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Antioxidant Status of Black Gram in Response to Bio Sulphur Granules Developed with *Methylobacterium thiocyanatum* in Sulphur Deficient Calcareous Vertisol

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ABSTRACT

Background: Sulphur (S) is an essential macronutrient required for growth and development of plants. This study was carried out to investigate the effect of Bio Sulphur Granules (BSG) developed with elemental sulphur and sulphur oxidizing bacteria (SOB) on the antioxidative defense system in blackgram under sulphur deficient calcareous soil.

Methods: Bio Sulphur Granule (BSG) was developed using ES plus SOB and its efficacy was tested in a pot experiment with blackgram as a test crop from April to June 2022. Plant samples were collected and analyzed in the laboratory for anti-oxidant status of blackgram, how sulphate stress affects the physiological and metabolic processes of plants, which in- turn affects crop yield.

Result: Photo assimilatory pigments were decreased and carbohydrates (sugar and starch) were accumulated in leaves of no sulphur treated plants. Hydrogen peroxide in without S supplied plant caused oxidative damage in plants, which also evident by the increase in activity of super oxide dismutase, catalase, peroxidase and ascorbate. The findings showed that by applying S as BSG granules (ES @ 40 kg S ha^{-1} + *Methylobacterium thiocyanatum* VRI7-A4) to S-deficient calcareous vertisols might prevent the oxidative damage of plant cells there by improve the growth of blackgram by S-oxidation.

Key words: Antioxidant status, Bio sulphur granules, Black gram, Calcareous soil, Sulphur deficiency.

INTRODUCTION

Blackgram (*Vigna mungo* L.) is one of the most highly prized pulse crops, cultivated in almost all parts of India. Being a proper leguminous crop, it is a mini-fertilizer factory, as it has unique characteristics of maintaining and restoring soil fertility through fixing atmospheric nitrogen in symbiotic association with Rhizobium present in the root nodules (Das, 2017). Sulphur (S) is an essential macronutrient required for growth and development of plants. It is indispensable for the synthesis of certain amino acids like cysteine, cystine and methionine besides being involved in various meta18) Sulphur deficiency is found all over the world including India and affects metabolic and physiological activities of plants, causes heavy losses in crop yield. Most of the S in soil environments (95% of total S) is bound to organic molecules and therefore not directly plant available. Use of S oxidizers enhances the rate of natural oxidation of S and speed up the production of sulphates (SO_4^{2-}) and makes them available to plants at their critical stages, consequently resulting in increased plant yield (Wainwright, 1986). Also, several studies have confirmed the importance of soil inoculation with sulphur oxidizing bacteria (SOB) to improve the S oxidation process in calcareous soil (Chaudhary *et al*., 2022).

Sulphur is found in essential compounds like cysteine (Cys), methionine (Met), thioredoxins and sulfolipids. Abiotic stress causes significant changes in electron transport in both chloroplasts and mitochondria, resulting in the formation of reactive oxygen species (ROS), which are partially reduced from O_2 excitation to form atmospheric oxygen (Apel and Hirt, 2004). They are formed, when O_2 is excited to form singlet oxygen (O_2^1), or when one, two, or three electrons are transferred to O_2 to form superoxide (O_2^{-2}), hydrogen peroxide (H_2O_2), or a hydroxyl radical (OH^-). The accumulation of reactive oxygen species, resulting in oxidative stress, is a common feature of several types of abiotic stress, including nutritional element deficiency or excess of nutrient element (Chandra and Pandey, 2014).

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Major ROS scavenging enzymes are super oxide dismutase (SOD), peroxidase (POD) and catalase (CAT). SOD accelerates the formation of hydrogen peroxide which is decomposed by catalase (Blokhina *et al*., 2003). Catalase is present in peroxisomes and responsible for removal of excess H_2O_2 during stress. There are very few reports an antioxidative response of plants to sulphur stress and most reports available are on the oxidative damage caused by SO_2 exposure. Changes in SOD were observed in pea cultivars exposed to SO_2 (Pandey *et al*., 2012). Increase in the concentration of antioxidants especially glutathione and in the activities of enzymes superoxide dismutase (SOD) and glutathione reductase (GR) involved in protection from oxidative stress were reported in wheat subjected to SO_2 fumigation (Soldatinl *et al*., 1992). In this study, the effect of bio sulphur granules (BSG) developed with elemental sulphur and sulphur oxidizing bacteria (SOB) on the antioxidative defense system in blackgram under sulphur deficient calcareous soil has been addressed.

