Physiological and Biochemical Responses of Summer Peanut (*Arachis hypogaea* L.) to Nitrogen, Sulphur and *Rhizobium* Combinations

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Research experiments were conducted to resolve the physiological and biochemical basis of the effect of nitrogen (N) sulphur (S) and *Rhizobium* application on summer *Arachis hypogaea* L. Six treatments were applied in different N and S combination. Nitrate reductase (NR) activity and ATP-sulphurylase activity in the leaves were determined at various phonological stages, as the two enzymes catalyze rate-limiting steps of the assimilatory pathways of nitrate and sulphate, respectively. The highest NR activity, ATP-sulphurylase activity and yield component were achieved with the treatment T_4. Slightly differences from this treatment decreased the activity of these enzymes, resulting in decreases of the seed yield in *Arachis hypogaea* L. The maximum seed yield achieved in *Arachis hypogaea* L. at treatment T_4 could be due to optimization of leaf soluble protein and photosynthetic rate, as these factors are influenced by S and N assimilation.

Key words: ATP-sulphurylase, nitrate reductase, nitrogen, *Rhizobium,*sulphur.

Peanut (*Arachis hypogaea*) is an important oilseed crops, grown as an edible or an industrial oil crops, and also used as a source of edible protein. The major total oilseed production in the world is 464.7 million metric tons and production of peanut 36.7 million metric tons. Contribution of peanut in world oilseed productivity is only 7.89% as compared with soybean 56.7% is very low (FAO, 2011). To convene the cumulative demand for oil and oil products in world for the teeming growing population, the production of oilseed crops should increase further. This will require additional attention in agricultural research and in the popularization of improved technologies, including appropriate fertilizer application.

With the improvement of crop output through the adoption of fertilizer use has become more important to increase crop productivity. S is an essential plant nutrient for crop production. S is a vital nutrient for all plants and animals, as it is a constituent of cysteine, methionine, several coenzymes (e.g. biotin, coenzyme A, thiamine pyrophosphate and lipoic acid), thioredoxins and sulpholipids. It is required along with nitrogen in the synthesis of proteins and enzymes (Jamal *et al.*, 2005, 2006a, 2006b, 2009, 2010a, 2010b). Therefore, they responded not only to applied S, but their requirement for S is also the highest among crop plants, indicating a role of the nutrient in oil biosynthesis (Fazli *et al.* 2005).

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S deficiency in soil and plant are recognized as a wide spread problem all through the world (Messick and Debrey, 2001). The deficiency are intensified by increased use of high analysis fertilizer and S free pesticide, increased crop production, and reduced industrial SO_2 emission into the atmosphere. Consequently, the crop suffers from S-deficiency during the most crucial reproductive phase due to high leaching losses as most of the S-fertilizers release S as SO_4^{2-} ions (an ionic species highly susceptible to the leaching) in the soil solution and this results into the low productivity. Furthermore, because of strong metabolic coupling between N and S assimilation, S also limits nitrogen utilization efficiency of the crop and ultimately affects growth and yield. Additionally, the yield and quality of oilseed crops have declined due to the S-deficiency, as it is required along with N for the synthesis of proteins and enzymes (Abdin *et al.*, 2003a, 2003b; Jamal *et al.*, 2005, 2006a, 2006b, 2009, 2010a, 2010b). N and S are equally involved to enhance the plant protein synthesis (Jamal *et al.*, 2009). The shortage in S supply to crops decreases the N use efficiency of the crop (Fazli *et al.*, 2008). In addition, the poor efficiency of N utilization caused by insufficient S needed to convert N into biomass production may increase N losses from cultivated soils (Schnug and Henkelaus, 2000). In this investigation, therefore, an endeavor was made to develop appropriate nutrition application technology of N and S to improve the productivity of seed, oil and protein yield in summer groundnut crop.

MATERIALS AND METHODS

A research field experiment, employing randomized block design, was conducted to study the interactive effect of N and S on physiological and biochemical responses of spreading type summer peanut crop. The plants were grown on sandy loam soil. The S and N content in the soil were 0.002% and 0.072%, respectively. The treatments consisted of six combinations of two levels of S (0 and 40 kg ha^{-1}) and two levels of N (23.5 and 43.5 kg ha^{-1}) : 0 S + 23.5 kg N ha^{-1} (T_1); 0 S + 23.5+20 kg N ha^{-1} (T_2); 40 S + 23.5 kg N ha^{-1} (T_3); 40 S + 23.5+20 kg N ha^{-1} (T_4); 20+20 S + 23.5 kg N ha^{-1} (T_5); 20+20 S + 23.5+20 kg N ha^{-1} (T_6). N and S were given in split applications (first dose at the time of sowing and second at 35 days after sowing). Each treatment had three replications. The plot size was 9 m^2 (3×3 m). Phosphorous and potassium were applied to all the plots as basal dressings at the rate of 60 and 40 kg ha^{-1}, respectively. Similar concentrations of *Rhizobium* cultured seeds were used in all six treatments. The source of N, phosphorous, potassium and S were urea, diammonium phosphate, murate of potash and gypsum, respectively. Irrigation was applied as per requirement of the crop. By regular weeding operations, the crop was kept free from weeds. At two weeks after sowing, seedlings were thinned to keep an intra-row spacing of 30 cm and plant to plant distance of 10 cm.

