

Effect of Nitrogen and Sulphur Application on Nitrate Reductase and ATP-sulphurylase Activities in Soybean

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ABSTRACTS : A number of field experiments were conducted to assess the role of combined application of nitrogen and sulfur to increase the seed and oil yield of non-nodulating soybean (*Glycine max* (L) Merr.) cv. PK-416 (V₁) and cv. PK-1024 (V₂). Six combinations of N and S in three replicates each were used for this purpose i.e. 0 S + 23.5 kg N ha⁻¹ (T₁); 0 S + 23.5+20 kg N ha⁻¹ (T₂); 40 S + 23.5 kg N ha⁻¹ (T₃); 40 S + 23.5 + 20 kg N ha⁻¹ (T₄); 20+20 S + 23.5 kg N ha⁻¹ (T₅); 20+20 S + 23.5+20 kg N ha⁻¹ (T₆). Nitrate reductase (NR) and ATP-sulphurylase activities in the leaves were measured at various growth stages as the two enzymes catalyze the rate limiting steps of the assimilatory pathways of nitrate and sulphate, respectively. The activities of these enzymes were strongly correlated with seed yield. The higher seed, oil and protein yields were achieved with the treatment T₆ in both the cultivars due to optimization of NR activity and ATP-sulphurylase activity, as these parameters were influenced by N and S assimilation. Any variation from this combination was observed to decrease the activity of these enzymes resulting in reductions in the seed, oil and protein yield of soybean.

Keywords: ATP-sulphurylase, nitrate reductase, nitrogen, oil yield, seed yield, sulphur

The requirements of nitrogen (N) and sulphur (S) are of vital importance for plants because S is part of amino acids (cystein and methionine), which in turn constitute the building blocks of proteins (Ceccoti, 1996). The evidence that these nutrients are involved in enhanced activity of nitrate reductase (NR, EC 1.6.6.1) and ATP-sulphurylase (ATP:sulphate adenylyltransferase, EC 2.7.7.4) enzymes resulting high seed production, and oil biosynthesis in oil seed crops have been widely reported (Ahmad *et al.*, 1999, Abidin *et al.*, 2003, Fazli *et al.*, 2005, Jamal *et al.*, 2005). It was also reported that absence of a readily assimilable external nitrogen source decreases ATP-sulphurylase activity (Smith, 1975). In addition, research on tobacco cell cultures

demonstrated that the induction of NR by nitrate was proportional to the initial sulphate concentration in case of sulphate limited growth and, the de-repression of ATP-sulphurylase was proportional to the initial concentration in case of nitrate limited growth. The interrelationship of the regulation of NO₃⁻ and SO₄²⁻ assimilation is an effective mechanism to co-ordinate and meets the requirements of net protein synthesis (Reuveny *et al.*, 1980). These studies suggest a metabolic coupling between N and S assimilation in the plants.

Keeping in view the above facts, experiments were carried out to investigate responses of NR and ATP-sulphurylase activities as well as seed, oil and protein yields of the two soybean cultivars to the combined applications of S and N.

MATERIALS AND METHODS

Experimental design

Field experiments were set up to investigate the interactive effects of N and S on seed and oil yield of non-nodulating soybean (*Glycine max* (L.) Merr.) cv. PK-416 (V₁) and cv. PK-1024 (V₂). The cultivars were grown in the experimental field of Hamdard University, New Delhi, during the rainy season. The initial N and S contents in the soil were 0.07 and 0.002%, respectively. The treatments consisted of two levels of S (0 and 40 kg ha⁻¹) and two levels of N (23.5 and 43.5 kg ha⁻¹) in different combinations: 0 S + 23.5 kg N ha⁻¹ (T₁); 0 S + 23.5+20 kg N ha⁻¹ (T₂); 40 S + 23.5 kg N ha⁻¹ (T₃); 40 S + 23.5 + 20 kg N ha⁻¹ (T₄); 20 + 20 S + 23.5 kg N ha⁻¹ (T₅); 20 + 20 S + 23.5 + 20 kg N ha⁻¹ (T₆). Each treatment had three replications. The sowing, culturing and harvesting of samples were carried out according to the method previously described (Jamal *et al.*, 2005).

