General Impact Factor (GIF): 0.875
Scientific Journal Impact Factor: 1.205

e- ISSN: 2394-5532
p- ISSN: 2394-823X

International Journal of Applied And Pure Science and Agriculture

www.ijapsa.com

Boron deficiency induced retardation in pollen-stigma interaction in soybean plants

Subtitle: Pollen-stigma interaction in soybean

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Abstract

To assess the boron deficiency induced changes in reproductive development, an experiment was conducted with soybean (Glycine max var. JS-335), in sand culture under glass house condition till maturity at deficient (0.033 mg BL^{-1}) and sufficient (0.33 mg BL^{-1}) boron supply. Deficient supply of boron delayed as well as decreased the number of flowers formed per plant and reduced the size of anthers, pollen producing capacity (PPC), the size and viability of pollen grains, pollen tube length, pistil size and number of ovules per ovary. Insufficient boron in plants also affected the activities of stigmatic enzymes- peroxidise, acid phosphatise and esterase which are involved in adhesion of pollen grains on stigmatic surface during fertilization phenomenon and this is indicative of a role of boron in pollen-stigma interaction.

Key words: deficient and sufficient boron, pollen-stigma interaction, soybean.

I. INTRODUCTION

Boron is an essential element for the normal growth and development of higher plants (Camacho-Cristobal *et al.* 2008). Many physiological and biochemical processes such as sugar transport, cell wall synthesis, lignification of cell wall structure, membrane integrity, carbohydrate, RNA, IAA and phenol metabolisms, in plants are directly or indirectly regulated by the boron (Cakmak and Romheld 1997). Boron deficiency is one of the most widespread micronutrient deficiencies in agricultural crops in world and leads to heavy losses in yield. In soil deficiency of boron is most prevailed due to its easily leachable property under high rainfall conditions (Camacho-Cristobal *et al.* 2008).

Boron requirement is generally higher for reproductive development than for vegetative growth in plants (Dell and Huang 1997). Restricted and delayed flowering with a marked reduction in their size and numbers in oilseed rape was observed under boron deficiency (Zhang *et al.* 1994). Agarwala *et al.* (1981) showed delayed emergence of tassels and lack of sporogenous tissue and formation of staminodes in place of stamens in B deficient maize. Boron deficient plants of oilseed rape developed smaller stamens with abnormally developed tapetum (Zhang *et al.* 1994) and showed arrest of microsporogenesis beyond the pollen mother cell stage (Xu *et al.* 1993). Requirement of B for pollen fertility has been demonstrated because poor *in vitro* germination of pollen grains in absence of B has been observed in a number of plant species including maize (Agarwala *et al.* 1981), avocado (Smith *et al.* 1997) and *Picea meyeri* (Wang *et al.* 2003). Besides microsporogenesis, development of ovule and embryo sac also remain arrested in flowers of oilseed rape deficient in boron (Xu *et al.* 1993).

Role of boron in reproductive development has been reported mainly to its involvement in pollen viability and pollen tube germination and rarely on the enzymatic activities of enzymes (Agarwala *et al.* 1981). No literature is available on the role of boron in pollen-stigma interaction as well as stigmatic proteins and ROS in plants. Hence the study in this direction was conducted to highlight the role of boron in pollen-stigma interaction, a precursor phenomenon for fertilization.

The study was conducted in soybean plants which is an important oilseed crop and suffers from upto 47% yield loss due to boron deficiency. Boron deficiency is the second most widespread micronutrient disorder after zinc in Indian soils and approximately 2-84 % soils in the total cultivated area in different agro-ecological zones are deficient in boron (Singh *et al.* 2009).