An area (1 m^2) of each plot was earmarked for the purpose of harvest, analysis of seed yield and its components. The remaining rows (except the border rows) were used for taking periodic plant samples. The sampling was done at 30, 45, 60, 75, 90, 105 days after sowing (DAS) and at harvest. Three plants were taken from each plot randomly. The samples were cut at root-shoot junction, brought to the laboratory in moist polythene bags and immediately weighed. Leaves and stems were cut into small pieces. The stem and leaf samples were kept separately in an oven at 80 ^{0}C for 72 h. The biomass, seed yield, biological yield and harvest index were determined at final harvest from an area of 1 m^2 from each plot. Harvest index was calculated by the following equation given by Donald & Hamblin (1976): Harvest index = [Seed yield (gm^{-2}) / Biological yield (g m^{-2})] x 100

Soluble protein content was estimated in leaves by the method given by Bradford (1976). Plant extract was prepared by grinding 1.0 g fresh leaf in 10.0 ml of 0.1M Tris-HCl (pH 8.0) containing 2 mM MgCl$_2$, 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 ^{0}C. 1.0 ml of 10 % TCA was added to 0.5 ml of the crude extract and allowed to age for 30 minutes in ice. After adding, the mixture was centrifuge at 5,000 rpm for 5 minutes. The pellet obtained was washed with 1 ml of 5% TCA. The pellets ware dissolved in 0.1N NaOH. To 0.1ml of aliquot, 5 ml Bradford's reagent was added and mixed vigorously. A blue color was observed to develop within 2 minutes. The absorbance of mixture was measured at 595 nm with uv-vis spectrophotometer

(Model DU 640B, Beckman, USA). Calibration curve was drawn using bovine serum albumin prepared similarly of aliquots to calculate protein content.

Fresh leaves were collected at 30, 45, 60, 75 and 90 days after sowing (DAS) and used for enzyme assays. The *in vivo* assay of NR activity in the leaves was performed according to the procedure of Klepper *et al.,* (1971) with slight modification by Ahmad *et al.,* (2007). Nitrite was estimated by the methods of Evans & Nason (1953). In brief, 0.3 gm of fresh leaf was taken in an assay vial containing 3.0 ml 0.2M phosphate buffer pH 6.8 and 3.0 ml 0.4M potassium nitrate. The evacuation of air from the reaction mixture was done by a vaccume pump for 1-2 min. After that vial was incubated at 33 ^{0}C in water bath 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% sulphanilamide and 1.0 ml 0.02% NEDD. Then it was kept for 20 min to developed color. 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) with slight modification by Ahmad *et al.,* (2007). Plant extract was prepared by grinding 1.0 g fresh leaf in 10.0 ml of 0.1M Tris-HCl (pH 8.0) containing 2 mM $MgCl_2$, 100 mM KCl, and 10 mM DTE in a glass homogenizer cooled with ice. The homogenate was centrifuged at 10,000 g for 10 min at 4 ^{0}C. The assay was started by adding 0.4 ml of the reaction mixture [8 ml DD H_2O, 4 ml $MgCl_2$- 6 H_2O (40 mM) 3 ml of 0.4 M Tris buffer (pH 8.00) 40 mg Na_2MoO_4, 45 mg Na_2ATP, and 20 µl of inorganic pyrophosphatase] to 0.1 ml of extract. Incubation was done at 30 ^{0}C for 10 min. The reaction was stopped by heating the vial. After stopping the reaction add 1.0 ml 5N H_2SO_4, 0.5 ml 2.5% ammonium molybdate and 0.1 ml of reducing solution (10 mg of 1-aminonephthol sulfonic acid, 30 mg of Na_2SO_3. 7 H_2O and 60 mg of $Na_2S_2O_5$ were dissolved in 4 ml DD H_2O) were 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.

