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Enzyme activity assessment of peanut (*Arachis hypogea* L.) under slow-release sulphur fertilization

Arshad Jamal[1*], Yong-Sun Moon[1], Malik Zainul Abdin[2]

[1]Department of Horticulture Science, College of Natural Resources, Yeungnam University, Gyeongsan 712-749, Republic of Korea
[2]Department of Biotechnology, Faculty of Science, Jamia Hamdard, New Delhi-110062, India

***Corresponding author: arsjamal@gmail.com**

Abstract

Field experiments were presently conducted to investigate the effect of slow release sulphur (S) nutrition on crop growth, enzyme activities and yield attributes in peanut cultivars. Two combinations of sulphur (in Kg/ha): 0S (-S) and 20S (+S) were used. In +S treatment, S was applied as a single basal dose in the field of peanut crop. The results showed that application of S (+S) significantly ($P<0.05$) increased the biomass accumulation in both the genotypes at all the growth stages compared with without S (-S). Rapid increases of nitrate reductase (NR) and ATP-sulphurylase were observed up to 45 days after sowing (DAS), and thereafter decline. Nodule weight and nitrogenase activity was increased up to 75 DAS and thereafter, these parameters declined. S fertilization favorably influenced NR, ATP-sulphurylase, nodule weight and nitrogenase activity. Seed yield, biological yield and harvest index were also enhanced by slow S-release fertilization.

Key words: *Arachis hypogea* L., nitrate reductase, ATP-sulphurylase, nitrogenase acitivity, sulphur glass fritz

Abbreviations: APR_Adenosine 5'-phosphosulphate reductase; AR_Acetylene reduction; DAS_Days after sowing; NR_Nitrate reductase; OAS_O-acetyl-serine; SGF_Sulfur glass fritz.

Introduction

Sulphur is considered to be the fourth major plant nutrient after nitrogen (N), phosphorous (P) and potassium (K), as it is an essential component of important metabolic and structural compounds. Most agronomic practices that have been developed for the cultivation of oilseeds and pulses do not include S. Consequently, these crops suffer from S-deficiency during the most crucial reproductive phase due to high leaching losses, as most of the S-containing fertilizers release S as SO_4^{2-} ion, which is highly susceptible to leaching. The metabolic requirement of plants for is closely related with N nutrition (Reuveny *et al.,* 1980). Nitrogen metabolism is strongly affected by the S status of the plant (Duke and Reisenauer, 1986; Fazli *et al.,* 2008). Sulphur deficiency decreases the concentration of N in the shoots of legumes (Andrew, 1977; Robson, 1983; Zhao *et al*., 1999). Whether this is due to a direct effect on symbiotic N_2-fixation or a more general effect on the host plant is not clear. If symbiotic N_2-fixation has a greater requirement for a nutrient than the growth of host plants, a negative interaction between the addition of that nutrient and inorganic N on plant growth is expected (Robson, 1983). Anderson and Spencer (1950) found no negative interaction between S and inorganic N on the growth of clover. This led them to conclude that the restricted growth of the S-deficient clover was not due to poor N_2-fixation, but to a deficiency in the host legume. Sulphur deficiency in legume crops affects not only yield, but also the nutritional quality of the seeds, since methionine is usually the most limiting essential amino acid in legume seeds (Friedman, 1996) Legume seeds also have relatively low concentrations of cysteine, which is regarded as conditionally indispensable for humans and animals. Different storage proteins in legume seeds vary considerably in their contents of S-containing amino acids. For example, the pea storage proteins vicilin and lectin contain no cysteine and methionine, whereas legumin has 1.7% S-containing amino acids (Spencer *et al.,* 1990). Many studies using different legumes have shown that S deficiency decreases the synthesis of S-rich storage proteins markedly, but increases the synthesis of S poor proteins concomitantly (Spencer *et al.,* 1990; Naito *et al.,* 1995). In comparison, little attention has been paid to the S nutrition of legumes. In particular, the question regarding the relative requirements of S by symbiotic N_2-fixation and by host plants has not been resolved unequivocally. In the present study, we investigated the effect of glass industrial waste material, sulphur glass fritz (SGF) as a slow sulfur-releasing fertilizer on peanut (*Arachis hypogea* L.).

