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Influence of Boron and Zinc on Nitrate and Nitrite Reductase Activity in Roots and Leaves, and Sulfur Containing Amino Acids, Protein and Oil Content in Seeds of Soybean [Glycine Max (L.) Merr.]

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ABSTRACT

Field Studies were conducted over two years on sandy loam soils, on the influence of boron (B) and zinc (Zn), on nitrate reductase (NR), nitrite reductase (NiR) activities in roots and leaves, and sulfur containing amino acids, viz., cysteine, cystine and methionine; protein, and oil content in seeds of soybean. Enzymatic activities were measured at 30, 60 and 90 days after emergence (DAE). Amino acids, protein and oil content were estimated in seeds after harvest. In absence of B and Zn applications, B and Zn symptoms such as distorted, chlorotic and puckered appearance of leaves, dwarfed plants, impaired flower development and seed set were noted. B and Zn applications were effective in overcoming the B and Zn deficiency symptoms. A significant increase in the nitrate reductase (NR) and nitrite reductase (NiR) activity in roots and leaves were noted. Sulphur containing amino acids (Cysteine, cystine and methionine), protein and oil content in seeds also significantly increased by B and Zn treatments. This work, in part, gives insight in mitigating environmental stresses through applications of micronutrients B and Zn
Keywords: Soybean seed, Boron, Zinc, Enzyme activity, Amino acids, Oil content

I. INTRODUCTION

Grain legumes like soybean [Glycine max (L.) Merr.] provide a rich and cheap source of protein as well as oil. In legume proteins the relative proportions of essential amino acids differ from crop to crop. The quality of a protein depends on its amino acid composition. The soybean protein is rich in sulphur containing amino acids, viz., cysteine, cystine and methionine. Although, nitrogen is present in atmosphere in plenty yet its availability in a form, which can be utilised by plants, is essential. Life on earth is dependent on the transformation of atmospheric nitrogen to a form in which it can be absorbed from the soil by plant for protein synthesis. The process can be accomplished industrially but at a very high-energy cost. Legume crops have unusual ability to be self-sufficient in nitrogen supply. Biological nitrogen fixation by symbiotic associations of legume plant with microorganisms is economically sounder and environmentally more acceptable than use of nitrogen fertilizer in agriculture. Both roots and shoots require organic nitrogen compounds but in which of these organs is nitrate reduced and incorporated into organic compounds can be estimated by activity of enzymes involved. Nitrate reduction occurs in two distinct reactions catalyzed by two enzymes, i.e., nitrate reductase (NR) and nitrite reductase (NiR). In some plants roots reduce almost no nitrate. So they depend upon amino acids translocated in the phloem from the leaves. In other plants, nearly all nitrates are absorbed and converted into amino acids and amides in the roots. Much more research is needed with the plants of different ages. Nutritional factor also plays an important role in altering the activity of different enzymes and manipulating the composition of seeds. With these ideas in mind this investigation was carried out to understand the influence of B and Zn on NR and NiR activities in leaves and roots as well as S-containing amino acids, protein and oil contents in seeds of soybean.

II. METHODS AND MATERIAL

Field studies were conducted at Agricultural Research Farm, Banaras Hindu University, Varanasi, in two successive years on a sandy loam soil. Soil test values prior to the establishment of experiments are given in Table 1. Seeds of soybean [*Glycine max* (L.) Merr.] Var. PK-327 treated with thiram @ 4.58 g kg^{-1} seed inoculated with (Brady) *Rhizobium japonicum* @ 0.5 kg ha^{-1} seed rate were sown during *kharif* (first week of July). Application of boron as boric acid @ 1.5 and 3.0 mg kg^{-1} soil and zinc as zinc sulphate @ 5.0 and 10.0 mg kg^{-1} soil were made with two modes of application, i.e., basal and foliar, in order to receive a timely and adequate amount of these micronutrients. The plants were treated in two split doses. The first half of the dose of B and Zn was applied at the time of sowing as basal application at the pre-flowering stage [i.e., 30 days after emergence (DAE)]. Basal application of N: P: K: S @ 40:60:40:20 kg ha^{-1} as N, P_2O_5, K_2O and S respectively was made uniformly in all plots irrespective of B and Zn application. A control lot of plants were also maintained where no additional supply of B and Zn was made. Weeds were constantly removed from experimental site to keep the crop free from unwanted plants.