Oil content was measured as described by Kartha and Sethi (1957). In brief, 0.3 g of seed was weighed and transferred to a glass mortar. 0.2 g each of glass powder (Pyrex glass washed with concentrated hydrochloric acid) and anhydrous sodium sulphate were added and the mixture reduced to fine powder. The mixture powder was transferred to a small glass percolator (20 cm long and 1.5cm in diameter). The mortar and pestle were washed twice with 0.5 g of anhydrous sodium sulphate and the washings were also packed over the seed powder. Finally, the mortar and pestle were washed with 3ml of fresh distilled petroleum ether (b. p. 70-90 ^{0}C) and washing was added to the packed meal powder. The mixture was allowed to remain as such for 5 minutes and then percolated was started by adding 7ml of solvent on the top of the column. The extract was collected in a weighed disk containing four one-inch square-strips of filter paper. The solvent was evaporated by keeping the disk in an oven at 60^{0}C for half an hour and disk was re-weighed. The difference in the weight of disk before extraction and after extraction of oil was the weight of extracted oil. The statistical analysis was done following the method of Nageswar (1983).

RESULTS AND DISCUSSION

N and S interaction significantly enhanced seed, biological and oil yields. Maximum seed, biological and oil yields were achieved in the treatment T_4 (Fig. 1). The increase rates of seed, biological and oil yields in the T_4 treatment compared to T_1 treatment 86.3%, 52.95% and 97.51% respectively. The increased of oil content, harvest index and protein content in T_4 was 6.91%, 20.98% and 15.26% respectively (Fig. 2). Biomass accumulation also achieved 57.15% higher in T_4 treatment compared to T_1 treatment (Fig. 3). Soluble protein increased until 60 DAS and there after decline slowly. At the stage of 60 DAS soluble protein content were 19.56% higher in compared to T_1 treatment (Fig. 4). The maximum response of peanut to the treatment T_4 may be due to the appropriate balance 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 N and S enhanced their use efficiency in crop plants (Jamal *et al*., 2009). In

the treatment T_5 and T_6 seed, biological and oil yields as well as oil content, harvest index and protein content was decreased because the imbalanced supply of N and S. Maximum seed and oil yield was obtained only when N and S applications were balanced for interact each other (Ahmad, *et al.*, 2007; Jamal *et al.*, 2005, 2006a). Similarly, a strong coupling between S and N has been established in many studies in terms of

biomass and seed as well as oil and protein yield in several crops (Fazli *et al.*, 2005, 2008; Jamal *et al.*, 2005). S is a basic 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). Fazli *et al.*, (2005) reported that, the N and S fertilization should be intimately associated with increases in acetyl-CoA
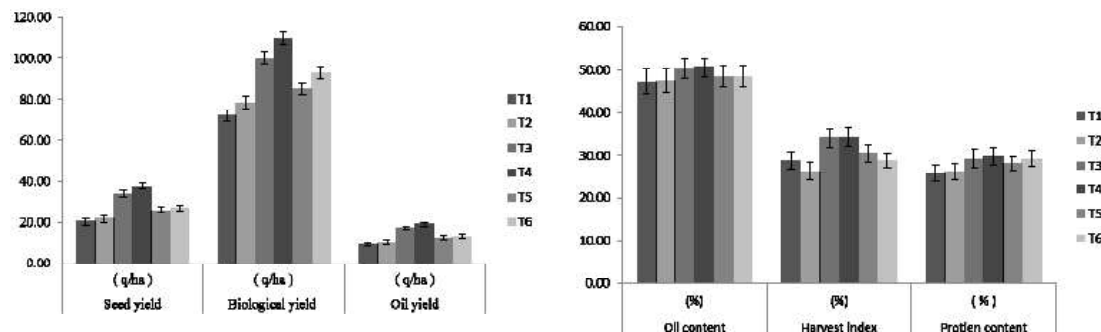

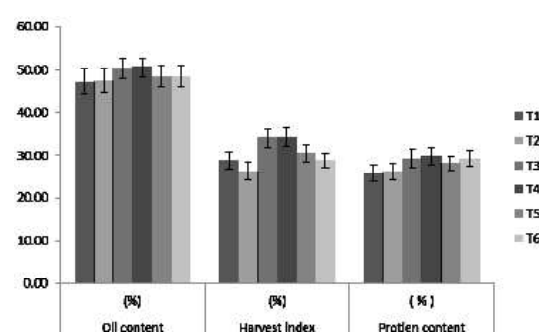**Fig. 1.** Effect of N and S on seed yield, biological yield and harvest index of peanut

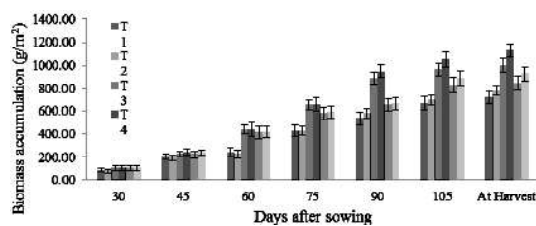**Fig. 2.** Effect of N and S on oil content, harvest index and protein content of peanut