Material and methods

Experimental materials

Two cultivars of peanut spreading type (V_1) and bunch type (V_2) were selected for the experiment. The cultivars were grown on sandy loam soil during the rainy season. The soil was a pH 7.1 sandy loam that was deficient in S (0.001%). S was recognized as an essential fertilizer and interacted with N. Two treatments, -S (0 kg S/ha) and +S (20 kg S/ha) were used. N, P and K were applied at the rate of 20, 60 and 40 kg/ha respectively, in both treatments. S was applied as a source of SGF. Similar concentrations of *Rhizobium* cultured seed were used in both treatments. Irrigation was applied as per requirement of the crop.

Determination of seed yield, biological yield and harvest index

A 1 m^2 area of each plot was earmarked for the purpose of harvest, analysis of seed yield and its components. The remaining rows (except border rows) were used to take periodic plant samples. Sampling was done at 30, 45, 60, 75, 90 and 105 days after sowing (DAS) and at harvest. Three plants were randomly taken from each plot. The samples were cut at the root-shoot junction, brought to the laboratory in moist polyethylene bags and immediately weighed. Leaves and stems were cut into small pieces, which were kept separately in an oven at 80^0C 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. Statistical analysis was done following the method of Nageswar (1983). Harvest index was calculated by the following equation Donald and Hamblin (1976): Harvest index = [Seed yield (g/m^2) / Biological yield (g/m^2)] × 100.

Nitrate reductase Assay

Fresh leaves were collected at 30, 45, 60, 75 and 90 DAS and used for enzyme assays. The assay of NR activity in the leaves was performed as described previously (Klepper *et al,* 1971) with slight modification. Nitrite was estimated as described previously (Evans and Nason, 1953). In brief, 0.3 g of fresh leaf was taken to an assay vial containing 3.0 mL of 0.2M phosphate buffer pH 6.8 and 3.0 mL of 0.4M potassium nitrate. Air was evacuated from the reaction mixture by a vaccum pump operating for 1-2 min. The evacuated vial was incubated with shaking at 33^oC in water bath shaken for 60 min, after which it was removed and the reaction was stopped by placement of the vial in hot water for 5 min. A 0.2 mL aliquot was dispensed in a test tube followed with the addition of 0.1 mL 1.0% sulphanilamide and 1.0 mL 0.02% N-(1-Napthyl)-ethylene diammonium dichloride. Color development occurred over the next 20 min. The volume was made up 6.0 mL by adding distilled water and the absorbance was measured at 540 nm using a spectrophotometer.

ATP-sulphurylase assay

ATP-sulphurylase in the leaves was performed as described previously (Wilson and Bandurski, 1958) with slight modification. In brief, 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 $MgCl_2$, 100 mM KCl, and 10 mM dithioerythritol in a glass homogenizer cooled with ice. The homogenate was centrifuged at 10,000 ×g for 10 min at 4^oC. The assay was started by adding 0.4 mL of the reaction mixture [8 mL double distilled water, 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^oC for 10 min. The reaction was stopped by heating the vial and 1.0 mL of 5N H_2SO_4, 0.5 mL of 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 $Na_2S_2O_5$ dissolved in 4 mL double distilled water) were added. The volume was made up 10.0 mL by adding distilled H_2O after 20 min and the absorbance was measured at 660 nm.

Table 1. Effect of SGF on seed yield, biological yield and harvest index of peanut (*Arachis hypogea* L.) cultivars

Treatment	Seed yield (mt/ha)	Bilogical yield (mt/ha)	Harvest index (%)
V_1-S	1.64±0.05	5.58±0.13	29.51±0.18
V_1+S	3.13±0.09	9.33±0.15	35.53±0.19
V_2-S	2.53±0.1	8.17±0.15	30.96±0.18
V_2+S	4.54±0.11	13.19±0.16	34.46±0.18

Acetylene reduction assay

The assay procedure was performed as described previously (Hardy *et al.* 1968) with slight modification. In brief, a legume plant was uprooted and adhering soil was gently removed using tissue paper (washing was not advisable).

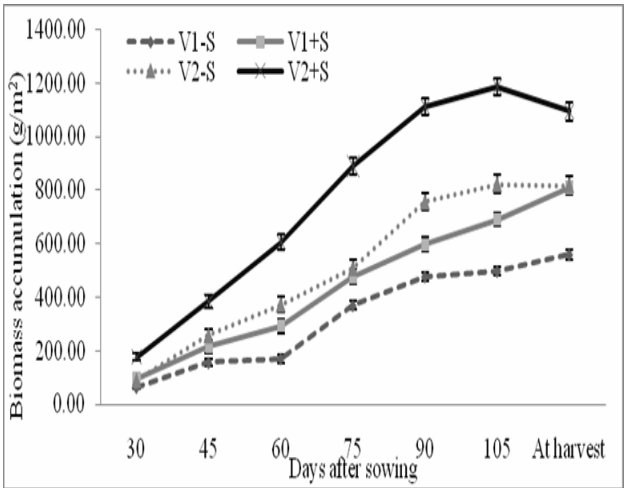

Fig 1. Effect of SGF on biomass accumulation of peanut cultivar at various growth stages