Enzyme assays

Fresh leaves were collected at 30, 45, 60, 75 and 90 days after sowing (DAS) and used for enzyme activity assays. The *in vivo* assay of NR activity in the leaves was performed

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according to the procedure of Klepper *et al.* (1971) with slight modification by Nair & Abrol (1977). Nitrite was estimated by the methods of Evans & Nason (1953). 0.3 gm of fresh leaf was taken in an assay vial containing 3.0 ml 0.2 M phosphate buffer pH 6.8 and 3.0 ml 0.4 M potassium nitrate. The evacuation of air from the reaction mixture done by a vacuume pump for 1-2 min. After that vial was incubated at 33 °C in water both shaken for 60 min. After the laps of incubation time, vial was taken out and the reaction was stopped by keeping the vial in hot water for 5 min. From the total assay mediam, 0.2 ml was taken in a test tube. It was followed with the addition of 0.1 ml 1.0% sulphanimide and 1.0 ml 0.02% NEDD. Then it was kept for 20 min to developed colour. The volume was made up 6.0 ml by adding distilled water after laps of 20 min the absorbance was measured at 540 nm using a spectrophotometer.

In vitro assay of ATP-sulphurylase was performed following the method of Wilson & Bandurski (1958). Plant extract was prepared by grinding 1.0 g fresh leaf in 10.0 ml of 0.1 M tris-HCl (pH 8.0) containing 2 mM MgCl₂, 100 mM KCl, and 10 mM DTE in a glass homogeniser cooled with ice. The homogenate was centrifuged at 10,000 g for 10 min at 4°C. The assay was started by adding 0.4 ml of the reaction mixture to 0.1 ml of extract. Incubation was done at 30°C for 10 min. The reaction was stopped by heating the vial. After stopping the reaction add 1.0 ml 5N H₂SO₄, 0.5 ml 2.5% ammonium molybdate and 0.1 ml of reducing solution was added. The volume was made up 10.0 ml by adding distilled water after lapse of 20 min and the absorbance was measured at 660 nm.

Determination of oil and protein content

The seed and oil yields were determined from the plants harvested from an area of 1 m² from each plot. Oil content was measured by rapid gravimetric method developed by Kartha & Sethi (1957). 0.5 g seed and 2.0 g of sodium sulphate were ground by grinder. Ground material was filled in column and oil was eluted by petroleum ether on filter paper. Paper was dried, weighed and used to calculates the % oil content in the seed. Oil yield was calculated on the basis of oil % and seed yield. The seed N content was determined by the micro-Kjeldahl procedure (Linder, 1944). The crude protein content in seeds was estimated by applying the factor N x 6.25 to the seed N content and was expressed as a percentage of seed dry weight. Protein yield was calculated on the basis of protein percentage and seed yield. The statistical analysis was performed following the method of Nageswar (1983).

RESULTS AND DISCUSSION

Application of S along with N significantly enhanced NR activities over the application of N alone in the two soybean varieties (Fig. 1). The NR activities increased until 45 DAS then decreased thereafter in both varieties. The NR activity was high at 45 DAS in both the cultivars by the N and S applications. The activities were increased by about 28.9% and 78.8% by the T6 treatment compared with T1 treatment (Fig. 1).

Application of S along with N also significantly enhanced ATP-sulphurylase activities over the application of N alone in the two soybean varieties (Fig. 2). The ATP-sulphurylase activities increased up to 45 DAS then decreased thereafter in both varieties. The ATP-sulphurylase activity was high at 45 DAS in both the cultivars by the N and S applications. The activities were increased by about 84.6% and 89.9% by the T6 treatment compared with T1 treatment (Fig. 2).

Similar increases of NR and ATP-sulphurylase activities with N and S applications indicate that the two enzymes interact in the metabolic activities related to N and S assimilation. These results are consistent with those reported in many different plant species (Clarkson *et al.*, 1989; Ahmad