MATERIALS AND METHODS

Growth conditions of SOB strains and development of Bio Sulphur Granules

Bio sulphur granules were created by combining ES, rice gruel (used as an adhesive) and SOB strains. For mass culture, strains of VRI 7-A4 (*Methylobacterium thiocyanatum*) and ASTB 16 (*Pandoraea thiooxydans*) were routinely cultured in nutrient broth (Himedia, India) for 48 h at 30°C under shaking conditions (120 rpm). ES was powdered, sieved through a fine mesh sieve (<0.5 mm) and sterilized. For 100 g of powdered elemental S, 250 ml of freshly prepared culture (1×10^8 cells/ml) were mixed and granulated by a pressure-less manual mixing method under aseptic conditions. By mechanical interlocking and cementing, ES powder, bacterial strains and rice gruel were combined and united into agglomerates (granules). Granulates of varied sizes are produced through manual preparation. Particle sizes ranging from 0 to 2 mm were separated using a 2 mm sieve. As a result, two separate bio-sulphur granules, *Pandoraea thiooxydans* ASTB 16 (BSG-I) and *Methylobacterium thiocyanatum* VRI 7-A4 (BSG-II), were created utilizing two different sulfur-oxidizing bacteria. The efficacy of these two granules was tested in pot experiments.

Pot culture experiment

The experiment was conducted during April to June (2022) in a greenhouse with opening windows at the experimental station of Department of Soil Science and Agricultural Chemistry, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India to study the efficacy of BSG on the blackgram in calcareous soil. S-deficient calcareous topsoil (0-20 cm) was collected from Eastern block, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India (11°0′37.66″N 76°56′23.85″E). The physical and chemical properties of surface soil (0-30 cm) were determined according to Page *et al.* (1982) which include; soil texture: sandy clay loam, clay: 25.4%, silt: 15.4% and sand: 58.6%, organic carbon: 0.58%, available N: 140 kg ha^{-1}, available P: 10.8 kg ha^{-1}, available K: 252 kg ha^{-1}, available S: 2.5 kg ha^{-1}, pH 8.3 and EC 0.03 dSm^{-1}.

The experiment was conducted in a completely randomized block design with ten different treatments (T_1- Absolute control; T_2- The recommended dose of NPK and Sulphur (RDF- 25:50:25 kg of N, P_2O_5, K_2O ha^{-1} and 40 kg S ha^{-1}) (Control); T_3-Soil test based NPK application; T_4-T_3 + Sulphur as Elemental Sulphur @ 40 kg S ha^{-1}; T_5-T_3 + Sulphur as Bio Sulphur Granules I @ 40 kg S ha^{-1}; T_6-T_3 + Sulphur as Bio Sulphur Granules II@ 40 kg S ha^{-1}; T_7-T_3 + Vermicompost @ 4 t ha^{-1}; T_8-T_4 + Vermicompost @ 4 t ha^{-1}; T_9- T_5 + Vermicompost @ 4 t ha^{-1}; T_{10}- T_6+ Vermicompost @ 4 t ha^{-1}) replicated thrice and 5 pots were maintained for each replication. Soil was air dried, crushed and passed through 2 mm sieve and 10 kg of soil was filled in earthen pots (11″ top diameter × 9.5″ base diameter × 9″ height). All the fertilizers and BSG were applied prior to sowing and all the pots received 25 kg N ha^{-1} as urea, 50 kg P_2O_5 ha^{-1} as DAP and 25 kg K_2O ha^{-1} as MOP except absolute control (T_1). Blackgram seeds (cv., VBN-8) obtained from Department of Pulses, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore were surface disinfected with 70% ethanol for 1 min, immersed in 0.5% NaOCl for 2 min and washed 4 times with sterilized distilled water and 10 seeds were sown in each pot and thinned to 5 plants per pot, two weeks after sowing. Throughout the experiment, soil moisture was maintained at field capacity on weight basis. An appropriate weeding and plant protection measures were undertaken as and when required.