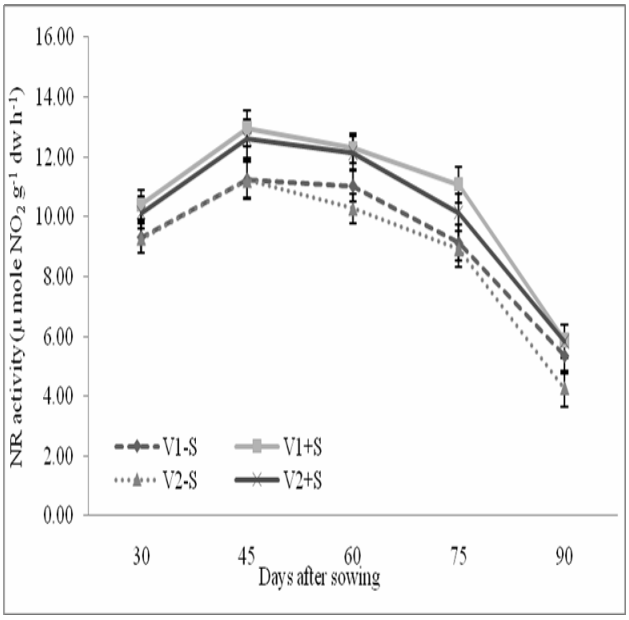

Fig 2. Effect of SGF on nitrate reductase activity in the leaves of peanut cultivar at various growth stages

The root system along with nodules was detached and the nodulated root was transferred to assay tubes. A serum stopper was inserted, the 10% of air was replaced with an equal volume of acetylene and the sample was incubated for 60 min at 28°C. A sample of gas (1 mL) was drawn from the tube and injected into Porapak N gas chromatography column equipped with a flame ionization detector for ethylene (C_2H_4) estimation. A volume of water equivalent to the volume of air space in the assay vial was added to the assay vial measured. The nodules were detached from the roots and the dry weight was determined. The produced C_2H_4 was determined using following equation: Nitrogenase activity (nmol C_2H_4 hr^{-1} g^{-1} dry weight of nodule) = (C × Ps × As × V) ÷ (Pstd × Astd × T × W) where C = concentration of ethylene in the standard in nmol, Ps = Peak height of sample, As = Attenuation used for sample, Pstd = Peak height of standard, Astd = Attenuation used for standard, T = Time of incubation in hrs, V = Volume of air space in the assay vial and W = Dry weight of nodules (g)

Results

Biomass accumulation

Fig. 1 shows the effect of +S and -S treatments on biomass accumulation of peanut cultivars at various growth stages. In cultivar V_1, biomass accumulation at 30 DAS varied from 64.40–97.44 g/m^2 and increased continuously, attaining peak values ranging from 558.7–933.33 g/m^2 at harvest with both the treatments. Corresponding figures for cultivar V_2 ranged from 89.10–159.0 g/m^2 at 30 DAS and 821.70–1452.33 g/m^2 at 105 DAS. There was a slight decline in biomass accumulation in cultivar V_2 at harvest.

Seed yield, biological yield and harvest index

Cultivar V_1 had low seed and biological yields (3.13 mt/h seed yield and 9.33 mt/h biological yield, respectively) and high harvest index (35.53%), as compared to V_2 (4.54 mt/h seed yield and 13.19 mt/h biological yield) and low harvest index (34.46%). The percent increase in seed yield of cultivars V_1 and V_2 in the +S condition was 90.85% and 79.44 %, respectively, when compared to the without S condition (Table. 1).

NR and ATP-sulphurylase activities

Figs. 2 and 3 show the effect of +S and -S treatment, respectively, on NR and ATP-sulphurylase activity in peanut. NR and ATP-sulphurylase activity increased until 45 DAS and declined thereafter in both cultivars. The percent increase of NR activity in peanut cultivars V_1 and V_2 at 45 DAS due to the addition of S was 42.22% and 35.91%, respectively, compared to –S. The magnitude of ATP-sulphurylase activity in V_1 and V_2 at 45 DAS was 73.05% and 70.74%, respectively.

