Effect of Sulfur on Nitrate Reductase and ATP Sulfurylase Activities in Groundnut (*Arachis hypogea* L.)

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Field experiments were conducted to determine the effect of sulfur (S) and Nitrogen (N) on nitrate reductase (NR) and ATP-sulfurylase activities in groundnut cultivars (*Arachis hypogea* L. cv. Ambar and Kaushal). Two combinations of S (in kg ha^{-1}): 0S (-S) and 20S (+S) were used with 20 kg ha^{-1} N. The application of S enhanced the NR and ATP-sulfurylase activities in both the cultivars at all the growth stages. The application of S also increased soluble protein and chlorophyll content in the all growth stages of both the cultivars. NR and ATP-sulfurylase 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 sulfate, respectively.

Keywords: ATP-sulfurylase, chlorophyll, leaf soluble protein, nitrate reductase, sulfur

The most frequent limitation of plant growth is the deficiency in macronutrients. General responses to carbon, nitrogen, and sulfur limitation are reduced growth and photosynthesis (Davies and Grossman, 1998). Sulfur is often referred to as the fourth major plant nutrient as it is an essential component of important metabolic and structural compounds. Sulfur is a constituent of proteins, lipids, carbohydrates, electron transport systems, and many other cellular constituents and intermediate metabolites (Leustek et al., 2000). Most sulfur is imported into cells as sulfate and then translocated into chloroplasts. Before sulfate is metabolized, it is adenylated by ATP-sulfurylase to form 5-adenylylsulfate, APS. APS is a branch point intermediate that can be phosphorylated by APS kinase and used in sulfation reactions, or used to synthesize cysteine (Cys). To form Cys, APS is reduced by sequential reactions catalyzed by APS reductase and sulfite reductase to form sulfide. Sulfide is subsequently combined with *O*-acetyl-serine (OAS) to form Cys by OAS (thiol) lyase. Cys is incorporated into proteins and is the precursor of methionine (Met) and *S*-adenosyl-L-Met in one set of reactions, and glutathione and phytochelatins in another.

Little is known about the interaction of N metabolism with S metabolism. Elements of this interaction may be the co-regulation of nitrate and sulfate uptake, the down-regulation of NR and changes in the level of OAS, the intermediate in Cys biosynthesis under S limitation (Clarkson et al., 1989; Giordano et al., 2000). Even less information is available on the interaction of sulfur and carbon metabolism. So far, mostly isolated steps of the corresponding pathways have been investigated during limitation of sulfate as a nutrient, although S is a key component of essential cell constituents (Hell, 1997).

Keeping in view the above facts, experiments were carried out to investigate nitrate reductase and ATP-sulfurylase activity as well as leaf soluble protein and chlorophyll content of groundnut cultivars to the application of S.

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MATERIALS AND METHODS

Plant Materials

The seeds of groundnut were collected from C.S.A. Agriculture & Technology University Kanpur, India. Two cultivars of groundnut namely Amber (V_1) spreading type and Kaushal (V_2) bunch type were selected for the experiment. The cultivars were grown on sandy loam soil at the experimental field of Hamdard University, New Delhi, India at 28° 38'N, 77° 11'E and 228 m altitude, during the rainy season. The sampling was done at 30, 45, 60, 75 and 90 d after sowing (DAS). The soil was a sandy loam with pH 7.1 and deficient in S (0.001%).

Nutrition Treatments

S was recognized as an essential fertilizer and interacted with nitrogen. Two treatments, -S (T_1) and +S 20 kg ha^{-1} (T_2) were used. N, phosphorus (P) and potassium (K) were applied at the rate of 20, 60 and 40 kg ha^{-1} respectively, in both treatments.

Enzyme Activity Assays and Protein and Chlorophyll Measurements

Fresh leaves were used for assay. The *in vivo* assay of NR activity in the leaves was performed according to Klepper et al. (1971) with slight modification by Nair and Abrol (1997). *In vitro* assay of ATP-sulfurylase was performed following the method of Wilson and Bandurski (1958). Soluble protein content was determined by the method of Bradford (1976). Chlorophyll was extracted using dimethylsulphoxide (DMSO) by the method of Hiscox and Israelstam (1979). The statistical analysis was performed following the method of Nageswar (1983).

