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Copper Stress Effect in Developing Wheat Caryopsis Cultured in Vitro

Ruby Srivastava, Nirmala Nautiyal
Botany Department, Lucknow University, Lucknow,
Uttar Pradesh, India

Abstract-

An in vitro experiment was conducted to study the effect of Cu stress, that is, low and excess Cu on the growth of wheat caryopsis at the active grain filling period. Fertilized ovules or developing kernels of wheat (Triticumaestivum L.) cv. WH 542, excised at 14 days after anthesis (DAA), were cultured aseptically in MS medium (without IAA and kinetin) at three levels of Cu, 0.01 (low), 0.1 (normal) and 0.5 µm (excess) Cu. At each Cu level ABA was supplied at nil (–ABA) and 0.1 mM (+ABA). After 8 days in culture growth of the caryopsis, measured as increase in the dry weight of kernels, was maximal at 0.1 µM Cu associated with an increase in starch, total and reducing sugars and nitrogen content. Compared to normal (0.1 µM) Cu, the size, dry weight, concentration of Cu, starch, total and reducing sugars, non-protein and protein nitrogen decreased and that of total phenols and activity of peroxidase increased in kernels at deficient Cu. Excess Cu, however, caused a decrease in the kernel size, fresh and dry weight, sugar fractions with an increase in the concentration of Cu, total phenols, both nitrogen fractions and a decrease in the peroxidase activity. The SDS-PAGE profile of total proteins shows that the expression of proteins of MW 89, 80 and 39 KDa was lesser at low and excess Cu as compared to normal Cu. ABA supply almost reversed the effect of both low and excess Cu on the kernel size, fresh weight, starch, sugars, protein and non-protein nitrogen content, activity of peroxidase and the expression of seed proteins.

Key Words- Triticumaestivum, in vitro culture, Cu stress, kernels,

I. INTRODUCTION

Copper is an essential micronutrient for plants indispensable for the function of a large number of enzymes catalyzing oxidation reduction reactions in a variety of metabolic pathways [17, 22]. Plants respond to both deficiency and excess of Cu [15].

Copper deficiency causes change in a wide range of physiological processes such as metabolism of carbohydrates, nitrogen and the cell wall [3, 21]. Plants suffering from Cu deficiency have lower content of soluble carbohydrates during vegetative growth [2]. However, these plants build up a higher concentration of soluble carbohydrates after anthesis in a few grains which remain green [23]. Graham [10] reported accumulation of soluble carbohydrates in the leaves and roots of Cu deficient wheat plants after anthesis. The effect of copper deficiency on grain, seed and fruit formation is more than the vegetative growth [22, 29, 30]. Higher Cu concentration in floral parts indicates the involvement of Cu in reproductive development [14]. Cu deficiency in wheat retarded the development of ears, anthers and pollen grains [1]. Magomedalier*et al.* [20] reported that Cu application caused an increase in the grain protein content and protein yield in wheat. Cu has been shown to participate in the grain filling process by affecting the enzymes of carbohydrate metabolism due to which starch and protein contents decreased in rice grain under Cu deficiency [29].

Excess Cu is highly toxic to the plants and causes a significant growth reduction [22]. The presence of Cu in excess amounts induces the oxidative stress due to over production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [38] and antioxidant response as increase in the activities of antioxidative enzymes such as catalase, ascorbate peroxidase [32], glutathione reductase[4] and phenylalanine ammonia lyase[9]. Peroxidase imparts rigidity to the cell wall and prevents its expansion involved in growth [8] by catalyzing the formation of cross linking among cell wall palymers[4]. An excess of Cu causes loss of membrane integrity due to accumulation of hydrogen peroxide in the intracellular spaces in between the cell wall and cell membrane [5].

Exogenous supply of ABA to cultured developing seeds prevents precocious germination of immature embryos [13] and regulates the gene expression [7].

Earlier study carried out on the *in vitro* development of hyacinth bean seeds revealed that Cu deficiency causes a reduction in seed reserve accumulation and loss in viability of embryo due to decrease in the activity of invertase and Cu containing enzymes, ascorbate oxidase and polyphenol oxidase [28]. This paper is an expansion of the similar work on the effect of both deficiency and excess of Cu on the development and physiology of wheat caryopsis cultured for 8 days at the active grain filling period i.e. 14 DAA in presence or absence of exogenous ABA.

II. MATERIALS AND METHODS

A. Explant and culture

Wheat (*Triticumaestivum* L.) cv. WH 542 was grown under natural conditions in soil till anthesis. Developing caryopsis or kernels were excised aseptically at 14 days after anthesis (DAA) and used as explant. The explants were

surface sterilized with 0.1 %(w/v) $HgCl_2$ for 5 min and washedseveral times in sterile double distilled water. The sterilized kernels were cultured *invitro* in MS [25] medium (without IAA and kinetin) at three levels of Cu i.e. 0.01 (low), 0.1 (normal) and 0.5 mm (excess) Cu in 10-12 ml MS medium in 8 cm petriplates. At each Cu level, ABA was supplied at nil (–ABA) and 0.1 mM (+ABA). Aseptic conditions were maintained throughout, that is, during sterilization and transfer of the explant to the media and culture. The petriplates containing developing seeds were placed in a BOD incubator at 25±1^oC provided with lights of 70 $Wm^{-2}$for 16 h photoperiod. Six petriplates were taken for each treatment and 15-20 kernels were cultured in each petriplates.