II. MATERIALS AND METHODS

The seeds of soybean (*Glycine max* L. var. JS-335) were surface-sterilized with 5% (v/v) mercuric chloride solution and washed properly with deionised manesty still water (MSW) before sowing. The composition of nutrient solution supplied was: 4 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1.33 mM NaH_2PO_4, 0.1 mM FeEDTA, 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 $NiSO_4$ with deficient (0.033 and 0.066 mg B L^{-1}) and sufficient boron supply (0.33 mg B L^{-1}) (Sharma 1996). The entire experimental studies were conducted in glasshouse under controlled conditions of light, humidity and temperature. During the period in which the experiment was conducted light (PAR) ranged between 980 and 1120 µmol $m^{-2}s^{-1}$ at 12:00 noon, relative humidity ranged between 68 and 90% at 9:30 A.M. and maximum and minimum temperature ranged between 34 to 42 ^oC and 25 to 28 ^oC. The plants were raised under sand culture till maturity and purified sand of particle size 0.25 to 0.84 mm, was used to carried out the experiment. The pots used for growing the plants were of high quality polyethylene and had a central drainage hole at the bottom. Nutrient solutions were supplied daily except on weekends when the pots were thoroughly flushed with distilled water to prevent accumulation of salts and root exudates in the rooting medium. The amount of nutrient solution supplied to the pots varied with the stage of growth and the weather conditions.

The plant material was separated into leaves, stem and roots after 55 days of sowing and washed thoroughly with distilled water, finely chopped and dried in a forced drought oven at 70^o C for 48 hours. The oven dried plant material was transferred to a desiccator, cooled and was weighed accurately. Tissue boron in vegetative parts (leaves, stem and roots) and reproductive parts (flower, pod and seeds) was estimated colorimetrically by Azomethine-H in wet digest (HNO_3, $HClO_4$ 10:1 v/v) of oven dried plant material by method of Wolf (1974).

For studying the morphology and determining the number of pollen grains and their viability, flowers were collected from plants receiving variable boron supply before 9 AM and kept in vials before use. A homogenous suspension was prepared from the anthers of mature flower buds by gently crushing the anthers in 10% glycerol. The number of pollen grains in the suspension was counted under compound microscope. Freshly dehisced pollen grains and pistils were collected and their length and width was calculated with the help of ocular and stage micrometer under compound microscope.

Viability of pollen grains was determined by germinating the pollen grains in a culture medium containing 10% sucrose, 0.01% boric acid, 0.03 % calcium nitrate, 0.02 % magnesium sulphate and 0.01% potassium nitrate by hanging drop method (Brewbaker and Kwack 1963). The pollen grains were suspended in a drop of culture medium on a cover glass hanging over a shallow depression of the cavity slide. To prevent evaporation of the medium the preparation was sealed with glycerine jelly and placed in a humid glass chamber at 22^{0}C. Pollen viability was assessed by per cent pollen germination as observed under a microscope. The pollen grains were considered as germinated when the length of pollen tube was more than the diameter of the pollen grain. Scoring was done in replicates of 10 sets of 20 pollen grains each, from each treatment.

For study of localization of enzymes, proteins and reactive oxygen species (ROS) on stigma surface, stigma from 10-20 flowers were gently excised without injuring the stigma and style. For localization of enzymes the stigmas were placed in a cavity slide so that the styles did not dip into the solution and incubated in the reaction solution as mentioned below for 10 –20 min at 25-35^{0}C in a humid chamber. After appropriate reaction each pistil was removed separately, rinsed thoroughly

in phosphate buffer and mounted in glycerin jelly. The stained samples were observed in a Nikon E-400 light microscope and were photographed by Nikon F-60 camera using a 35 mm (100 ASA) color film

Staining for POD was done by the method of Raa (1973). The stigma were stained for 30 min in 0.5 % paraphenylenediamine and 0.5% H_2O_2 and washed thoroughly in phosphate buffer before mounting. For localization of APase the stigma were placed for 15 min in a reaction mixture containing α- naphthyl phosphate (sigma) as the substrate, fast garnet GBC (sigma) as the coupling agent in 0.1M acetate buffer (pH 4.0) and 10% $MgCl_2$ (Shivanna and Rangaswamy 1992). Staining for localization of esterase was done in a freshly prepared solution of α-naphthyl acetate (Sigma) in 0.15M phosphate buffer, 10% sucrose and 25 mg Fast Blue B salt (Sigma). For control α- naphthyl acetate was excluded from the reaction mixture (Shivanna and Rangaswamy 1992).