Data acquisition

Plants from each replication were sampled on 45th days after sowing (DAS) and the experimental analysis was carried out to determine the response of variable sulphur supply on photo assimilatory pigments, carbohydrates (sugar and starch), proteins and antioxidative enzymes (SOD, CAT and POD). Finely chopped leaves were ground in a pestle mortar and extracted in 80% acetone with a pinch of calcium carbonate and this extract was centrifuged at 5000×g and spectrophotometric measurements for carotenoids were taken at 480 and 510 nm and 645 and 663 nm for chlorophylls, as described by (Lichtenhaler, 1987). At 500 nm, sugars were estimated calorimetrically using (Nelson, 1944) method. Montgomery's phenol method was used to calculate starch (Montgomery, 1957). The Brenan and Frenkel method (1977) were used to calculate hydrogen peroxide (H_2O_2) and Ascorbate (ASA).

Assay of antioxidative enzymes

For enzymes, the leaf tissues were stored in liquid nitrogen at -80°C. A fresh leaf samples (500 mg) were homogenized

in pestle and mortar using 50 mM Na_2HPO_4, pH 7.0 contains 1 M Sodium chloride, 1 mM EDTA and 1% polyvinylpyrrolidone. Enzyme activities were determined using supernatant of enzyme extract produced from sample solution after centrifugation (20,000 × g, 15 min) at 4°C. CAT activity was determined by monitoring the degradation of hydrogen peroxide (H_2O_2) as described by Chance and Maehly, (1955). POD activity was estimated spectrometrically at 480 nm as described by Chance and Maehly, 1955. The activity of SOD was recorded using the method of Van Rossun *et al*. (1997).

Statistical analysis

The data has been presented as the mean of observations (n=3) and the data were analyzed by an analysis of variance (ANOVA) using the general linear model version 9.1; SAS institute Inc, Cary, NC, USA. Means were compared by Tukey's post hoc test.

RESULTS AND DISCUSSION

Photo assimilatory pigments and carbohydrates

The photosynthetic pigments (Chl a, chl b and total chl) in leaves of blackgram were found to be significantly (*p<0.05) decreased by sulphur stress and the decrease was more pronounced in no S applied plants (S deficient) as compared to S applied (normal) plants (Table 1). Reduction in chlorophyll was observed in without sulphur treated plant, which is in consonance with results of Lunde *et al*. (2008) in rice. Concentration of carotenoid was also found to be depleted more in no sulphur applied plants (Table 1). In the short term, a possible correlation between photosynthesis and sulphur assimilation is also important, because photosynthesis in the field can vary significantly depending on environmental conditions and thus plant sulphur nutrition could be affected accordingly. Furthermore, sulphur metabolism in illuminated leaves is involved in electron consumption and redox metabolism, which can affect leaf photosynthetic capacity (Chandra and Pandey, 2014). In leaves, both reducing sugar and non-reducing sugar showed accumulation in sulphur stressed plants (Table 1). As compared to BSG II (T_6) treated plants, accumulation of non-reducing sugars was more in no sulphur applied plants (T_1) (Table 1). The accumulation of sugar is probably due to poor translocation resulting in poor growth and development. Starch was also accumulated in no sulphur received plants (T_1). The accumulation of starch is a known general response to nutrient deficiency and indicates that carbohydrate utilization is restricted under stress conditions. Because the oxidized Fd: thioredoxin system regulates both starch synthesis and degradation, starch metabolism is dependent on it. Thioredoxin is the primary regulator of carbon assimilation, changing the thiol disulpide redox state (Geigenberger *et al*., 2005). Plants'

Table 1: Effect of BSG on photo assimilatory pigments and carbohydrates of blackgram in sulphur deficient calcareous vertisol.