Nodule weight and nitrogenase activity

Figs. 4 and 5 show the nodule weight and nitrogenase activity of the peanut cultivars at various growth stages. Both nodule weight and nitrogenase activity increased continuously up to 75 DAS in both cultivars and decreased thereafter with both treatments. The percent increase of nodule weight in V_1 and V_2 at 75 DAS due to the +S condition was 150% and 143.85%, respectively, compared to the -S condition. The increase of nitrogenase activity in V_1 and V_2 at 75 DAS was 184.89% and 169.2%, respectively.

Discussion

Plants grown on S-deficient soils have suppressed development of reproductive organs that, in rapeseed, can even lead to pod abortion (Fismes *et al.,* 2000). Reproductive growth and the proportion of the reproductive tissues in total dry matter are significantly increased by the application of S during pod development (McGrath and Zhao, 1996). In this experiment, the -S treatment resulted in S deficiency during the period of early plant growth. Plant growth was also reduced later in the growing season (Fig.1). The presence of S maximizes the seed and oil yields of other plants (Ahmad *et al.,* 2007; Fazli *et al.,* 2008). Under S-deficient conditions, an excess of unassimilated NO_3^--N or free amino acids accumulates in leaves (Hue *et al.,* 1991). Presently, NR and ATP-sulphurylase activities were maximum during different growth stages of the crop in the presence of SGF as a source of S (Figs. 2 and 3), consistent with the findings in different plants (Jamal *et al*., 2006; Ahmad *et al.,* 2007) when they applied calcium sulphate (gypsum) as a source of S. The activities of ATP sulphurylase, APR, and OAS (thiol) lyase decline under N-deficient conditions in *Lemna minor* and cultured tobacco *(Nicotiana tobacum)* cells (Reuveny *et al.,* 1980; Smith, 1980; Brunold and Surer,

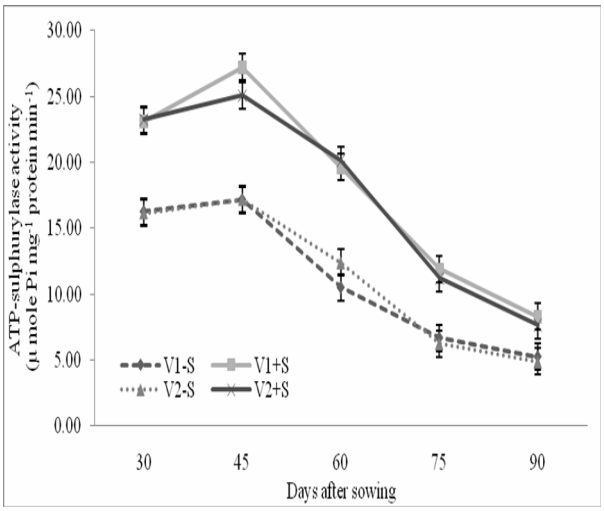

Fig 3. Effect of SGF on ATP-sulphurylase activity in the leaves of peanut cultivar at various growth stages

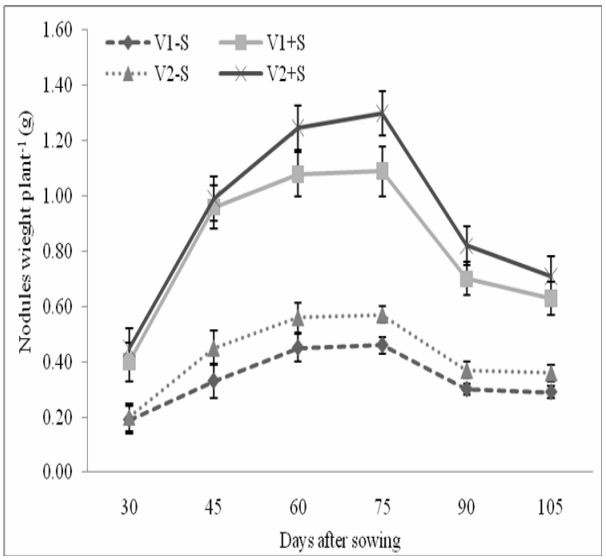

Fig 4. Effect of SGF on nodule weight of peanut cultivar at various growth stages