Protein localization in pollinated stigma was carried out by immersing the stigma directly in 0.25% Coomassie Blue in 5% trichloroacetic acid (Fazekas et al. 1963). For the localization of ROS (H_2O_2), the excised pistils were immersed in a solution containing the ROS indicator dye TMB (3,3', 5,5'-tetramethylbenzidine-HCl, in TRIS-acetate buffer, pH 5.0) until a blue colour was observed (Barceló et al. 2002).

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=10). Differences between treatments means were compared by using LSD at the 0.05 probability level.

III. RESULTS AND DISCUSSION

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 and 0.066 mg B L^{-1} supply showed growth reduction and deficiency symptoms such as irregular chlorotic areas between the veins in young leaves. 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.

Table 1- Effect of boron supply on the dry weight and boron concentration in vegetative and reproductive parts of soybean (Gycine max var. JS-335).

		Boron supply : mg B L^{-1}		
Plant parts	DAS	0.033	0.066	0.33
		Dry weight: g $plant^{-1}$		
Leaves	55	5.00[b]	5.65[a]	6.13[a]
Stem	55	6.97[b]	7.34[b]	9.95[a]
Root	55	2.17[c]	3.87[b]	4.96[a]
		B concentration: µg B g^{-1} dry wt.		
Leaves	55	18.25[c]	25.68[b]	30.10[a]
Flowers	55	28.15[c]	48.75[b]	56.70[a]
Pods	115	18.30[c]	22.89[b]	30.75[a]
Seeds	115	17.70[c]	21.63[b]	25.21[a]

Differences between group means with different letters in the same row are significant at $P \geq 0.05$

Reproductive growth was found to be more sensitive to boron nutrition than vegetative growth. The increased susceptibility of plants to boron deficiency during reproductive phase is exhibited by particularly marked effects of deficiency on the flowering. Flowering was delayed by 10 days in plants supplied deficient boron 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 flowers formed per plant (Table 1). Tissue boron concentration in flowers was found to be more than in leaves and indicates a higher boron requirement for reproductive development (Table 1). High boron concentration in reproductive parts such as stamens, stigma, style and ovaries has been reported earlier in oilseed rape but in our best knowledge no work in this direction is reported in soybean (Zhang *et al.* 1994).

Table 2- Effect of boron supply on flower formation and male and female reproductive parts of soybean (Glycine max var. JS-335).

	Boron supply : mg B L^{-1}		
Parameters	0.033	0.066	0.33
Flowers no. [$plant^{-1}$]	159^{b}	341^{a}	358^{a}
Anther size [µm] L	375^{b}	458^{a}	465^{a}
B	465^{b}	489^{a}	495^{a}
Pollen producing capacity [No. $anther^{-1}$]	371^{c}	521^{b}	624^{a}
Pollen size [µm]	61.25^{b}	72.89^{a}	78.75^{a}
Pollen viability [%]	47.5^{c}	65.60^{b}	77^{a}
Pollen tube length [µm]	225^{c}	295^{b}	339^{a}
Stigma size [µm] L	105^{c}	139^{b}	165^{a}
B	180^{c}	223.8^{b}	247.5^{a}
Stylar length [mm]	2.00^{b}	2.11^{a}	2.11^{a}
Ovary length [mm]	1.55^{a}	1.62^{a}	1.62^{a}

Differences between group means with different letters in the same row are significant at $P \geq 0.05$

There was improper development of male and female reproductive parts in flowers of plants subjected to deficient boron supply. Flowers of deficient plants of soybean were poorly developed, deformed and showed small sized anthers with very poor pollen producing capacity (Table 2). Size of stigmatic head was also affected under boron deficiency. Reduced stigmatic head size (Table 2) decreased the receptive area for the pollen grains on stigmatic surface. Number of ovules formed per ovary was found to be decreased in plants subjected to insufficient boron supply (Table 2), which might be the cause of reduced seed formation per pod, resulting in poor seed yield.

