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Research Article

Impact of Low Boron Treatment on Germination Capacity, Membrane Permeability and Antioxidant Machinery in Soybean Seeds

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Abstract

To detect the boron deficiency induced changes in germination capacity and seed quality an experiment was conducted on soybean (Glycine max var. JS-335) plants. For this soybean plants were grown till maturity at deficient (0.033 mg L-1) and sufficient (0.33 mg L-1) boron supply. Seed yield was determined at 112d when plants were harvested. To study the effect of low boron concentration on seed quality- concentration of carbohydrates (sugars and starch), phenols, proteins and oil content were determined in the post harvested seeds of soybean. Membrane permeability was determined by the electrical conductivity and MDA content in seeds. Antioxidant responses of seeds were studied by estimating the parameters of CAT, PPO and POD enzymes.

Keywords: CAT; Boron Stress; Electrolyte Leakage; POD; PPO; Soybean Seed

Introduction

The soybean (U.S.) or soya bean (UK) (Glycine max) is a species of legume native to East Asia and originated in China. It is also known as a 'miracle crop' with over 40% protein and 20% oil. The plant is classed as an oilseed rather than a pulse. Soybean oil contains significantly greater amount of omega-6 fatty acids in the oil: 100g of soybean oil contains 7g of omega-3 fatty acids to 51g of omega-6: a ratio of 1:7. Soybeans also contain the isoflavones genistein and daidzein, types of phytoestrogen, that are considered by some dietitians and physicians to be useful in the prevention of cancer and by others to be carcinogenic and endocrine disruptive. Soy's content of isoflavones is as much as 3 mg g-1 dry weight. Isoflavones are polyphenol compounds, produced primarily by beans and other legumes, including peanuts and chickpeas [1]. Soybean seed is a source of protein and oil for human nutrition and as well as for livestock feed. Soybean seed quality is determined by the quantity and quality of protein and oil content.

Boron plays an important role in the formation of flowers and fruit and seed setting process in higher plants. There was improper development of male and female reproductive parts in flowers of plants subjected to boron deficiency [2]. Flowers of deficient plants showed poorly developed, deformed and small sized anthers with very poor pollen producing capacity. Pollen grains were also underdeveloped and malformed. Boron deficiency symptoms during reproductive stage include shedding of buds, flowers and developing fruits and seeds as well poor fruit/seed quality and low viability of seeds.

Seed germination is a complex phenomenon, which involves finely regulated catabolic and anabolic processes where hormones and ions play a key role. Intracellular bodies of lipids, proteins, carbohydrates, organic phosphate and various other inorganic compounds support the process of germination of seeds and the growth of seedlings [3].

Material and Methods

Sand culture experiments using the technique developed at the Long Ashton Research Station, Bristol, U.K. [4] and standardized for Indian conditions by [5] were carried out on soybean (*Glycine max* var. JS-335). The composition of nutrient solution used for growing the plants excluding B was: 4 mM KNO_3, 4 mM Ca $(NO_3)_2$, 2 mM $MgSO_4$, 1.33 mM NaH_2PO_4, 0.1 mM Fe EDTA, 10 µM $MnSO_4$, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, 0.1 µM Na_2MoO_4, 0.1 mM NaCl, 0.1 µM $CoSO_4$ and 0.1 µM $NiSO_4$. Boron was supplied as H_3BO_3 at varying levels. The soybean plants were grown till maturity at 0.033 and 0.33 mg L^{-1} B supply. The effect of deficient boron supply on the growth and visible symptoms were studied during the period of plant growth. For studying the effect of boron deprivation on reproductive development in soybean- number of inflorescence per plant, number of flowers per inflorescence and total number of flowers per plant, length, number and weight of pods and seed formed per plant were determined.

The entire experimental studies were conducted in glasshouse under controlled conditions of light, humidity and temperature. Visual observations of the plant growth and symptoms to the low supply of boron were recorded daily.