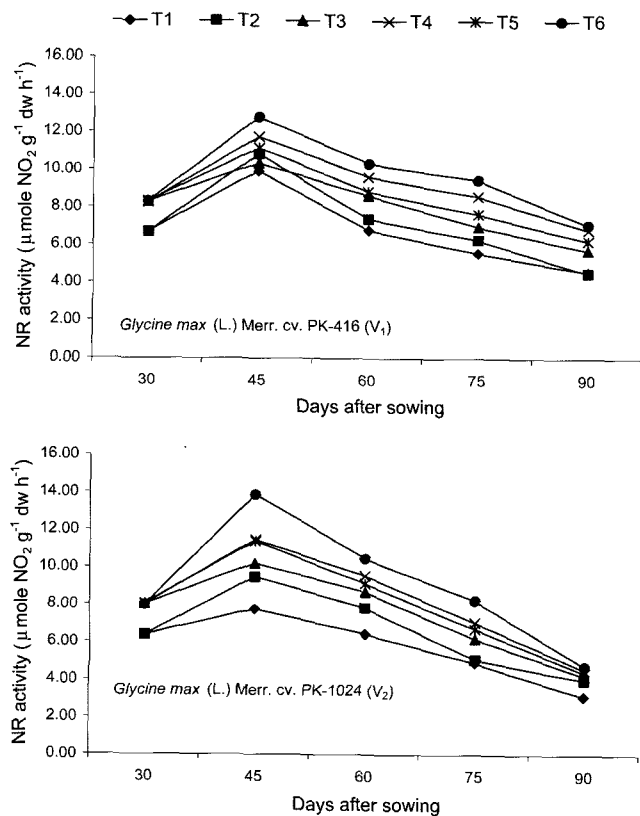


Fig. 1. Effect of sulphur and nitrogen on nitrate reductase activity of soybean cultivars at various growth stages.

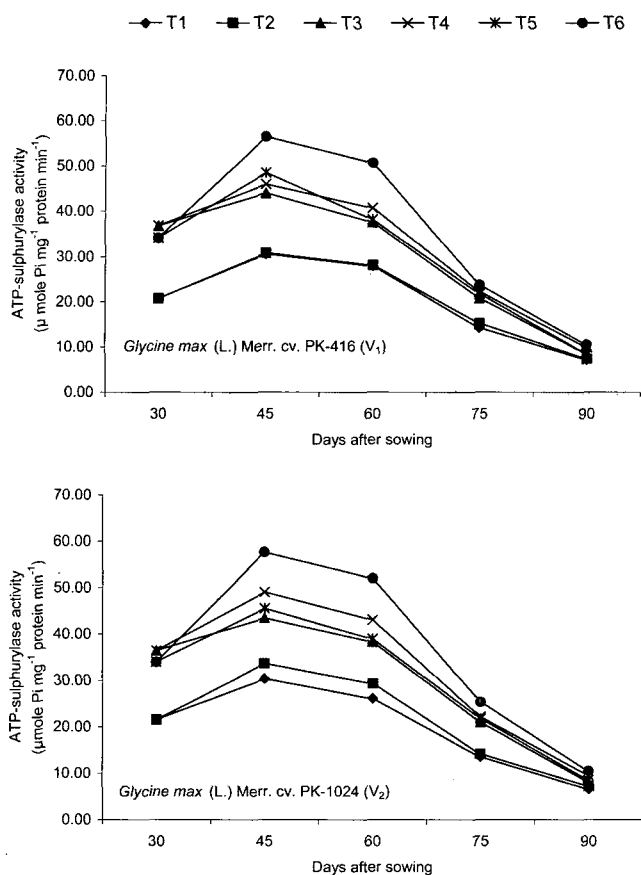


Fig. 2. Effect of sulphur and nitrogen on ATP-sulphurylase activity of the two soybean cultivars at various growth stages.

et al., 1999). Barney & Bush (1985) reported that tobacco plants treated with N but without S (+N-S) had very low NR activity because of the lack of S. When plants were transferred from +N-S to -N+S, NR activity remained very low because of the lack of N. Similarly, plants treated with -N+S had very low ATP-sulphurylase activity, perhaps the limited N supply in the plant prevented SO_4^{2-} translocation from the roots to the shoots. Clarkson *et al.* (1989) demonstrated that the apparent supply matching to demand is accompanied by an apparent linkage of nitrate and sulphate uptake at the whole plant level in barley.

The synthesis of cysteine as a result of the incorporation of sulphide moiety into O-acetyl serine appears to be the meeting point between N and S metabolism. The naturally occurring thiol compounds (cysteine and glutathione) were shown to influence NR activity in wheat and *Brassica* species (Lakkineni & Abrol, 1992). Reuveny *et al.* (1980) reported that in tobacco, induction of NR activity by nitrate was proportional to the initial sulphate concentration. Therefore, our result provides supporting evidence that the assimilatory pathways of both S and N are functionally convergent and there is a metabolic coupling between S and

Table 1. Effect of sulphur and nitrogen on protein, oil and seed yield of soybean (*Glycine max* (L.) Merr.) cultivars.

