. In present studies, higher GS activities were observed in cytosol as compared to bacteroid fraction and increased with the nodule development from 80 to 90 DAS that was followed by the induction of leghemoglobin in all the treatments. The observed GS activity in the roots indicated that substantial amount of absorbed ammonium was incorporated into glutamine. GOGAT catalyzes the conversion of glutamine to glutamate which further acts as a source for the formation of various amino acids. Sufficient levels of GOGAT in both bacteroid and cytosolic fraction of soybean nodules were observed. An increase in the level of GOGAT in cytosol fraction over growth period suggested that GS/GOGAT cycle operates in cytosol fraction for assimilation of ammonia produced by bacteroids.

Increase in GDH and GS activities in both bacteroid and cytosol fractions of nodules from 70 to 90 DAS and GOGAT from 80 to 90 DAS by N treatment alone or in combination with S suggested that the release of bacteria or bacteroids occurring at the initiation of N fixation (Egli *et al.,* 1989) might act as a signal for enhanced enzyme activity. Furthermore, the highest nodule number found at 90 DAS which was accompanied by enhanced activities of GDH and GS, thereby suggesting that more availability of fixed N might modulate the enzyme gene expression (Chopra *et al.,* 2003).

Highest GDH activity at 90 DAS might be relevant to its role in the detoxication of high NH_4^+ concentration present at this stage of nodule development (Chopra *et al.,* 2003). GDH has low affinity for NH_4^+ and effectively act in ammonia assimilation only when NH_4^+ concentration is high (Malencic *et al.,* 2006). From the pattern of GDH, GS and GOGAT observed in present studies, it was clear that although GS/GOGAT was the main route for the ammonia assimilation, GDH present in large proportion in nodule cytosol, might also play an important role under some nutritional and environmental conditions. The substantial activities of GDH and transaminases in soybean nodules in this study support the above statement. GS and GOGAT activities

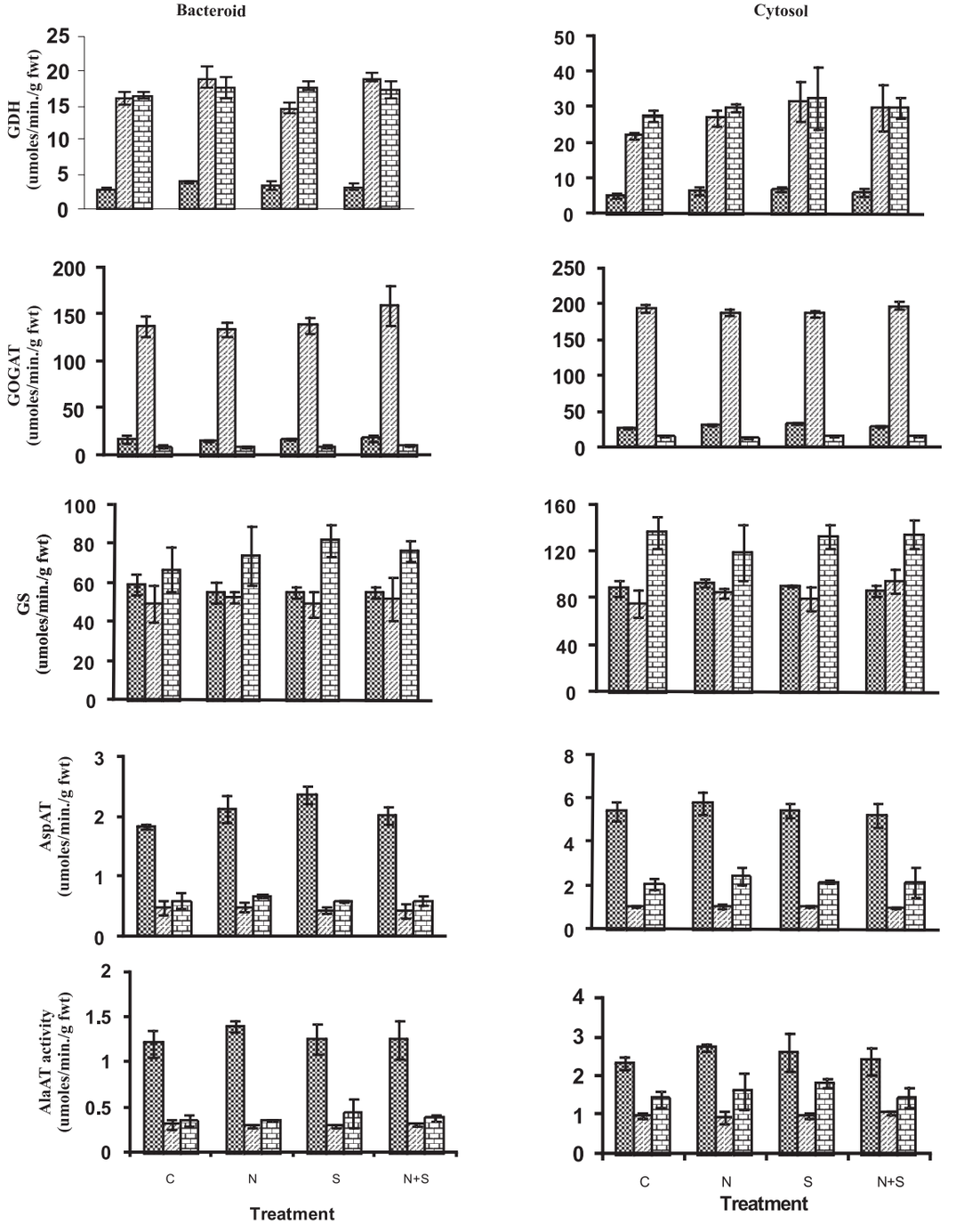

Fig 1. Effect of nitrogen and sulphur supply on various ammonia assimilating enzymes in bacteroid and cytosolic fractions of soybean nodules during crop development (GDH: Glutamate dehydrogenase; GOGAT: Glutamate synthase; GS: Glutamine synthetase; AspAT: Aspartate aminotransferase; AlaAT: Alanine aminotransferase)