Pollen grains were also underdeveloped and malformed in soybean plants under boron deficiency (Fig. 1). Observed results were in consonance with the work of earlier workers (Rerkasem *et al.* 1993, Cheng and Rerkasem 1993, Chatterjee and Nautiyal 2000). Pollen viability is known to be impaired by boron deficiency (Cheng and Rerkasem 1993) and pollen germination and pollen tube growth is particularly sensitive to boron supply. In wheat, Rerkasem *et al.* (1993) reported that boron deficiency results in a failure of fertilization due to the impaired development of anthers and poor pollen germination. They also found reduced fertility of both male and female parts of wheat flowers of boron deficient plants. In our study also we observed poor pollen viability and pollen tube germination at deficient boron supply reiterating the role of boron in pollen germination and microsporogenesis. Accumulation of phenolics is a characteristic feature of the tissues subjected to boron stress, resulting in an injury to membrane structure and cellular functions (Cakmak and Römheld 1997). Thus, poor pollen germination and pollen tube growth might be due to the enhanced accumulation of phenolics, resulting in morphological and structural alterations in pollen tubes as observed earlier by Wang *et al.* (2003).

Kuruvilla and Shah (1988) reported the role of pollen and stigma surface borne proteins including various enzymes, polysaccharides and lipids in the recognition and rejection reactions by the cytochemical studies. Receptive stigma should have an adhesive surface because its first function is to trap pollen grains. After adhesion, pollen-stigma interaction is initiated and many events take place, of which first one is the hydration of the pollen grain and release of wall proteins that bind to receptors on the stigma surface (Clarke *et al.* 1979). Low protein content at stigmatic surface of the plants of soybean supplied with deficient boron supply (Fig.1) indicated the poor receptivity of stigma for pollen grains resulting in poor fertilization.