| Treatment | Protein yield | Oil yield | Seed yield |
|--|---------------|-----------|------------|
| | Kg/ha | Kg/ha | Kg/ha |
| <i>Glycine max</i> (L.) Merr. cv PK-416 (V ₁) | | | |
| T ₁ | 674 | 383 | 1798 |
| T ₂ | 796 | 452 | 2077 |
| T ₃ | 1023 | 570 | 2536 |
| T ₄ | 1399 | 786 | 3380 |
| T ₅ | 1272 | 707 | 3107 |
| T ₆ | 1598 | 903 | 3837 |
| <i>Glycine max</i> (L.) Merr. cv PK-1024 (V ₂) | | | |
| T ₁ | 743 | 396 | 1966 |
| T ₂ | 849 | 444 | 2186 |
| T ₃ | 1124 | 588 | 2742 |
| T ₄ | 1564 | 845 | 3735 |
| T ₅ | 1430 | 767 | 3443 |
| T ₆ | 1674 | 909 | 3967 |
| L.S.D. (0.05) | | | |
| Cultivars (V) | 13.9 | 12.9 | 54.5 |
| Treatment (T) | 25.1 | 22.3 | 94.4 |
| V x T | 33.2 | 31.6 | 133.5 |

N assimilation in soybean (Clarkson *et al.*, 1989).

Application of S and N also significantly enhanced protein, oil and seed yields. Maximum protein, oil and seed yields were achieved in the treatment T₆ in the two varieties (Table 1). The increase rates of protein, oil and seed yields in the T₆ treatment compared to T₁ treatment ranged from 125-137%, 129-135% and 101-113% in both varieties. The high response of soybean cultivars to the treatment T₆ may be due to the balanced application of N and S. Since both of these nutrients are involved in the biosynthesis of the proteins and thiol compounds, a balanced application of S and N enhanced their use efficiency in crop plants. In the treatment T₅ seed, oil and protein yield was decreased because the imbalanced supply of N and S. Maximum seed and oil yield was obtained only when S and N applications were balanced (Ahmad, *et al.*, 1998; Jamal *et al.*, 2005). Similarly, a strong coupling between S and N has been established in many studies in terms of dry matter and seed as well as oil and protein yield in several crops (Abdin *et al.*, 2003; Fazli *et al.*, 2005; Jamal *et al.*, 2005).

Interestingly, apparent positive relationships are indicated between the levels of NR and ATP-sulphurylase activities and seed yield in the two varieties. A strong positive correlation between NR activity and seed yield ($r = 0.9887$ in V₁ and $r = 0.9328$ in V₂) is observed in the two soybean culti-

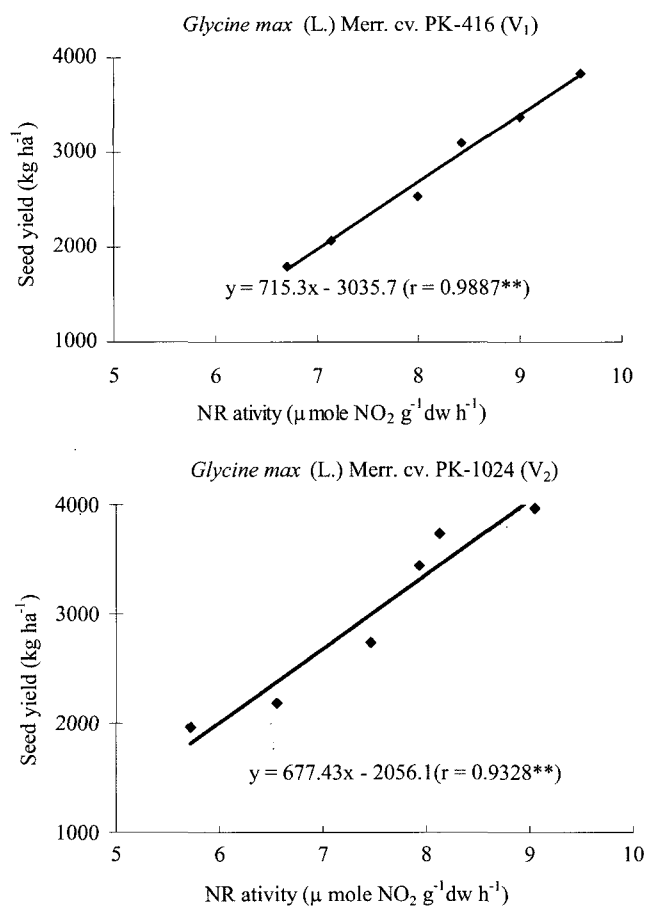


Fig. 3. Relationship between seasonal nitrate reductase activity in leaves and seed yield of soybean cultivar.