Seed yield was determined at 112d when plants were harvested. To study the effect of boron deprivation on seed quality- concentration of carbohydrates (sugars and starch), phenols, proteins, oil content, lipid peroxidation, membrane leakage and antioxidant enzymes-CAT, POD, PPO were determined in the post harvested seeds of soybean.

- **Seed germination:** The percentage germination and embryonic axes length of post harvested seeds in distilled water were recorded at 48 h [6].
- **Vigor index:** was calculated according to the formula [7].
- VI = germination % at 48 hr X length of embryonic axes at 48 hr
- **Harvest index:** was calculated by the formula of-

  Grain yield (dry mass of harvested components)

  Harvest Index (HI) = Biological yield (total shoot dry mass)
- **Carbohydrates and Total phenols:** For the estimation of sugar, starch, and phenols, plant material (post harvested seeds, cotyledons and embryonic axis) was fixed in boiling 80% ethanol in the proportion of 1:10. The fixed material was crushed in a pestle and mortar. Alcohol soluble and insoluble fractions were separated by repeated centrifugation at 800 xg. In the alcohol soluble fractions, sugars and phenols were estimated while alcohol insoluble fraction was used for the determination of starch. Sugars were determined colorimetrically by the method of [8]. Starch was estimated by the method of [9]. Total phenols were determined in alcohol soluble fractions by the method of [10].
- **Oil content:** Oil extraction was done in mature seeds by the solvent extraction method. The seeds were crushed in solvent hexane and extracted in the solvent system by repeated extraction as described in [11]. From this mixture solution was separated from the residue and oil was recovered from solution through evaporation in vaccum. Results were expressed as percent oil content per unit seed weight.
- **Seed protein:** Seed protein was extracted by the method of [12]. After the harvesting of crops seeds were collected and seed coat was removed and seeds were ground in acetone. The extract was centrifuged 3-4 times at 11,500 xg for 10 min. portions of air dried seed flour (200 mg) were extracted with water for albunins, 5% NaCl for globulins, 0.1N NaOH for glutenins and 70% ethanol with 2 drops of mercaptoethanol for prolamines at room temperature. The proteins in the above extracts were estimated by the method of [13]. The optical density of the reaction mixture was measured on spectrophotiometer at 750 nm. The readings were referred to a standard calibration curve prepared from crystalline bovine serum albumin.
- **Lipid peroxidation**: Lipid peroxidation was measured in terms of thiobarbituric acid reactive substances (TBARS) formation [14]. Post harvested seeds were homogenized with 0.1% trichloroacetic acid and centrifuged at 10,000 xg for 5 min. The supernatant was treated with 0.5% thiobarbituric

acid (TBA) prepared in 20% TCA and the mixture was incubated at 95°C in water bath for 30 min. Samples were cooled immediately in ice bath and centrifuged at 10,000 xg for 10 min. The absorbance was read at 532 nm and adjusted for non-specific absorbance at 600 nm. The concentration of TBARS was estimated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

- **Electrical conductivity:** Leakage of ions from seeds was measured according to the method of [15] and expressed as MSI (membrane stability index) percentage. 15 seeds were placed in 20 ml of deionised water in two sets. Test tubes in one set were kept in water bath at constant temperature of 40 ^{0}C for 30 min and its conductivity (CI) was measured with an Electrical Systronics Conductivity Meter Model conductivity meter 306. Test tubes in the second set were kept in a boiling water bath (100 ^{0}C) for 10 minute and conductivity was recorded (C2). Electrolyte leakage (EC) was expressed in percentage using the formula: EC = (1 - C1 / C2) × 100.
- **Enzyme assay: Catalase** (EC 1.11.1.6) was assayed by an adaptation of the permanganate titration method [16]. For peroxidase (EC 1.11.1.7) estimation 0.1 M phosphate buffer pH 6.0, 0.01% H_2O_2 and 0.5% p-phenylene diamine was added to the suitable enzyme extract. Reaction was stopped by adding 4 N H_2SO_4. The colour intensity was read at 485 nm [17]. Polyphenol oxidase (EC 1.14.18.1) (DOPA oxidase) was assayed [18]. in the enzyme, extracted in 0.01M phosphate buffer pH 7.5 containing 0. 2 M KCl, 0.001 M EDTA, 0.01 M sodium ascorbate and 1g of polvinylpyrrolidone at 4° C. The assay mixture for polyphenol oxidase contained 0.1 M phosphate buffer pH 6.5 and suitably diluted enzyme extract. The reaction was initiated by the addition of 0.01 M DL-DOPA (3, 4 –dihydroxy 1- phenol alanine). The reaction was allowed to proceed for 30 minutes at 30°C and 0.25 M lead acetate was added to stop the reaction. The contents were centrifuged and optical density of the supernatant was measured on a spectrophotometer at 470 nm.
- **Statistical analysis**: Standard analyses of variance (ANOVA) were used to assess the significance of treatment means. The data are presented as mean values ± standard error (SE, n=3). Differences between treatments means were compared using LSD at the 0.05 probability level.