Treatments	Photo assimilatory pigments				Carbohydrates (% fresh weight)			
	Chlorophyll (mg g^{-1})			Carotenoids	Reducing	Non reducing	Total	Starch
	Chl a	Chl b	Total chl	(mg g^{-1})	Sugar	sugar	sugars	
T_1 - Absolute control	0.80±0.02^{l}	0.20±0.002^{l}	1.00±0.06^{l}	0.245±0.006^{i}	0.160±0.003^{a}	0.082±0.006^{a}	0.242±0.003^{a}	2.53±0.04^{a}
T_2 - NPK+S (Control)	1.16±0.01^{i}	0.26±0.005^{i}	1.42±0.02^{h}	0.361±0.008^{h}	0.120±0.011^{b}	0.080±0.009^{b}	0.200±0.001^{b}	2.42±0.03^{b}
T_3 - Soil test based NPK	1.62±0.03^{g}	0.37±0.007^{h}	1.99±0.02^{g}	0.437±0.002^{f}	0.085±0.034^{f}	0.059±0.001^{d}	0.144±0.007^{d}	1.92±0.01^{d}
T_4 - T_3 + ES	1.29±0.03^{h}	0.70±0.001^{g}	1.99±0.03^{g}	0.386±0.009^{g}	0.100±0.033^{c}	0.071±0.001^{c}	0.171±0.006^{c}	2.31±0.04^{c}
T_5 - T_3 + BSG I	2.11±0.04^{d}	1.11±0.010^{d}	3.22±0.06^{d}	0.555±0.005^{c}	0.076±0.033^{g}	0.042±0.007^{g}	0.118±0.007^{e}	1.18±0.01^{f}
T_6 - T_3 + BSG II	2.51±0.06^{a}	1.38±0.005^{a}	3.89±0.10^{a}	0.637±0.013^{a}	0.051±0.001^{j}	0.022±0.002^{j}	0.073±0.007^{h}	0.96±0.01^{h}
T_7 - T_3 + Vermicompost	1.87±0.04^{f}	0.84±0.001^{f}	2.71±0.02^{f}	0.521±0.012^{d}	0.090±0.003^{e}	0.051±0.003^{e}	0.141±0.001^{d}	1.29±0.01^{e}
T_8 - T_4 + Vermicompost	1.93±0.01^{e}	0.92±0.012^{e}	2.85±0.01^{e}	0.502±0.005^{e}	0.094±0.002^{d}	0.049±0.001^{f}	0.143±0.001^{d}	1.35±0.02^{e}
T_9 - T_5 + Vermicompost	2.23±0.02^{c}	1.19±0.009^{c}	3.42±0.01^{c}	0.589±0.006^{b}	0.069±0.001^{h}	0.036±0.001^{h}	0.105±0.001^{f}	1.12±0.03^{f}
T_{10} - T_6 + Vermicompost	2.34±0.06^{b}	1.28±0.011^{b}	3.62±0.01^{b}	0.601±0.011^{b}	0.063±0.001^{i}	0.025±0.003^{i}	0.088±0.010^{g}	1.05±0.02^{g}

Values are the mean of three replications ±SE (standard error). Within columns, values followed by different letters are significantly different at *p <0.05. Same letters are not different at *p<0.05 level, according to post hoc Tukey's test.

utilization of starch is thus inhibited in plants not supplied with S, leads to its accumulation.

Hydrogen peroxide and ascorbate

The current study demonstrates that sulphur stress affects the oxidative metabolism of black gram. When compared to S-treated plants (T_6), sulphur stress significantly increased reactive oxygen species (ROS) content in non-sulphur-treated plants (T_1), as evidenced by an increase in H_2O_2 concentration in these plants' leaves (Fig 1a). This result was in consistent with previous reports of Tiwari *et al*. (2004). Increased concentrations of O_2 and H_2O_2 cause lipid peroxidation, resulting inmembrane damage and electrolyte leakage. The plant has a variety of protective mechanisms and repair systems that can reduce the occurrence of oxidative damage caused by ROS (Apel and Hirt, 2004). Ascorbic acid is the most important ROS detoxifying compound in the aqueous phase because; it donates electrons in numerous enzymatic and non-enzymatic reactions. Through the ascorbate peroxidase reaction, ascorbic acid can directly scavenge superoxide,