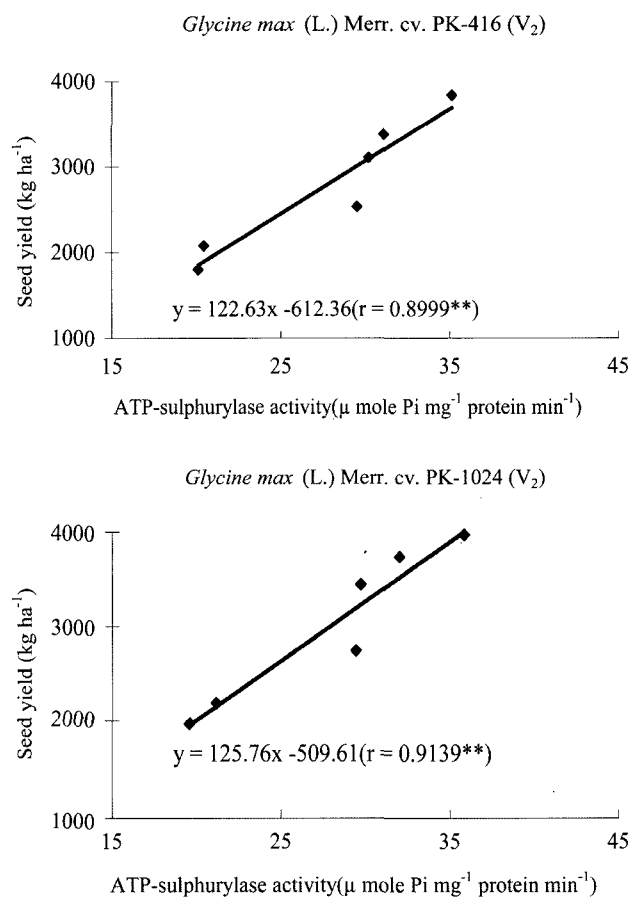


Fig. 4. Relationship between seasonal ATP-sulphurylase activity in leaves and seed yield of soybean cultivar.

vars (Fig. 3). A similar correlation between the NR activity and seed yield was also observed in other crop species, e.g. maize (Balasubramanian *et al.*, 1977), Brassica species (Ahmad *et al.*, 1999) and wheat (Nair & Abrol, 1982). The result from this study also supports that the strategic role of NR in nitrogen metabolism has profound effects on the productivity and quality of soybean.

A highly significant positive relationship is also apparent between the ATP-sulphurylase activity and seed yield ($r = 0.8999$ in V₁ and $r = 0.9139$ in V₂) (Fig. 4). Ahmad *et al.* (1999) observed a similar correlation between ATP-sulphurylase activity and seed yield in Brassica species. ATP-sulphurylase catalyses the first step of the S assimilation pathway. Thus, the enzyme plays a key role in regulating the inflow of S, just as NR regulates the inflow of reduced N in plants. Furthermore, there is a strong metabolic coupling between N and S assimilation.

Carbon and nitrogen are key elements of biomolecules such as amino acids and proteins. Thus, the carbon assimilation pathway is closely associated with nitrate assimilation. Nitrogen assimilation is also closely linked to

S metabolism through S-containing amino acids and thiol compounds. The role of S is a link to the function of NR, the enzyme responsible for conversion of NO₃⁻-N taken up by the crop in to amino acid and subsequently in to protein (Reuveny *et al.*, 1980; Barney & Bush, 1985). Furthermore, S is a constituent of the initiation amino acid methionine, which is essential for protein in eukaryotes. Thus, an imbalance in N and S may have an adverse effect on protein metabolism (Beaton & Wagner, 1985). Increase in oil content with increased N and S fertilization should be closely associated with increases in acetyl-CoA carboxylase activity coupled with increase in acetyl-CoA concentration. In the developing seeds of rapeseed, S fertilization resulted in increased acetyl-CoA carboxylase activity. Furthermore, sugar content was also increased with S fertilization providing carbon source for oil biosynthesis (Ahmad *et al.*, 1999). Thus, the activities of NR and ATP sulphurylase, which are critically affected by the availability of N and S, influence significantly on the productivity and quality of soybean seeds.

It is concluded from the results obtained in this study that

increases in NR and ATP-sulphurylase activities due to application of balanced doses of N and S fertilizers lead to higher seed, oil and protein yield in soybean cultivars. Sulphur must be included in the nutrient management package for optimum yield and quality attributes of soybean cultivars.

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