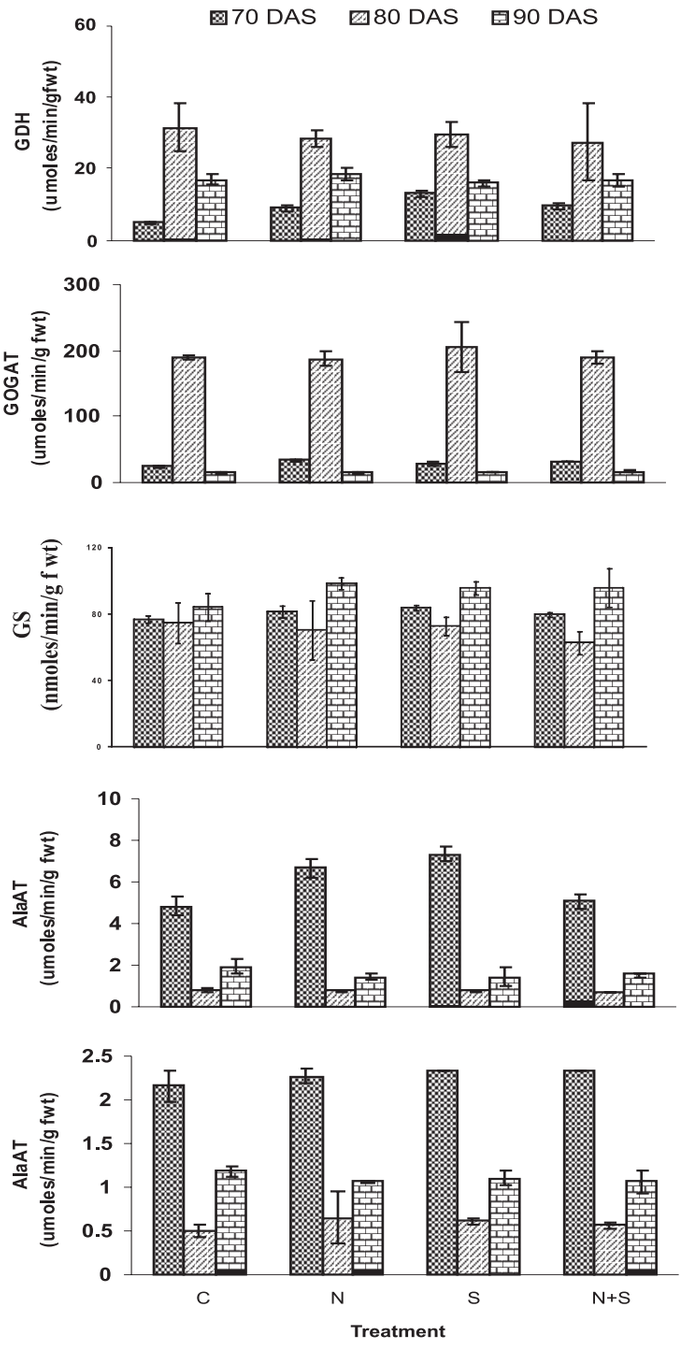

Fig 2. Effect of nitrogen and sulphur supply on various ammonia assimilating enzymes in soybean roots during crop development (GDH: Glutamate dehydrogenase; GOGAT: Glutamate synthase; GS: Glutamine synthetase; AspAT: Aspartate aminotransferase; AlaAT: Alanine aminotransferase)

were unaffected by the applied N/S in the present study. Similar effects of applied N have been reported in alfalfa nodules, where specific activity of both GS and GOGAT were unaffected by N treatment (Groat and Vance, 1982). Roots retained significant GDH activity throughout the crop progression from 70 to 90 DAS, in all treatments, which suggested the possible role of this enzyme in N assimilation. Non-significant changes in GS and GOGAT in response to applied fertilizers suggest that factors in addition to N or S supply are involved in the induction of high levels of these enzymatic activities.

Aspartate aminotransferase was more active than alanine amionotransferase in roots, bacteroid and cytosol fractions of soybean nodules in present studies. The occurrence of both GS/GOGAT system and GDH alongwith aminotransferases in soybean roots indicated that roots were capable of making the amino acids they may need for growth as well as export to the upper parts of the plant. However, these enzymes activities are not influenced by the N and S doses used in the present studies.

It is concluded that the recommended doses of N alone or in combination with S increased ammonia assimilating enzymes and photosynthates in roots/nodules non-significantly but these doses were effective in increasing vegetative growth and seed yield (data not given).

Acknowledgements

The authors are thankful to Dr B S Gill, Plant breeder and Dr G Singh, Senior Agronomist for sowing the crop and management of field experiment.

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