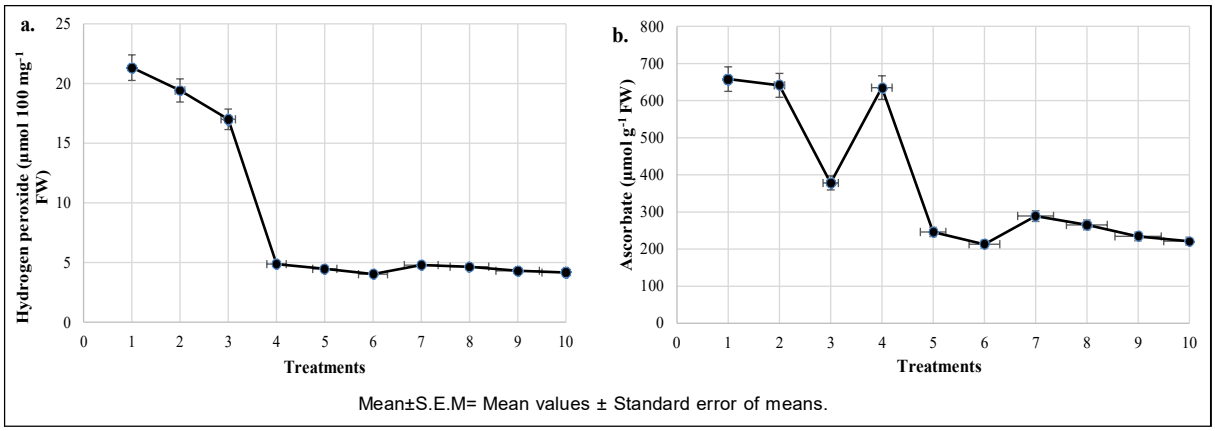


Fig 1: Effect of BSG on (a) hydrogen peroxide (H_2O_2) and (b) Ascorbate concentration of blackgram leaves on day 45 under pot culture condition in S deficient calcareous vertisol.