Assay of nitrate reductase (NR) and nitrite reductase (NiR) activity in vivo in roots and leaves:

The NR and NiR activities were measured in fresh roots and fresh leaves at 30, 60 and 90 DAE. The third leaf from the top was taken for use in the assay of enzymatic activities. The third leaf from the top represents both the mature and young leaves with respect to growth and age. Moreover, it was also convenient to keep sample of leaf position on the plant constant throughout the study.

NR and NiR activities in the root and leaf samples were determined by *in vivo* method as described by Nicholas *et al.* (1976) and Ferari and Varner (1971), respectively.
Determination of sulphur containing amino acids:

Sulphur containing amino acids, viz., cysteine, cystine and methionine in seeds were determined by the method of Bieleski and Turner (1966). Samples (50 –100 mg) were extracted with methanol: chloroform: water (MCW) 12: 5: 3 (by vol.) overnight at – 20°C, then stirred for 1 min using an Ultra-Turrax. The extract was centrifuged at 10000 *g* for 15 min and the supernatant (MCW extract) collected. The chloroform layer of the MCW extract was separated from the aqueous layer and discarded. The pellet was re-extracted with cold 80% (v/v) ethanol for 4 min in an Ultra-Turrax, and centrifuged as above. The supernatant of the ethanol extract was combined with the aqueous layer of the MCW extract and evaporated to dryness at 40 – 50°C. After dissolving the residue in 5.0 cm^3 100 mol m^{-3} HCl, analysis of free amino acids was performed using a HPLC system consisting of two pumps, a controller for gradient programming and a modified auto-injector with a 20 mm^3 filling loop for automatic on-line derivatization. Fluorescence was monitored with a spectromonitor at an excitation wavelength of 330 nm and an emission wavelength of 450 nm. The derivatization was performed as described by Graser *et al.* (1985). For the separation of amino acids a Hyperchrome HPLC column (125 x 4.6 mm i.d.) and a guard column (10 x 4.6 mm i.d.) were used, both packed with Spherisorb ODS II, 3.0 μm. A two-component gradient system was used (Solvent A: 1% tetrahydrofuran + 5% methanol in 12.5 mol m^{-3} sodium phosphate buffer, pH 7.2; Solvent B: 4.5% tetrahydrofuran + 35% acetonitrile + 14.5% methanol in sodium phosphate buffer, pH 7.2). The solvent programme used was: 0 min 85% A; 12 min 73% A; 16 min 60% A; 21 min 55% A; 38 min 0% A; 40 min 70% A. The flow rate was 1.2cm^3 min^{-1} and the separation was performed at room temperature.

Protein estimation:

500-mg seed sample was homogenised with 5 ml of 80% ethanol and centrifuged at 4000 r.p.m. for 20 min. Supernatant was kept aside and the residue was re-extracted twice with 5 ml ethanol (80%) each and centrifuged. Supernatants were discarded. The residue was left after 80% ethanol extraction was hydrolysed in 5 ml of 1 N NaOH for overnight and centrifuged at 4000 r.p.m. for 20 min. Supernatant was kept aside and residue was again extracted with 5 ml of 1 N NaOH for 1 hr. and centrifuged. Both supernatants were pooled and volume was made to 10 ml. The total protein was determined in this supernatant by folin ciocalteau reagent by the method of Lowry *et al.* (1951). To the sample (1.0 ml) was added 10 ml of 2% Na_2CO_3 in 1 N NaOH + 0.5% $CuSO_4$ in 1% in sodium potassium tartarate (50:1 $^{V}/_{V}$) and was kept for 10 min. 0.5 ml of 1 N folin ciocalteau reagent was added and it was immediately shaken vigorously. It was kept for 30

minutes and thereafter absorbance reading was taken at 750 nm on spectrophotometer. Protein content was determined from a standard curve drawn with the help of bovine sernum albumen (BSA).