Results

Growth and visible symptoms

The growth difference in deficient plants became perceptible after 22 days of growth. Compared to plants receiving 0.33 mg B L^{-1} supply plants receiving 0.033 mg B L^{-1} supply showed growth reduction and deficiency symptoms such as irregular chlorotic areas between the veins in young leaves (Plate 1). After 33 days of growth severe deficiency symptoms appeared and there was shortening of the internodes, stem thickening and reduction in leaf area. After 40 days young emerging leaves failed to unroll and showed twisting and curling of margins. Young leaves of deficient plants also showed downward as well as upward cupping. Apical meristem ceased to grow under severe deficiency (Plate 1).

Plate 1: (A) Effect of boron on the growth of soybean plant supplied with 0.33 and 0.033 mg B L^{-1} supply. (B) Boron deficient (0.033 mg B L^{-1}) plants showing chlorosis and cupping of young leaves

Plant height, internodal length and leaf area was reduced in plants receiving low boron supply compared to control. Reduction in plant height and leaf area was more at earlier stage (55 DAS) than later stage (112 DAS) of growth, whereas length of internodal region was found to be reduced more at later stage of growth (Table 1).

Parameters	Boron supply : mg B L^{-1}	
	0.033	0.33
Plant height (cm)	112.75 ± 3.81	185.50 ± 3.73
Internodal length (cm)	5.06 ± 0.78	8.33 ± 1.15
Leaf area (cm^2)	32.39 ± 1.35	46.87 ± 1.73
No. of inflorescence $plant^{-1}$	20 ± 2.41	34 ± 2.60
No. of flowers inflorescence $^{-1}$	8 ± 1.40	12 ± 1.73
No. of flowers $plant^{-1}$	159 ± 5.31	358 ± 9.80

Table 1: Effect of boron supply on the vegetative and reproductive growth of soybean (*Gycine max* var. JS-335).

Floral development

Flowering was delayed by 10 days in boron deficient plants compared to plants receiving sufficient (0.33 mg B L^{-1}) boron supply. Flower formation was not only delayed but also reduced in number per plant. There was reduction in number of inflorescence per plant and number of flowers per inflorescence (Table 1).

Reproductive yield

Number as well as length and weight of pods produced by boron deficient plants were reduced compared to control plants (Table 2, Plate 2). As compared to control plants receiving 0.33 mg B L^{-1} supply deficient plants receiving 0.033 mg B L^{-1} supply showed upto 53% reduction in seed yield. Weight of seeds was also reduced in boron deficient plants (Table 2).

Post harvested seeds of boron deficient plants were reduced in size and showed poor rate of germination (Plate 2) and vigor index as compared to seeds of boron sufficient plants. Decreased harvest index under low B supply was reported. Length of embryonic axes of germinating seedlings was also decreased upto 50% in seeds of plants grown with 0.033 mg B L^{-1} supply (Table 2; Plate 2).