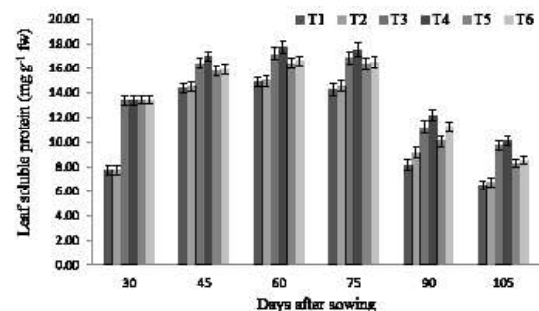**Fig. 3.** Effect of N and S on biomass accumulation of paenut at various growth stages

Fig. 4. Effect of N and S on leaf soluble protein content of peanut at various growth stages
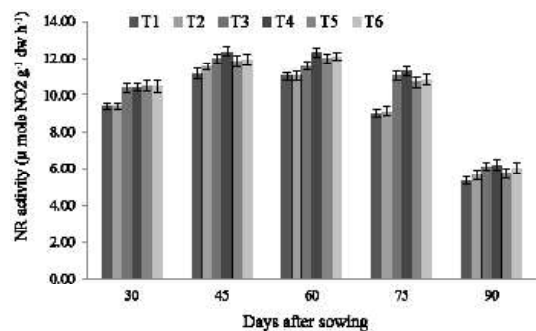

Fig. 5. Effect of N and S on Nitrate reductase activities of peanut at various growth stages
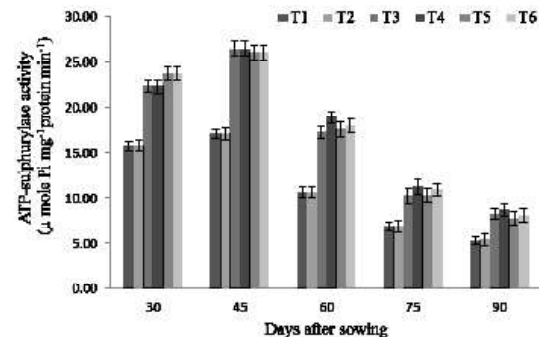

Fig. 6. Effect of N and S on ATP-sulphurylase activities of peanut at various growth stages

carboxylase activity attached with increase in acetyl-CoA concentration. In the developing seeds of rapeseed, S fertilization resulted in increased acetyl-CoA carboxylase activity.

Application of S along with N significantly boosted NR activity over the application of N alone in the peanut plant leaves (Fig. 5). The NR activities increased until 45 DAS then decreased thereafter. The NR activity was high at 45 DAS by the N and S applications. The activity was 12.35 µ mole NO_2 /gram dry weight/ hour increased by 10.76% in the T_4 treatment compared with T_1 treatment 11.15 µ mole NO_2 /gram dry weight/ hour. N along with S also significantly enhanced ATP-sulphurylase activities over the application of N alone (Fig. 6). The ATP-sulphurylase activities increased up to 45 DAS then decreased thereafter. The ATP-sulphurylase activity was high at 45 DAS by the N and S applications. The activity was 26.46 µ mole Pi/ mg protein/ min increased by about 55.19% by the T_4 treatment compared with T_1 treatment 17.05 µ mole Pi/ mg protein/ min. 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. Tobacco plants treated with N but without S (+N-S) had very low NR activity in the view of the fact that the lack of S. When plants were transferred from +N -S to –N +S, NR activity remained very low for the reason of the lack of N (Barney and Bush, 1985). In the same way, 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. The apparent supply matching to demand is accompanied by an apparent linkage of nitrate and sulphate uptake at the whole plant level in barley, rapeseed, soybean and groundnut (Clarkson *et al.* 1989, Abdin *et al.*, 2003, Jamal *et al.*, 2005, 2006a). Under S-deficient conditions, reduced protein synthesis is escorted by the accumulation of organic and inorganic nitrogenous compounds. Plants starved of S accumulate arginine and asparagine with reduced levels of S-containing amino acids including cysteine and methionine (Prosser *et al.*, 2001; McCallum *et al.*, 2002; Nikiforova *et al.*, 2003). S uptake and assimilation have been revealed to be dependent upon a continuous supply of the precursor of cysteine,

O-acetylserine, which in turn, is dependent upon adequate nitrogen and carbon availability (Koprivova *et al.*, 2000). Excess cysteine or another reduced S-compound represses the uptake and assimilation of S, either when excess S or limited N conditions pertain (Zhao *et al.*, 1999).

The high response of summer groundnut cultivars to the treatment T_4 may be due to the balanced application of N, S and *Rhizobium*. Subsequently, these nutrients are involved in the biosynthesis of proteins and numerous other important biomolecules, as well as enhanced their use efficiency in crop plants. Similarly, a strong metabolic coupling between N and S has been established through many studies in terms of dry matter, seed and oil yield in several crops (Zhao *et al.*, 1999; Abdin *et al.*, 2003a, 2003b; Fazli *et al.*, 2005).

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