Oil Extraction:

Oil was extracted by the cold percolation method of Kartha and Sethi (1957). A small glass percolator (20 cm long and 0.5 cm in diameter) was prepared by drawing a taper on one end of a glass tube; a perforated glass plate was inserted just above the taper. A thin wad of glass wool was adjusted over the glass plate. A layer of coarsely powdered anhydrous sodium sulphate (0.25 – 0.31 inches thick) was packed over the glass wool wad. 0.3g of seed material was transferred to a porcelain mortar. 2.0g of each of glass powder (Pyrex glass washed with concentrated hydrochloric acid) and anhydrous sodium sulphate were added and the mixture was reduced to fine powder. The mixture was transferred to percolator. The mortar and pestle were washed twice with 0.5g of anhydrous sodium sulphate and the washings were also packed over the seed powder. Finally the mortar and pestle were washed with 3-4 cc of freshly distilled petroleum ether, B.P. 70 – 90°C and this was transferred to the packed meal powder. This initial 3 – 4 cc of distilled water solvent served to wet the mixture. This was allowed to remain as such for 5 min. and the percolation started by adding measured quantity of solvent on the top of the percolator. The oil present in the seed powder is soluble in the solvent. The percolated solvent was collected below the percolator in a weighed dish containing four; 1 inch square strips of filter paper. The percolated solvent collected in the dish brings with it oil content present in the seed powder. Keeping the dish in an oven at 90 – 100°C for half an hour evaporated the solvent. The constant weight of the dish was obtained after the complete evaporation of the solvent. The difference in the initial and final weight of the dish is equal to the amount of oil present in the seed sample.

Statistical Analysis:

The experimental design was a randomised complete block with three replications. The treatments were a factorial combination of the levels of B and Zn. The results were statistically processed for calculating Least Significant Difference (LSD) at P=0.05 (Gomez and Gomez, 1984).

III. RESULT AND DISCUSSION

The chemical analyses of soil, as presented in Table 1, indicate inadequate B and Zn for optimum growth and development of soybean plants, which were found to be improved considerably by the addition of these two micronutrients at two different levels and their combinations. B and Zn at the threshold individual levels of 1.5 and 10.0 mg kg^{-1}, respectively and threshold combined level (B + Zn) of 1.5 + 10.0 mg kg^{-1} soil significantly increased NR and NiR activities in roots and leaves (Tables 2, 3, 4 & 5). This was followed by enhancement in amino acids cysteine, cystine and methionine percentage in seeds (Table 6 and Figure 1), and protein and oil content in seeds (Table 6 and Figure 2) of soybean.

In a comparison of nitrogen metabolism of cocklebur (*Xanthium pennsylvanicum*) with that of field pea Wallace and Pate (1967) concluded that each exemplified a group of plants with a characteristic distribution of nitrate reducing capacity. Whereas both the roots and leaves of field pea displayed vigorous NR activity, such activity in *Xanthium* was restricted wholly to the leaves. Soybean appears to resemble the field pea in that the present (Table 2 and 3) and previous studies (Evans and Nason, 1953; Weissman, 1972) give evidence of active nitrate reduction centres in both roots and leaves. Significant increase in NR activity by B and Zn application indicates the involvement of these two micronutrients in the activity of this enzyme either directly or indirectly. Maximum activity recorded in coincidence with the pod setting stage, a crucial phase for improving yield of this proteinaceous crop implicitly indicates the utility of B and Zn for nitrogen metabolism. Previously individual foliar spray of B to B-deficient sorghum at reproductive stage increased the nitrogen content and NR activity (Misra *et al.*, 1991) as found in the present study by splitting the dose. Similarly Zn also exerts a beneficial effect on the N-assimilation indirectly through its influence on NR activity (Rossel and Ulrich, 1964; Garg *et al.*, 1986). In B-deficient sugar beet and tomato plants a decrease in the NR activity (Bonilla *et al.*, 1980; Ramon *et al.*, 1989) is in conformity with the present findings.