Reproductive yield	Boron supply : mg B L^{-1}	
	0.033	0.33
No. of pods $plant^{-1}$	52 ± 4.32	79 ± 5.11
Wt. of pods $plant^{-1}$ (g)	39.05 ± 1.51	65.54 ± 1.65
Pod length (cm)	3.51 ± 0.38	4.89 ± 0.56
No. of seeds pod^{-1}	3 ± 0.081	4 ± 0.098
No. of seeds $plant^{-1}$	150 ± 1.62	318 ± 1.81
Wt. of seeds $plant^{-1}$ (g)	12.41 ± 9.31	32.67 ± 1.68
100 seed wt. (g)	8.05 ± 0.31	10.25 ± 0.66
Tissue B in seeds (μg^{-1} dry wt.)	17.7 ± 1.21	25 ± 1.26
Seed germination (%)	60 ± 3.65	95 ± 4.01
Length of embryonic axes (cm)	1.06 ± 0.21	2.13 ± 0.23
Vigour index	63.60 ± 1.82	202.3 ± 1.93
Harvest index	1.032 ± 0.26	1.175 ± 0.53

Table 2: Effect of boron supply on the pods, seed yield and vigor of soybean (*Gycine max* var. JS-335) after harvesting.

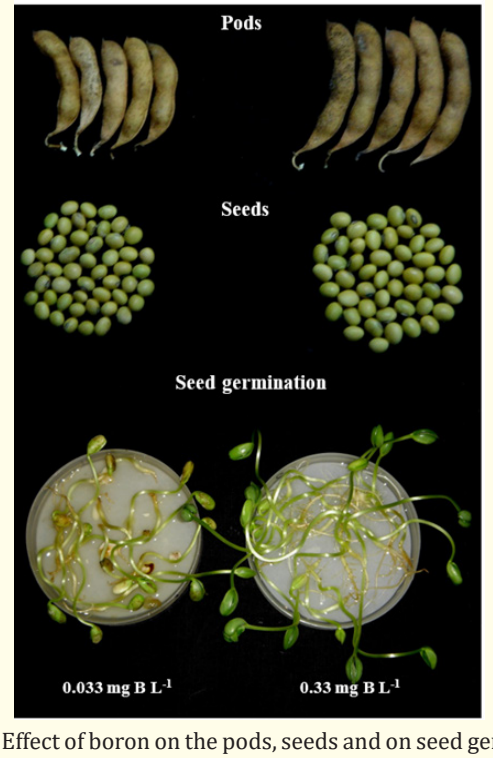

Plate 2: Effect of boron on the pods, seeds and on seed germination of soybean plant supplied with 0.033 and 0.33 mg B L^{-1} supply.

Seed carbohydrate, phenols and oil content

Compared to control plants the reducing and non-reducing sugars were decreased in seeds harvested from boron deficient plants. Non-reducing sugars was found to be decreased more than reducing sugars in seeds of boron deficient plants. In these plants starch content of seeds was also decreased but this decrease was not as pronounced as the decrease in sugars.

Phenols were found to be accumulated in seeds of boron deficient plants. Oil content of seeds was also affected and showed decreased upto 37% compared to seeds of control plants (Table 3). Electrical conductivity (EC) was found to be more in boron deficient seeds as compared to seeds received sufficient boron supply (Table 3).

Seed quality	Boron supply : mg B L^{-1}	
	0.033	0.33
% fresh weight		
Reducing sugars	0.324 ± 0.068	0.393 ± 0.055
Non-reducing sugars	2.306 ± 0.080	2.936 ± 0.092
Total sugars	2.630 ± 0.074	3.329 ± 0.057
Starch	5.004 ± 0.121	5.808 ± 0.119
Phenols	0.051 ± 0.001	0.027 ± 0.001
Electrical conductivity	48.91 ± 1.75	38.89 ± 1.31

Table 3: Effect of boron supply on the carbohydrate, phenols, oil content and electrolyte leakage of post harvested seeds of soybean (*Gycine max* var. JS-335).

Seed protein

Soluble proteins of seeds were decreased upto 66% compared to seeds of control plant. All the storage protein fractions- albumins, globulins, glutelins and prolamins were decreased in seeds of boron deficient plants as compared to those of control plants. Albumins were found to be decreased more than other fractions and glutelins were least decreased (Table 4).