1984). On the other hand, S deficiency results in a reduction of NR activity and an accumulation of amino acids in a variety of plants (Reuveny *et al.,* 1980; Migge *et al.,* 2000; Prosser *et al.,* 2001). The latter study reported that the reduction of NR activity and mRNA levels seemed to be a relatively late process in plant adaptation to S-limiting conditions (Yoshimoto *et al.*, 2002). The replenishment of the nitrogen-deficient medium by nitrate or ammonia restored the activities of these enzymes. Ammonia also increased the flow of S-assimilation intermediates, measured as the radioactive sulphate incorporated into proteins (Brunold and Suter, 1984). Koprivova, *et al.,* (2000) demonstrated that, the sulphate reduction is regulated by nitrogen nutrition on the transcriptional level, and OAS plays an important role in this regulation. Additionally, the - S response of the β-conglycinin gene was dependent on the N/S status and accumulation of OAS (Kim *et al.*, 1999). The yield response to optimum S application, however, differs among crop species, being lower in *Medicago sativa* and *Pisum sativum* as compared to *Trifolium pratense* and *Vicia faba*, suggesting that legumes differ in their S requirement (Scherer and Lang, 1996). S supply only increases the S concentration of the plants without enhancing the yield (Gupta and McLeod, 1984). Although these studies show a link between the S supply of legumes and N_2 fixation, only a few experiments have been conducted to investigate the influence of S nutrition on this process. Shock *et al.* (1984) reported that applied S increases the percentage of symbiotically-derived N in subclover. With S-deficiency, amino acids and other N forms accumulate due to the impaired protein synthesis. This could be due to the feedback repression of N fixation (Janssen and Vitosh, 1974). Meanwhile Lang (1998) suggested that S affects leguminous species through its influence on N fixation by *Rhizobium* species. Accordingly, the present observations strongly support the view that biological N_2- fixation, nodulation and yield of peanut crops are reduced with S-deficiency. As compared to subterranean clover supplied with S, nodulation was markedly decreased in S-deficient clover. This is attributed to the decline in the requirement for N with reduced S supply. However, the observed increase in the number of nodules by S fertilization of legumes was not the result of increased nodulation per unit length of roots, but rather due to enhanced root growth (Gilbert and Robson, 1984; Scherer and Lang, 1996). Circumstantial evidence indicates that S-deficiency greatly diminishes carbon (C)-fixation of *M. sativa* (Mertz and Matsumoto, 1956), which is assumed to be caused by the reduction in the synthesis of key carbon metabolic enzymes as a result of reductions in the pools of the free S-containing amino acids, cysteine and methionine (DeBoer and Duke, 1982).

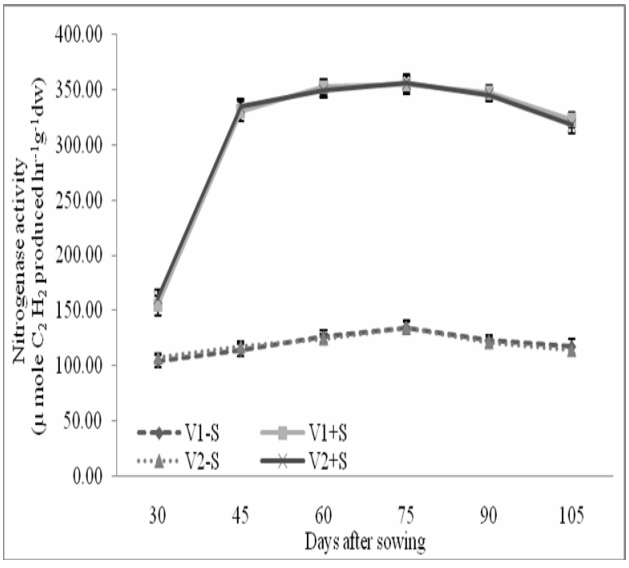

Fig 5. Effect of SGF on nitrogenase activity in the nodules of peanut cultivar at various growth stages

Scherer and Lang (1996) investigated the effect of S nutrition on the activity of key enzymes of the C and N metabolism of *Vicia faba* and *Pisum sativum*. Nitrogenase and ferredoxin, which play vital roles in N_2-fixation, are rich in S and contain Fe-S clusters (Duke and Reisenauer, 1986; Ali *et al.,* 2004). Based on the results obtained in this investigation, it can be concluded that biological N_2-fixation and yield attributes of peanut are strongly influenced by SGF application and it can be used as a source of S fertilizer. Addition of S in cultivation of peanut cultivars can result in the significant increase of the growth, yield, nodules weight, NR and ATP-sulphurylase activities, as well as nitrogenase activity; because metabolic coupling between S and N enhances assimilation of S and N by the plant.

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