The pattern of NiR activity in the roots and leaves follows closely the distribution of NR in these tissues. In roots and leaves an obvious enzymatic activity is present (Table 4 and 5). Beevers and Hageman (1969) have noted the limited demonstration of NiR in the roots, but in the present study increased NiR activity was obtained which might be characteristic of genotype taken as well as influence of treatments applied. Indirect evidence for the presence in soybean roots of a system capable of converting nitrite to ammonia comes from post studies (Woodhouse and Hardwick, 1966) indicating an increase in the ammonia content in the root exudes of plants provided with nitrate. In the present study B and Zn application found that enzymatic system seems to be in active form and an increase in activity. The decrease in activities of both the enzymes by higher level of B might be due to toxic effect at higher concentration.

In absence of Zn, protein content decreases with a simultaneous increase in the total free amino acids (Cakmak *et al.*, 1989), but in the present study only three sulphur containing amino acids, viz., cysteine, cystine and methionine were estimated and were found to be increased by B and Zn treatment (Table 6), of which methionine content was greater than cysteine and cystine. There might be a simultaneous decrease in other free amino acids. In wheat, boron application improved amino acid composition and protein synthesis (Iqtidar and Rehman, 1984). Boron has a role in the process of atmospheric nitrogen fixation (Bolanos *et al.*, 1994). Increased nitrogen metabolism in turn helps in the process of protein synthesis. Zinc is also required for protein synthesis because in higher plants under conditions of Zn deficiency several metabolic processes are impaired such as RNA metabolism and protein synthesis (Sharma *et al.*, 1981; Kitagishi and Obata, 1986). Therefore, fairly high Zn concentrations are required in plant tissues for extensive synthesis of proteins (Cakmak and Marschner, 1993). This specific role of these micronutrients is also evident in present study (Table 6) in the form of increased protein content of seeds. Generally at given latitude, variations in protein and fat contents are inversely proportional to about two weights of proteins equivalent to one of fat (Hanson *et al.*, 1961). Increase in both protein and oil content (Table 6) might be due to efficient protein synthesis as well as fat synthesis in the presence of required nutrients.

On the basis of overall findings, as discussed in the preceding pages, it may also be assumed that B and Zn help better growth of bacteroids in root nodules. Through the chemical analyses of experimental soil presented in Table 1, it is apparent that Zn and B contents are below the required level for the growth of pulse crop, in general. The same may be limiting to provide a congenial environment to the N_2-fixing microbial partner inside the root nodules, which require the needful supply of the micronutrients, including B and Zn for their optimal activities of N_2-fixation and release of growth promoting substances. These micronutrients help a higher rate of N_2-fixation not especially because of their direct role in N_2-fixation but because of their possible role in producing a sufficient bacteroid mass. Consequently, the total fixed N output expected to be more by the increased bacterial population and this may be one of the good reasons why the total N content of plant is significantly enhanced. Similarly requisite supply of micronutrients including B and Zn has a positive impact on the activities of nitrate reductase and nitrate reductase enzyme systems, which collectively add to the assimilable N-status of the plant. An increase in the total plant N content through the above processes facilitates better amino acid synthesis and protein assembly. The latter is well reflected through the increased protein content of the treated plants (Table 6; Figure 2). A better amino acid synthesis under B and Zn treatment is apparent from the higher methionine, cysteine and cystine contents in the treated plants. May be that the synthesis of other amino acids has been also similarly increased, although the same was not tested in this series of experiments. However, the increase in the amount of these three amino acids is a reflection of the qualitative increase in the amino acid contents of plants under B and Zn treatments. The findings provide enough scope, therefore, for detail biochemical analyses of the qualitative and quantitative status of various other amino acids in plants with graded application of B and Zn.