PPO, LPO, POD, and CAT Concentration

Polyphenol oxidase (PPO), Peroxidase (POD), Lipid Peroxidation (LPO) and catalase (CAT) activity was found to be enhanced in boron deficient seeds as compared to seeds received sufficient boron supply (Table 5).

Protein fractions	Boron supply : mg B L^{-1}	
	0.033	0.33
mg 100 mg^{-1} fresh weight		
Albumins	1.785 ± 0.057	3.260 ± 0.084
Globulins	1.982 ± 0.040	3.323 ± 0.052
Glutelins	1.981 ± 0.049	3.176 ± 0.063
Prolamins	0.115 ± 0.012	0.239 ± 0.014
Soluble proteins	0.887 ± 0.019	2.618 ± 0.052

Table 4: Effect of boron supply on the protein content of post harvested seeds of soybean (*Gycine max* var. JS-335).

Parameters	Boron supply : mg B L^{-1}	
	0.033	0.33
mg 100 mg^{-1} fresh weight		
PPO	0.041 ± 0.005	0.021 ± 0.001
LPO	21.62 ± 0.981	17.59 ± 0.751
POD	0.716 ± 0.089	0.546 ± 0.068
CAT	321 ± 2.05	274 ± 1.95

Table 5: Effect of boron supply on PPO, LPO, POD and CAT conc. in post harvested seeds of soybean (*Gycine max* var. JS-335).

Discussion

In the sand culture experiments, deficiency symptoms observed in soybean, incorporate growth retardation, reduced leaf area, shortening of internodes, stem thickening, death of apical growing points, curling and cupping of young leaves. These boron deficiency traits were earlier set out in other crops [19,20].

The post harvested pods and seeds of soybean showed lower concentration of tissue boron than the flowers. This might be due to low grade boron translocation from flowers to pods and seeds. From the present study, it was constituted that the sufficient range of boron concentration for soybean is 30-60 µg B g^{-1} dry weight. Post harvested seeds of plants receiving low B supply showed low germination capacity and vigor index. Increased source:sink size ratio and decreased harvest index under low B supply indicated that reproductive growth is more affected than vegetative growth by B deficiency.

This fact is very well known and reported by several workers including us that, sugars both reducing and non-reducing and starch were accumulated under deficiency and excess of boron in leaves [21,22]. However, in present study in seeds there was reduced concentration of sugars both reducing and non-reducing and starch under deficiency of boron. In case of low boron supply, there might be inhibition in the formation of borate-sugar complex, which resulted in poor translocation of carbohydrates from source (leaves) to sink (pods/seeds) and hence their accumulation occurred in the leaves.

Decreased concentration of starch in seeds of plants [23] grown under boron deficiency could be due to weak activity of starch synthetase involved in starch synthesis [24]. This decrease in starch and sugar contents of seeds conceivably the cause of undeveloped and deformed seeds under boron deficiency.

In seeds, reserve proteins can be located in the cotyledonous embryo or endosperm region [25]. Seed protein content in soyabean is upto 40%. In leguminosae seeds, globulins are more pronounced than albumins. Globulins are storage proteins whereas albumins are mosly enzymatic and non storage proteins [26]. Plants grown under low boron conditions showed decrease in all protein fractions. This might be due to enhanced activity of ribonuclease which disturbed the protein synthesis mechanism via influencing the RNA content of a cell. An increase in ribonuclease activity in plants under boron stressed condition was earlier observed by many workers [27,28].

Seeds of boron deficient plants have low oil content, suggesting the role of boron in lipid biosynthesis and a study on fatty acid composition of oils would provide valuable information. The decrease in lipid content in the germinating seeds probably caused by the involvement of a lypolytic enzyme, which is responsible for hydrolysis of triacylglycerol that ultimately generate sugars for the growth of germinating embryo. This finding is in accordance with those reported by [29]. The seed storage substances gradually decrease with the increase of germination time. The decrease in different types of nutrient content in germinating seeds probably caused by involvement of the hydrolytic enzymes, which hydrolyses seed storage nutrients, a process that generates amino acids and sugars for the development of embryo and seedling growth.