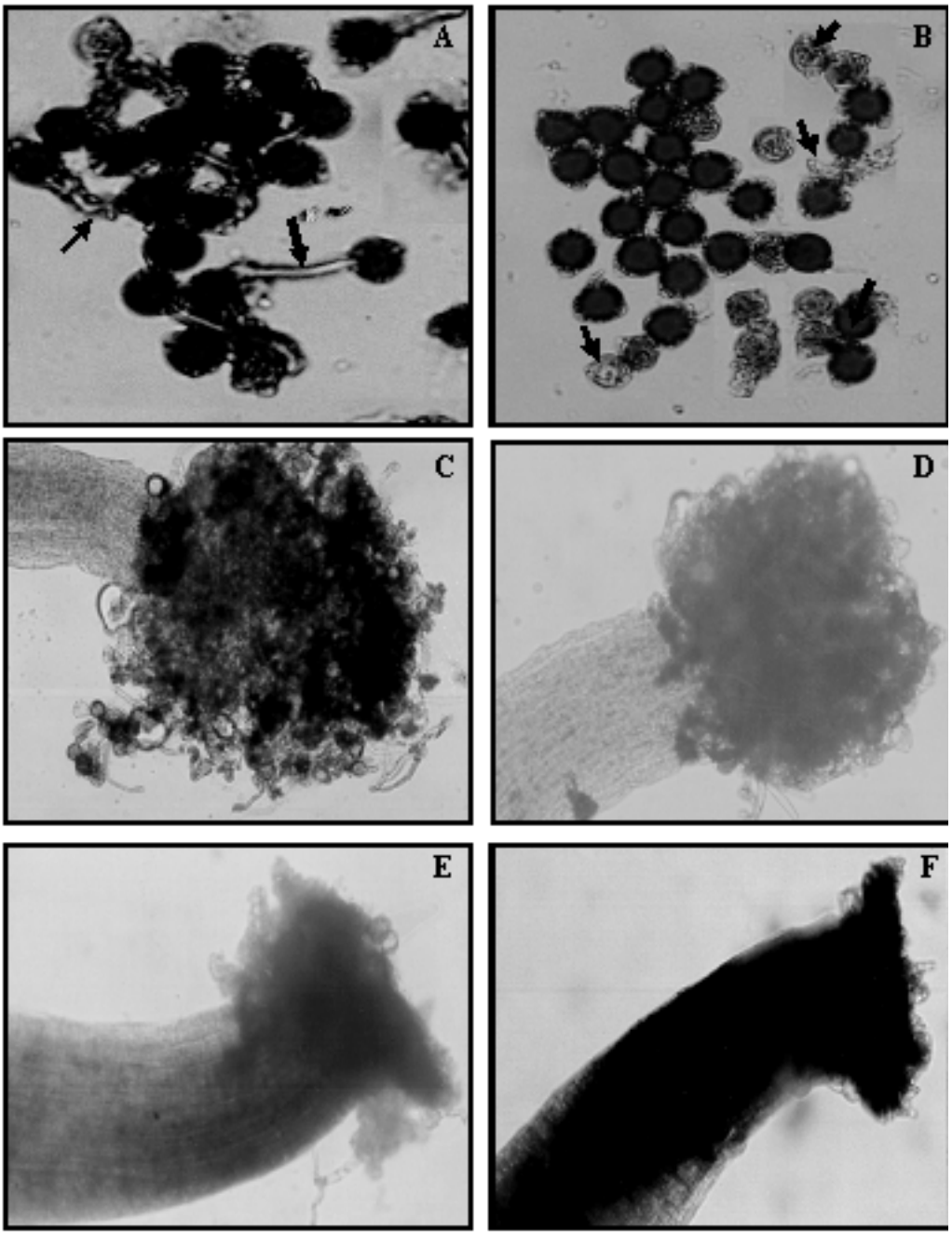

Fig.1

Fig. 1. Effect of boron sufficient- 0.33 mg BL^{-1} (A, C, E) and deficient- 0.033 mg BL^{-1} (B, D, F) supply on pollen viability (A, B), protein (C, D) and ROS/H_2O_2 (E, F) in soybean plants. Arrows indicate pollen tube germination (A) and non-viable pollen grains (B).