TABLE 1. Soil Test Values Prior to the Study

1.	Soil Extractable Nutrients (mg kg^{-1} soil)				
(a)	Nitrogen	84.50	88.50	Alkaline Permanganate	Subbiah and Asija (1956)
(b)	Phosphorous	11.00	10.75	0.5 N $NaHCO_3$ extractable	Oslen *et al.* (1954)
(c)	Potassium	104.00	102.00	Ammonium acetate extractable flame Photometer	Jackson (1973)
(d)	Sulfur	8.50	8.25	Turbidity	Chesnin and Yien (1950)
(e)	Boron	0.89	0.80 boron	Hot water soluble Ervco (1977)	Sippola and
(f)	Zinc	1.20	1.00	DTPA	Lindsay and Norvell (1978)
2.	Soil pH (1: 2.5 soil water ratio)	7.48	7.50	Glass electrode pH meter	Jackson (1973)
3.	Electrical Conductivity d Sm^{-2} (1:2.5 soil water Ratio)	0.15	0.16	Systronics electrical conductivity meter	Jackson (1973)

TABLE 2. Effects of Boron (B) and Zinc (Zn) Levels on Nitrate Reductase (NR) Activity (µ mol.NO_2.hr^{-1}.g^{-1}) in Fresh Roots of Soybean [*Glycine max*(L.) Merr] at different growth stages

Treatments		First Year Age of plants (DAE)			Second Year Age of plants (DAE)		
B (mg kg^{-1} soil)	Zn	30	60	90	30	60	90
0.0	0.0	0.830	1.590	0.976	0.940	1.670	1.010
1.5	0.0	0.930	1.740	0.990	0.990	1.810	1.070
3.0	0.0	0.620	1.310	0.580	0.710	1.370	0.630
0.0	5.0	1.130	1.950	1.140	1.200	1.970	1.210
0.0	10.0	1.190	2.010	1.210	1.220	2.086	1.350
1.5	5.0	1.270	2.173	1.273	1.340	2.170	1.430
1.5	10.0	1.330	2.260	1.390	1.430	2.256	1.490
3.0	5.0	0.740	1.520	0.730	0.830	1.510	0830
3.0	10.0	0.753	1.660	0.810	0.890	1.570	0.910
LSD (P=0.05) for B or Zn		0.043	0.010	0.020	0.045	0.032	0.035
LSD (P=0.05) for B x Zn		0.075	0.018	0.035	0.078	0.055	0.062

TABLE 3. Effects of Boron (B) and Zinc (Zn) Levels on Nitrate Reductase (NR) Activity (μ mol.NO_2.hr^{-1}.g^{-1}) in Fresh Leaves of Soybean [*Glycine max*(L.) Merr] at different growth stages

Treatments		First Year Age of plants (DAE)			Second Year Age of plants (DAE)		
B (mg kg^{-1} soil)	Zn	30	60	90	30	60	90
0.0	0.0	1.620	3.090	1.610	1.690	3.190	1.750
1.5	0.0	2.810	3.506	1.790	1.870	3.430	1.910
3.0	0.0	1.200	2.560	1.070	1.270	2.660	1.200
0.0	5.0	2.220	3.770	2.190	1.280	3.840	2.280
0.0	10.0	2.320	3.920	2.330	2.370	4.010	3.460
1.5	5.0	2.473	4.120	2.530	2.530	4.220	2.690
1.5	10.0	2.620	4.486	2.743	2.690	4.426	2.870
3.0	5.0	1.410	2.790	1.310	1.490	2.930	1.470
3.0	10.0	1.480	2.910	1.420	1.560	3.030	1.570
LSD (P=0.05) for B or Zn		0.020	0.144	0.030	0.034	0.011	0.030

TABLE 4. Effects of Boron (B) and Zinc (Zn) Levels on Nitrite Reductase (NiR) Activity (μ mol.NO_2.hr^{-1}.g^{-1}) in Fresh Roots of Soybean [*Glycine max*(L.) Merr] at different growth stages