RESULTS

Nitrate Reductase Activity

The cultivars, V_1 and V_2 had shown slight differences in

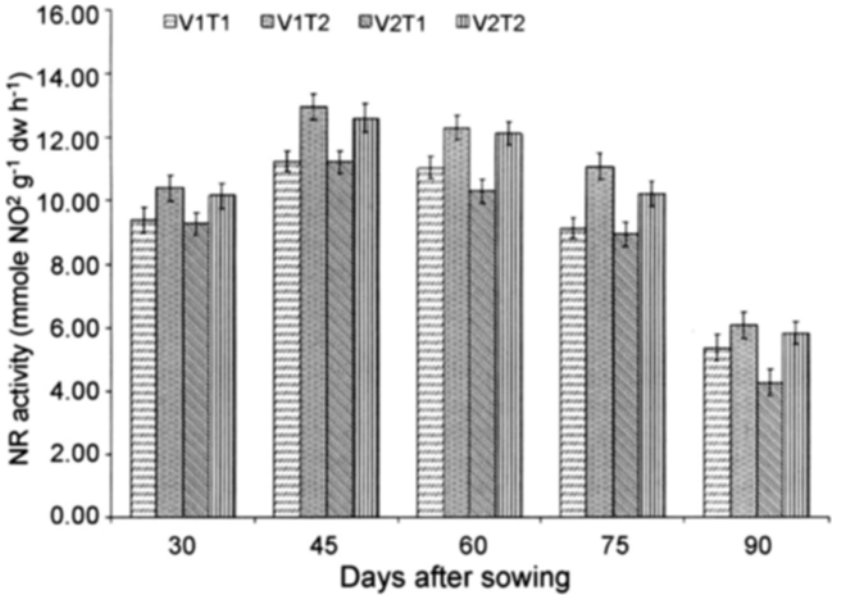

Figure 1. Effect of sulfur on nitrate reductase (NR) activity of groundnut cultivar at various growth stages.

the nitrate reductase activity and these differences were significant. The magnitude of NR activity in V_1 at 30 DAS varied from 9.37-10.40 μmole NO_2^- g^{-1} dw h^{-1} and increased continuously attaining peak values ranging from 11.25-12.97 μmole NO_2^- g^{-1} dw h^{-1} at 45 DAS with these treatments. Corresponding figures for V_2 ranged from 9.27-10.14 μmole NO_2^- g^{-1} dw h^{-1} and 11.22-12.61 μmole NO_2^- g^{-1} dw h^{-1}. The NR activity increased till 45 DAS and declined thereafter in both cultivars. Amongst the treatments, V_1 and V_2 responded maximum to the treatment, T_2 (10.40, 12.97 and 6.06, and 10.14, 12.61 and 5.83 μmole NO_2^- g^{-1} dw h^{-1} at 30, 45 and 90 DAS) (Fig. 1).

ATP-Sulfurylase Activity

Significant differences in the ATP-sulfurylase activity were observed in the cultivars, V_1 and V_2, during various growth stages with the treatment of S. The magnitude of ATP-sulfurylase activity in V_1 at 30 DAS varied from 15.73-22.33 μmole Pi mg^{-1} protein min^{-1} and increased continuously attaining peak values ranging from 17.05-26.46 μmole Pi mg^{-1} protein min^{-1} at 45 DAS with these treatments. Corresponding figures for V_2 ranged 15.39-21.98 μmole Pi mg^{-1} protein min^{-1} and 16.94-25.26 μmole Pi mg^{-1} protein min^{-1}. The ATP-sulfurylase activity increased till 45 DAS and declined thereafter in both the cultivars. Amongst the treatment, V_1 and V_2 responded maximum to the treatment, T_2 (22.33, 26.46 and 8.22, and 21.98, 25.26 and 7.72 μmole Pi mg^{-1} protein min^{-1} at 30, 45 and 90 DAS) (Fig. 2).