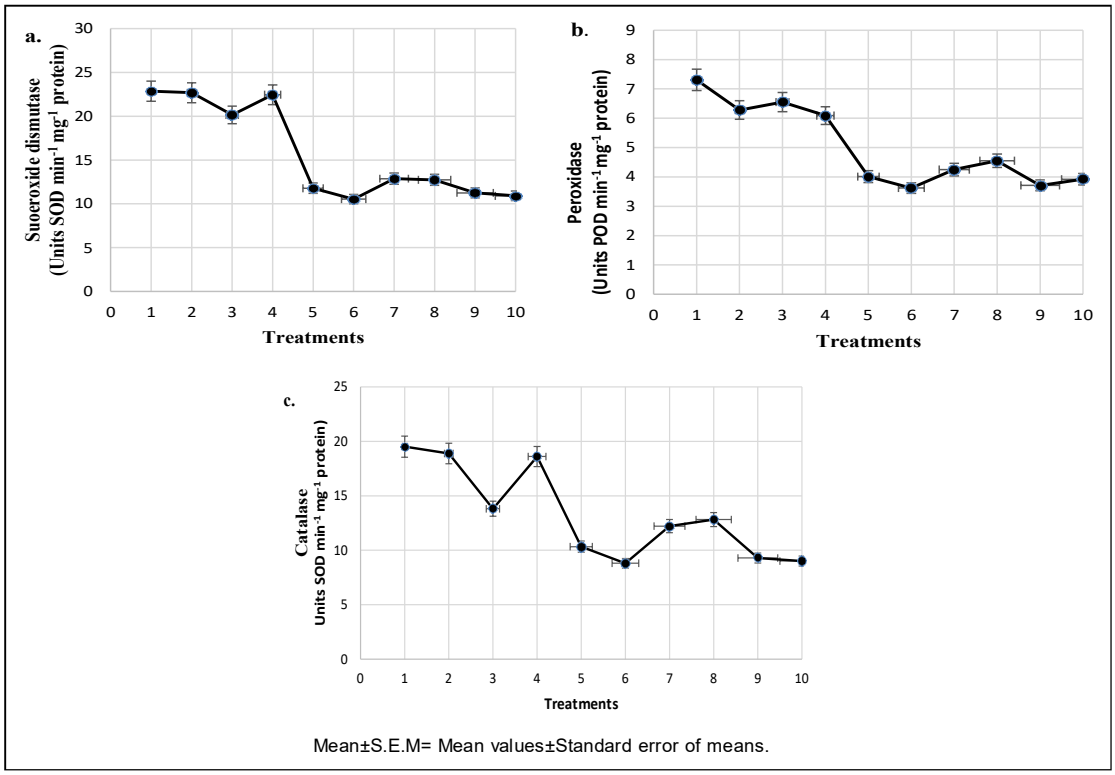


Fig 2: Effect of BSG on anti oxidant status (a) super oxide dismutase (SOD), (b) peroxidase (POD) and (c) catalase (CAT) of blackgram leaves on day 45 under pot culture condition in S deficient calcareous vertisol.

hydroxyl radicals and singlet oxygen and reduce H_2O_2 to water. Ascorbate also produces the lipophilic antioxidant tocopherol and removes H_2O_2 from chloroplasts, which lack catalase Tewari *et al*. (2004). No sulphur supplied (T_1) plants have been shown to have higher ascorbic acid concentrations, as observed in this study. Plants that received 40 kg S ha^{-1} (T_6) S supply had lower ascorbate levels than deficient plants, most likely due to higher POD activity in the former (Fig 1b).

Antioxidative enzymes

The induction of ROS scavenging enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) during the stress response is important for ROS detoxification. Plants lacking in sulphur (T_1) had varying effects on the activity of antioxidative enzymes in blackgram. In the current study, compared to sulphur-treated plants, sulphur non treated plants (T_1 and T_3) had higher CAT, POD and SOD activities (Fig 2). Adequate S supply helps to counteract the drastic effects of ROS on nucleic acids and proteins through up regulation of antioxidant enzymes such as CAT, POD and SOD. Activity of SOD was found significantly higher in plants grown in S without condition (T_1 and T_3) (Fig 2a). In sulphur non treated plants, SOD increased, which is consistent with reports of Howarth *et al*. (2003). First line of defense against reactive oxygen species is SOD, which converts the extremely poisonous O_2 to H_2O. CAT involved in H_2O_2 removal (Gill and Tuteja, 2010). H_2O_2 is changed by CAT into H_2O and O_2. Increased CAT and POD activity during a sulphur deficit condition, as seen in the current experiment and previously reported by Halliwell, (2007). High SOD activity in these plants led to increased CAT and POD activity, which increased the amount of H_2O_2 detoxification (Fig 2c).

CONCLUSION

In the present investigation, as a result of increased H_2O_2 accumulation in plants grown under S deficient conditions, we can conclude that the effect of sulphur deficiency was more pronounced in those plants. The leaves of sulphur deficient plants acquired more carbohydrates (sugar and starch) than healthy plants accomplished in terms of biomass and photo assimilatory pigments. H_2O_2 buildup led to oxidative damage in plants, which was also demonstrated by an increase in SOD, CAT, POD and ascorbate concentration. This will demonstrate unequivocally how sulphate stress impacts the physiological and metabolic processes of plants, which in turn affects the yield. The physiological activities of the plant in calcareous soil were strongly altered by the application of *Methylobacterium thiocyanatum*VRI7-A4 and elemental sulphur at 40 kg S ha^{-1}. Therefore, Bio Sulphur Granular inoculants with *Methylo bacterium thiocyanatum* VRI7-A4 and elemental sulphur at 40 kg S ha^{-1} can be advised to increase the productivity of blackgram plants in sulphur deficient calcareous soils.

Conflict of interest

All authors declared that there is no conflict of interest.

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