Treatments		First Year Age of plants (DAE)			Second Year Age of plants (DAE)		
B	Zn						
(mg kg^{-1} soil)		30	60	90	30	60	90
0.0	0.0	1.770	3.720	1.750	2.100	4.090	2.050
1.5	0.0	1.990	4.080	1.920	2.220	4.430	2.190
3.0	0.0	1.300	3.020	1.100	1.560	3.310	1.260
0.0	5.0	2.440	4.600	2.220	2.710	3.840	2.490
0.0	10.0	2.580	4.760	2.370	2.760	5.160	2.790
1.5	5.0	2.760	5.180	3.703	3.180	5.357	2.970
1.5	10.0	2.910	5.400	3.750	3.270	5.700	3.110
3.0	5.0	1.560	3.520	1.390	1.840	2.660	1.670
3.0	10.0	1.610	3.860	1.550	1.980	3.830	1.840
LSD (P=0.05) for B or Zn		0.048	0.040	0.651	0.021	0.032	0.035
LSD (P=0.05) for B x Zn		0.082	0.069	1.127	0.036	0.056	0.060

TABLE 5. Effects of Boron (B) and Zinc (Zn) Levels on Nitrite Reductase (NiR) Activity (μ mol.NO_2.hr^{-1}.g^{-1}) in Fresh Leaves of Soybean [*Glycine max*(L.) Merr] at different growth stages

Treatments		First Year Age of plants (DAE)			Second Year Age of plants (DAE)		
B	Zn						
(mg kg^{-1} soil)		30	60	90	30	60	90
0.0	0.0	2.340	5.060	1.980	2.600	5.510	2.320
1.5	0.0	2.640	5.510	2.210	2.890	5.960	2.550
3.0	0.0	1.690	4.120	1.290	1.910	4.520	1.560
0.0	5.0	3.260	6.250	2.730	3.550	6.720	3.070
0.0	10.0	3.430	6.540	2.930	3.720	7.050	4.700
1.5	5.0	3.660	6.920	3.210	3.657	7.460	3.780
1.5	10.0	3.930	7.300	3.460	4.270	7.880	3.960
3.0	5.0	2.000	4.510	1.590	2.260	5.010	1.920
3.0	10.0	2.110	4.740	1.740	2.440	5.210	2.070
LSD (P=0.05) for B or Zn		0.037	0.027	0.032	0.184	0.022	0.059
LSD (P=0.05) for B x Zn		0.064	0.048	0.056	0.318	0.038	0.103

TABLE 6. Effects of Boron (B) and Zinc (Zn) Levels on Sulfur containing Amino Acids, Protein and Oil Content (per cent) in Seeds of Soybean [*Glycine max* (L.) Merr.]

Treatments		**First Year Age of Plants (DAE)**					**Second Year Age of Plants (DAE)**				
B	**Zn**										
(mg kg^{-1} soil)		Cysteine	Cystine	Methionine	Protein	Oil	Cysteine	Cystine	Methionine	Protein	Oil
0.0	0.0	1.410	2.150	7.910	32.097	20.900	1.480	2.230	8.010	32.483	20.970
1.5	0.0	1.470	2.190	7.980	35.600	22.750	1.540	2.290	8.100	39.083	23.000
3.0	0.0	1.210	1.980	7.510	32.287	22.670	1.250	2.050	7.650	32.607	22.850
0.0	5.0	1.540	2.270	8.210	38.160	21.900	1.630	2.370	6.663	35.296	21.650
0.0	10.0	1.590	2.320	8.300	38.847	22.200	1.690	2.430	8.420	35.523	21.900
1.5	5.0	1.610	2.430	8.530	41.223	23.150	1.720	2.540	8.610	41.603	23.200
1.5	10.0	1.680	2.510	8.650	42.037	23.350	1.800	2.620	8.750	42.356	23.450
3.0	5.0	1.300	2.080	7.680	33.830	21.100	1.360	2.130	7.830	33.413	21.260
3.0	10.0	1.340	2.130	7.740	33.415	21.210	1.420	2.180	7.873	33.790	21.350
LSD (P=0.05) for B and Zn		0.037	0.029	0.037	0.823	0.176	0.027	0.028	0.965	0.242	0.201
LSD (p=0.05) for B x Zn		0.065	0.051	0.065	1.350	0.305	0.047	0.049	1.672	0.419	0.349