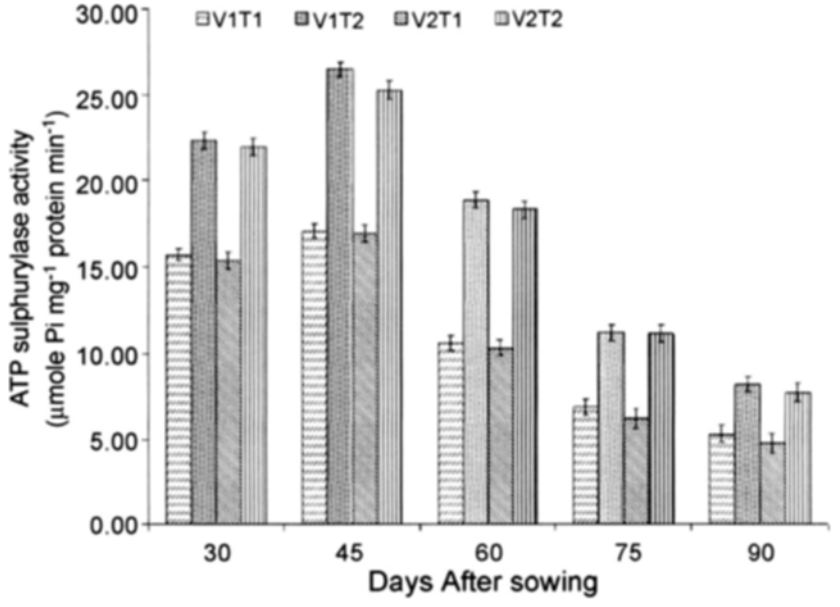

Figure 2. Effect of sulfur on ATP-sulfurylase activity of groundnut cultivar at various growth stages.

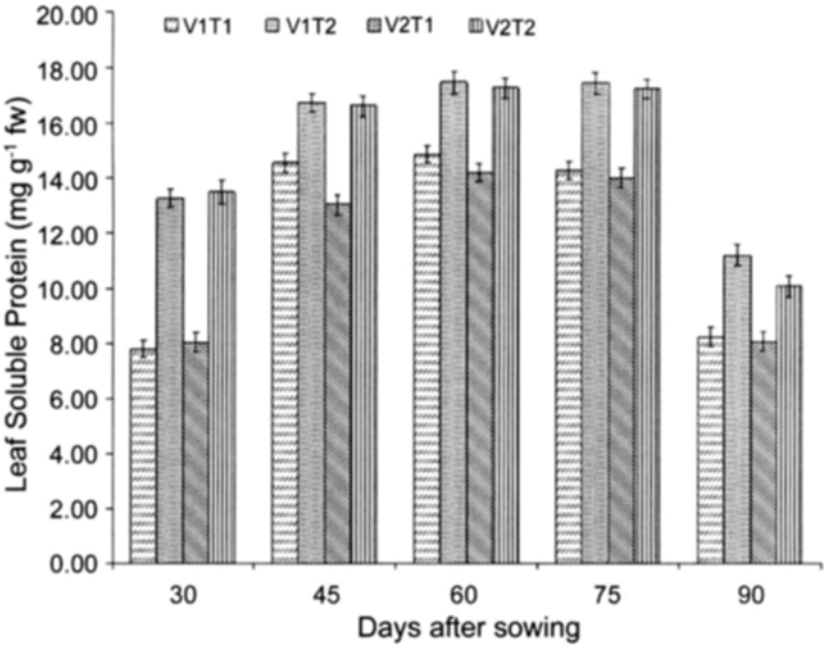

Figure 3. Effect of sulfur on soluble protein content in the leaves of groundnut cultivar at various growth stages.

Leaf Soluble Protein

Significant differences in the leaf soluble protein were observed in the cultivars, V_1 and V_2, during various growth stages with the treatment of S. The magnitude of leaf soluble protein in V_1 at 30 DAS varied from 7.77-13.27 mg g^{-1} fw and increased continuously attaining peak values ranging from 14.87-17.48 mg g^{-1} fw at 60 DAS with these treatments. Corresponding figures for V_2 were 8.01 to 13.49 mg g^{-1} fw and 14.21-17.27 mg g^{-1} fw. The leaf soluble protein increased till 60 DAS and declined thereafter in both the cultivars. Amongst the treatment, V_1 and V_2 responded maximum to the treatment, T_2 (13.27, 17.48 and 11.20, and 13.49, 17.27 and 10.07 mg g^{-1} fw at 30, 60 and 90 DAS) (Fig. 3).