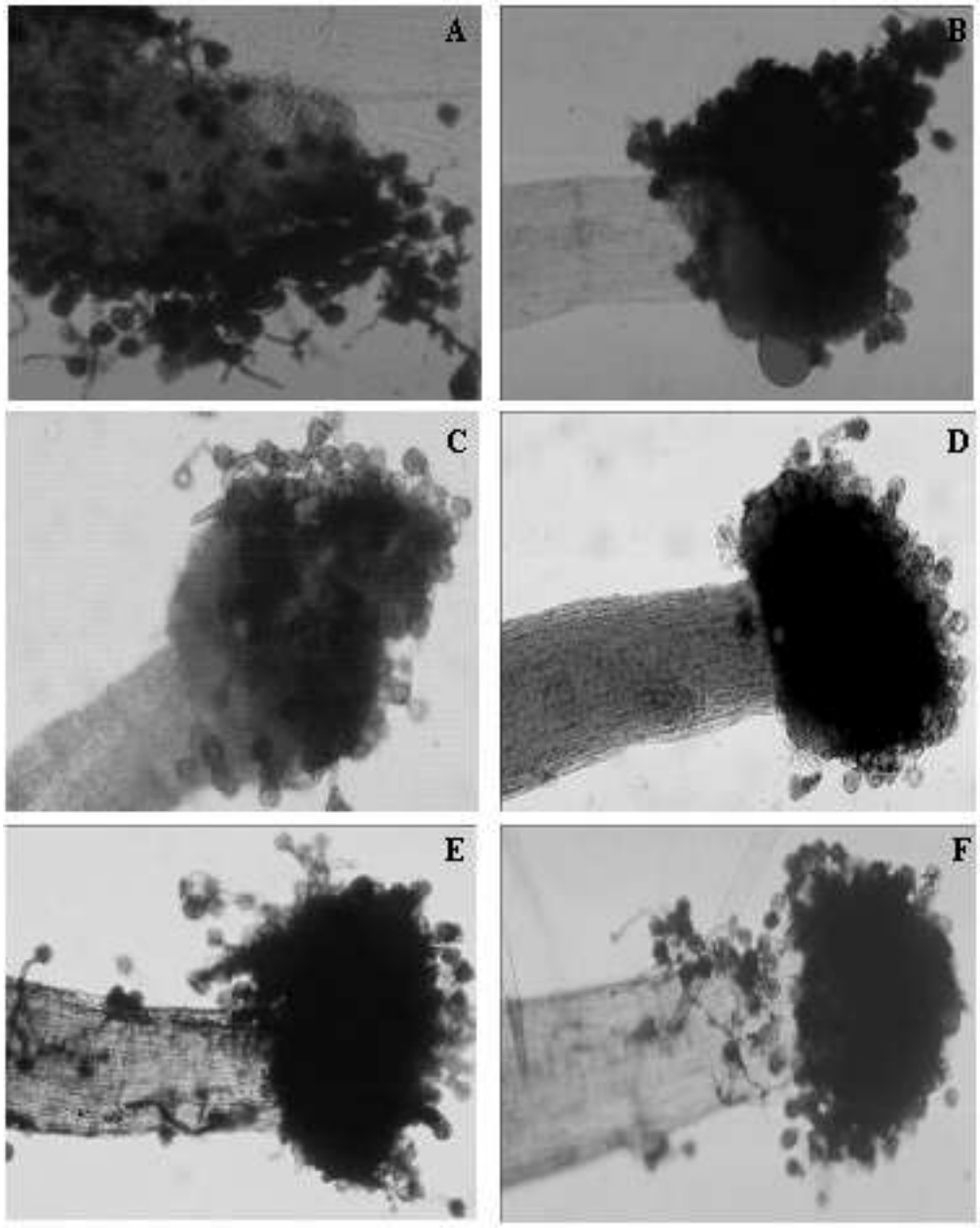

Fig. 2

Fig. 2. Effect of sufficient- 0.33 mg BL^{-1} (A, C, E) and deficient- 0.033 mg BL^{-1} (B, D, F) boron supply on peroxidase (A, B), acid phosphatase (C, D) and esterase (E, F) in stigma of soybean plants.

Enzymic activities are characteristic feature of the stigmatic receptivity. The presence of several enzymes is found to be essential for the pollen-stigma interaction. Enzymes acid phosphatase (AcPh) and peroxidase (POD) showed changes in their activities under insufficient boron supply. There was increased activity of AcPh in soybean (Fig. 2) as also observed by Agarwala *et al.* (1981) in boron deficient pollen grains of maize. The activity of POD in stigma was also found to be increased in soybean plants subjected to boron deficiency (Fig. 2). POD generated by the pollens may function in the formation of pollen tube wall and its regulation and that of stigmatic POD may facilitate the pollen tube growth in the transmitting tissue of the style (Bredemeijer 1984). Thus POD may have regulatory function in pollen-stigma interaction and also facilitate the fertilization phenomenon. McInnis *et al.* (2006) reported that the peroxidases may provide enhanced protection against pathogen attack when the stigma is primed to receive pollens. They have also suggested that

peroxidases generally catalyze the breakdown of H_2O_2 and produced highly oxidizing intermediates which in turn oxidizes a variety of organic and inorganic reducing substrates. Enhanced ROS/H_2O_2 generation as observed by us for the first time, in the stigma of boron deficient plants (Fig. 1) creates oxidative stress condition and increased POD activity might be to overcome this by detoxification of H_2O_2. It has been investigated by several workers (Neill *et al.* 2002, Foreman *et al.* 2003, Rentel and Knight 2004) that H_2O_2 and other reactive oxygen species (ROS) are involved in cell signaling in plants and regulate diverse aspects of plant metabolism and cell growth. Enhanced generation of ROS/H_2O_2 might be response of plants induced stress created by low boron availability.

Pollen and/or stigma cutinases are serine esterases and mediate pollen tube penetration. It is suggested that the activities of cutinases (Edlund *et al.* 2004) and esterases (Hiscock *et al.* 2002, Pandey *et al.* 2009) are possibly involved in the rupture of the stigmatic cuticle. The most important role of esterase enzyme is hydrolysis of cutin and dissolution of the stigmatic cuticle prior to entry of the pollen tube into the papillar cell wall (Hiscock *et al.* 2002). As observed in the present study decreased esterase activity in stigma of boron deficient plants (Fig. 2) would diminish the pollen germination and the growth of pollen tube within the transmitting tissue of style.

IV. CONCLUSION

In our study it was concluded that the observed changes in the activities of stigmatic and pollen peroxidase, acid phosphatase and esterase as well as concentration of proteins and H_2O_2 induced in response to boron deficiency creates an unfavourable situation for the pollen-stigma interaction and limits fertilization leading to poor reproductive yield.

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