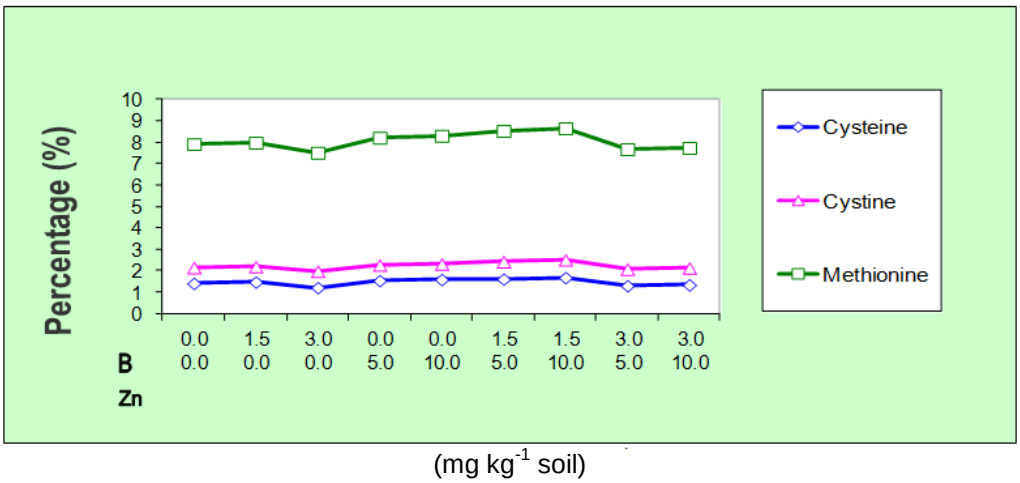

Figure 1. Effects of Boron (B) and Zinc (Zn) Levels on Sulfur Containing Amino Acids (Cysteine, Cystine and Methionine in per cent) in Seeds of Soybean [*Glycine max* (l.) Merr]

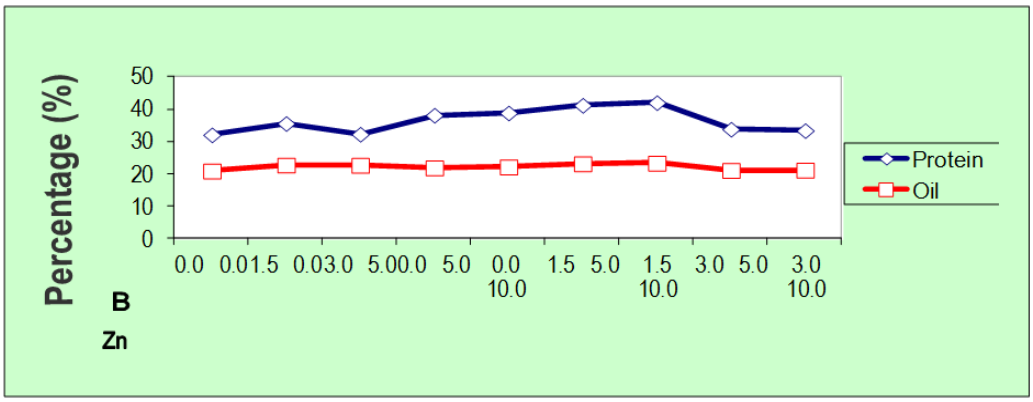

Figure 2. Effects of Boron (B) and Zinc (Zn) Levels on Protein (%) and Oil (%) in Seeds of [*Glycine max* (L.) Merr]

IV. CONCLUSION

Physiological significance of B and Zn application in relation to nitrate and nitrite reduction, protein and fat synthesis in this particular crop is explicit. Nitrate and nitrite reduction in accordance with the need of plant depends on the activity of enzymes involved, which in turn are regulated by genetic as well as nutritional factors. Improvement in sulphur containing amino acids, protein and oil content, which are the quality parameters of soybean seeds, by B and Zn treatments expounds the importance of these micronutrients for seed quality. Further, this work gives insight and keeps relevance in mitigating environmental stresses through applications of micronutrients B and Zn.

V. ACKNOWLEDGEMENTS

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