Chlorophyll Content

Significant differences in the total chlorophyll content were observed in the cultivars, V_1 and V_2, during various growth stages with the treatment of S. The magnitude of chlorophyll contents in V_1 at 30 DAS varied from 1.40-1.67 mg g^{-1} fw and increased continuously attaining peak values ranging from 1.73-2.16 mg g^{-1} fw at 60 DAS with these treatments. Corresponding figures for V_2 were 1.34-1.65 mg g^{-1} fw and 1.73-2.05 mg g^{-1} fw. The chlorophyll contents increased till 60 DAS and declined thereafter in both the

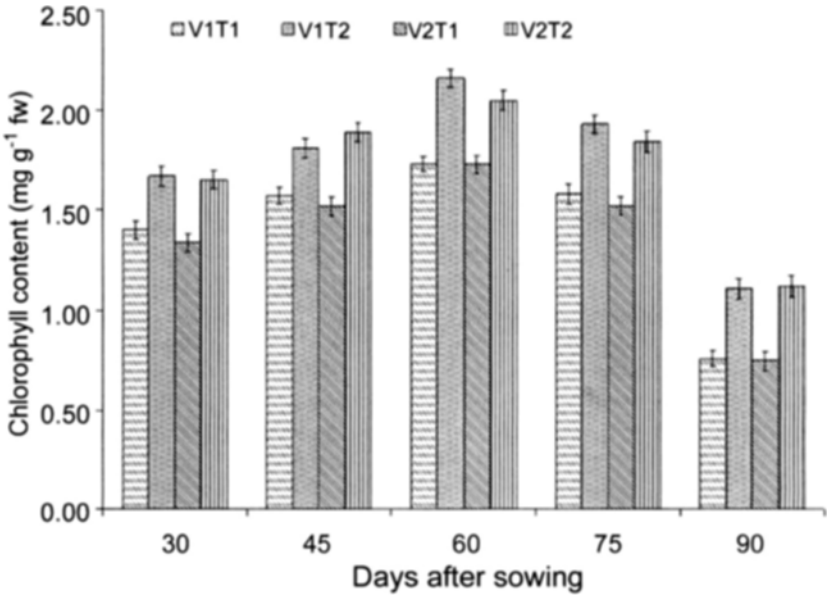

Figure 4. Effect of sulfur on chlorophyll content in the leaves of groundnut cultivar at various growth stages.

cultivars. Amongst the treatment, V_1 and V_2 responded maximum to the treatment, T_2 (1.67, 2.16 and 1.11, and 1.65, 2.05 and 1.12 mg g^{-1} fw at 30, 60 and 90 DAS) (Fig. 4).

DISCUSSION

In view of the key role played by the enzyme nitrate reductase in nitrate assimilation and of ATP-sulfurylase in sulfate assimilation, it is to be expected that the activities of these enzymes are related to seed and oil yield (data not shown). The highest NR and ATP-sulfurylase activities were observed during the various growth stages of the crop with the +S treatment when compared with the application of without S (-S) (Fig. 1, 2). These results are in consistent with those reported in different plants (Reuveny et al., 1980; Barney and Bush, 1985; Clarkson et al., 1989; Ahmad et al., 1999). The activities of ATP sulfurylase, APR, and OAS (thiol) lyase decreased under nitrogen-deficient conditions in *Lemna minor* and cultured tobacco (*Nicotiana tobacum*) cells (Reuveny et al., 1980; Smith, 1980; Brunold and Suter, 1984). The addition of nitrate or ammonia to the N-deficient medium quickly restored the activity of these enzymes. Supplementing ammonia or amino acids (Arg, Asn, and Gln) to normal nutrient solution caused 50 to 110% increase in extractable APR activity in *L. minor* and increased the flux through the sulfate assimilation measured as incorporation of ^{35}S in proteins after feeding with [^{35}S] sulfate (Brunold and Suter, 1984; Suter et al., 1986). In *Arabidopsis*, deprivation of a nitrogen source for 3 d led to 30 and 50% decrease of APR activity in leaves and roots, respectively, whereas the concentrations of cysteine and glutathione were not affected (Koprivova et al., 2000). The decrease of APR activity correlated with decreased mRNA and enzyme levels. On the other hand, in plants, sulfur deficiency results in a reduction of NR activity and an accumulation of amino acids (Reuveny et al., 1980; Migge et al., 2000; Prosser et al., 2001), whereas in cyanobacteria, NR is decreased and nitrite accumulates (Kramer and Schmidt, 1989). However, the reduction of NR activity and mRNA levels seems to be a relatively late process in plant adaptation to sulfur-limiting conditions (Prosser et al., 2001).