[30] reported that the phenols protect the germinating seeds by an antimitotic effect on soil microorganism. [31] suggested that accumulation of phenolic compounds particularly caffeic acid and quinones, which are highly reactive leads to enhanced generation of superoxide ions (O_2^-), which are known to cause peroxidative damage to the vital components of cellular membranes such as lipids and proteins. Seeds from plants raised under boron stress condition showed the enhancement in phenolics. This increased phenol may be the cause of reduced oil and proteins in seeds which in turn are responsible for deterioration of quality of seeds.

The amount of electrolyte leakage, an indicator of membrane damage, was found to be more in boron deficient than sufficient plants. Enhanced electrolyte leakage under low boron indicated the direct role of boron in cell integrity. Failure of boron deficient seeds to germinate might be due to lipid peroxidation, mitochondrial impairment and less ATP production [32]. Many studies it was observed that peroxidative changes in fatty acid composition of membrane lipids lead to abnormal functioning of cellular membranes associated with increased viscosity and permeability of bilayers [33]. Thus the increase in solute leakage is due to changes in membrane lipids [34]. The decrease in seed reserve mobilization rate was the cause of decreased seedling growth.

ROS production occurs during various stages of seed development such as embryogenesis, seed maturation, aging and germination. According to [35], ROS in seed physiology performs dual function, a detrimental role that cause the oxidative damage of cell components by excessive production of ROS and beneficial role by participating in the cellular signalling pathways. [35], has reported the involvement of ROS in endosperm weakening, protection against pathogens, redox regulation, and programmed cell death in the aleurone layer during seed germination.To describe the requirements for the balanced production and catabolism of ROS during seed germination the concept of an “oxidative window for germination” is proposed by [35]. When the ROS level is maintained between the upper and lower limits of this oxidative window the dormancy of seed will be broken and germination started. Less ROS production result in the maintenance of dormancy, whereas more ROS production influencing the oxidative damage and accelerating the deterioration of seeds.

POD has been localized in the integument of soybean seeds [36]. During embryo axis growth and cotyledon expansion the increase in peroxidase activity perhaps related to cell wall growth and lignification of xylem elements of growing seedlings.The maintenance of a correct size and shape of protoderm cells, apparently indispensable for normal embryo growth and to the catabolism of IAA in higher plants are mainly associated with the activity of peroxidases. This might show the involvement of peroxidases in the management of plant growth and development [37].

Catalase is heme containing tetrameric enzyme involved in the removal of H_2O_2 [38]. Plant catalases are involved in photo respiraotory functions [39], and scavenging of H_2O_2 during β- oxidation of fatty acids in germinating seeds [40]. Hence CAT activity plays a vital role in the early events of seedling growth in oily seeds. In the present study, we found that the CAT activity enhanced under boron deficiency. Increased CAT activity could be an indication of the enhanced ROS production in tissues during stress condition.

Conclusion

Deformed seed structure and poor storage capacity for reserves such as- carbohydrate, proteins and oil contents and highly accumulated toxic phenolic compounds might be the cause of retarded seed viability and vigor in post harvested seeds of plants subjected to boron stress. It was also reported that B deficiency resulted in enhanced activity of phenolic compounds and polyphenoloxidase, contributing to ion leakage, phenols oxidation and generation of reactive quinones which generate toxic oxygen species, cause of peroxidative damage to cell components such as membrane lipids and proteins. The observation made on the basis of results obtained in the present study suggested the role of boron in enhancing the properties of seeds, such as germination, viability and vigour. Carbohydrate, protein and lipid metabolism of seeds is also influenced by the boron nutrition. It is also concluded that boron influenced the seed reserve mobilization, lipid peroxidation and oxidative damage in germinating seeds. Thus an optimum concentration of boron is required for the appropriate germination of seeds and growth of seedlings. Thus boron deficiency is a major factor responsible for low seed yield and quality of oil yielding crops widely cultivated on boron deficient soils world over.

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