Data on leaf soluble protein content (Fig. 3) and chlorophyll content (Fig. 4) revealed that the application of +S resulted in significant enhancement in both the soluble protein and the chlorophyll content over the application of -S (T_1). When plants are grown with insufficient S, non-protein N accumulates in the vegetative tissue at the expense of protein N and growth is retarded (Eppendorfer, 1971). The increase in non-protein N in S-deficient plants is characterized by an accumulation of amides, usually asparagines (Stewart and Porter, 1969). The decline in soluble protein due to either low S supply is mainly a consequence of the linkage of N and S metabolism at the level of protein synthesis. Since Rubisco (ribulose-1,5-biosphosphate carboxylase/oxygenase) constitutes 50-70% of total soluble protein content, a change in soluble protein content affects the rate of photosynthesis. The observed strong reduction of photosynthesis under sulfur-limited growth correlates with a substantial decline of Rubisco and chlorophyll *a*/*b*-binding proteins. Yet the lowered photosynthetic response to carbon cannot be attributed to periplasmic carbonic anhydrase, whose activity was independent of sulfur concentration in the growth media. This decline in photosynthesis could therefore be the consequence of a combined reduction of major components of photosynthetic light and dark reaction. At the same time, due to its high abundance, Rubisco constitutes a large reservoir of reduced sulfur that has been estimated to reach 50 mM in the chloroplast (Ferreira and Teixeira, 1992). Therefore, lower ratios of Rubisco protein to total protein could facilitate the allocation of reduced sulfur and carbon skeletons to proteins specifically induced by sulfur limitation without an aggravation in sulfur and carbon consumption, as has been suggested for acclimation of wheat leaves to sulfate deprivation (Gilbert et al., 1997). The difference in cell volume between sulfur-limited and sulfur-sufficient cells enhances this process: On a volume basis, in fact, the amount of resources made available for sulfur-limited cells by the decrease of Rubisco would be even larger. At the onset of sulfur limitation, Rubisco may act as a source of fixed sulfur and carbon; in acclimated cells, the reduced synthesis of Rubisco would allow to shift resources to other more needed proteins.

The molecular mechanisms of the coordination of sulfate and nitrate assimilation are not yet completely understood. OAS is considered to connect these pathways as it regulates sulfate uptake and assimilation. In the presence of excess sulfate, OAS seems to be limiting for cysteine synthesis (Rennenberg, 1983). Overexpression of serine acetyltransferase, the enzyme synthesizing OAS, led to increased cysteine and glutathione (GSH) concentrations in transgenic potato (*Solanum tuberosum*) and tobacco (Blaszczyk et al., 1999; Harms et al., 2000). OAS accumulates during sulfur starvation and may thus act as a signal of the sulfur status (Kim et al., 1999). The addition of OAS increases sulfate uptake and APR activity and mRNA level also at normal sulfate levels (Koprivova, et al., 2000; Hesse et al., 2003). It is apparent that sulfate reduction is regulated by nitrogen nutrition on the transcriptional level, and OAS plays a major role in this regulation (Koprivova, et al., 2000).

From these observations, it can be concluded that the application of sulfur may result in increased nitrate reductase and ATP-sulfurylase activities in groundnut. This increase may be associated with the increase in leaf soluble protein content and chlorophyll content. Also, a strong metabolic coupling exists between sulfur and nitrogen assimilation at the cellular level. The most important role of S in plant metabolism comes from its essential participation in the making of proteins. Firstly, S is a constituent of methionine (21%), the first amino acid incorporated during the protein synthesis. Secondly, S is linked to the proper functioning of NR (Ahmad et al., 1999), the enzyme regulating the flow of NO^-_3-N into the amino acid and subsequently into protein.

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