B. Growth parameters

Growth parameters were studied as change in size and shape of kernels after 8 days ofculture. The fresh and dry weights were determined before and after drying the harvested kernels in an electric oven at 80±1^o C for 48 h.

C. Tissue analysis

Oven dried plant material was digested in di acid (HNO_3:$HClO_4$, 10:1) mixture and used for the analysis of tissue Cu concentration by atomic absorption spectrophotometry [28].

The material for the determination of carbohydrate and nitrogen fractions was fixed in 80% (v/v) boiling ethanol (1:10) and was ground at room temperature and centrifuged at 1000xg for 15 minutes. Sugars, phenols and non-protein nitrogen were determined in the alcohol soluble fraction, while the alcohol insoluble fraction was used for the estimation of starch and protein nitrogen content as described earlier [27].

Total proteins from the kernels were extracted using the powdered alcohol insoluble fraction in extraction buffer containing 50 mMtrisHClbuffer pH 6.8, 10 mM $MgCl_2$, 2% (w/v) sodium dodecyl sulphate, 5% (v/v) β- mercaptoethanol and 10% (v/v) glycerol. The extract was centrifuged at 5000 x g for 15 minutes at 4^oC and total protein concentration was estimated in a suitable aliquot of the supernatant [18]. The remaining extract was stored in a freezer at 0^oC and used for SDS-PAGE.

D. Enzyme activity

Peroxidase was extracted from freshly harvested kernels in 0.1M phosphate buffer pH 7.0. The enzyme activity was measured as increase in OD at 485 nm after 5 min of reaction at 25^oC [19]. The reaction mixture (10 ml) contained 500 moles phosphate buffer pH 6.0, 3 µmoles H_2O_2, 50 mg p-polyenylnediamineand a suitable dilution of the enzyme extract. The soluble protein content of the enzyme extract was measured according to the method of Lowry *etal*. [18].

E. SDS-PAGE

The SDS-PAGE of total proteins was carried out after denaturation of the crude extract of proteins for 3 min at 100^oC. Approximately 20-40 µl of protein sample containing 40 µg proteins was loaded in each well and the proteins were resolved in 12% gel in continuous buffer system in a vertical mini gel electrophoresis apparatus as described by Laemmli [16]. After electrophoresis, the gels were stained with Coommassie brilliant blue- R250 and photographed. The markers were run simultaneously and were used for calibration of the molecular weight of proteins according to the method of Neilsen and Reynolds [31].

F. Statistical analysis

All determinations were made in triplicate and the mean values are presented ± SEM. The data were subjected to the analysis of variance (ANOVA) and LSD values were calculated at P=0.05.

III. RESULTS AND DISCUSSION

A. Growth parameters

The developing wheat *(Triticumaestivum* L.) cv.WH542 caryopsis or kernels, excised at 14DAA showed growth induction and response to variable Cu supply, after 8 days of *in vitro* culture. The growth enhancement was measured as increase in size i.e. length, breadth, thickness and fresh and dry weight of the kernels from that of before culture (Table I) both in presence and absence of ABA. The caryopsis growth was maximum at 0.1 µM (normal) Cu and was decreased both at low (0.01 µM) and excess (0.5 µM) Cu in presence of ABA.

Compared to normal Cu, the increase in fresh weight at low Cu in absence of ABA was associated with a decrease in dry weight and size of the kernels(Table I). This could be due to high moisture content and immaturity of kernels in Cu deficiency. These kernels appeared shriveled at maturity due to lower accumulation of reserves as has been reported in rice and sunflower [29, 30]. Supply of ABA reduced both fresh and dry weight of wheat kernels especially at deficient and excess Cu (Table I). The results are in accord with the previous reports on reduction of seed setting in maize [26] and wheat [37] in response to exogenous ABA application to leaves and ears.

B. Tissue analysis

The increase in dry weight of the kernels at normal Cu in presence of ABA was also associated with an increase in the tissue Cu, starch, total and reducing sugars content (Fig.1 and 2; Table II). Compared to normal Cu, decrease in the level of both sugar fractions at low Cu in wheat kernels might be attributed to the reduction in sink capacity in Cu deficiency [10]. The results on decrease in reducing and non-reducing sugars in Cu deficient kernels are in consonance with the earlier reports on wheat plants grown in soil [24] and *in vitro* cultured hyacinth bean seeds [28]. The increase in

the total sugar content in presence of ABA at each Cu level in wheat kernels (Fig. 2) shows induction in the uptake of sugars by ABA due to increased permeability of plasma membrane [22]. The results are in accord with Sharma *et al*. [34], who observed accumulation of reserves, starch, total and soluble sugars, proteins and phenols in somatic embryos of tea at different stages of development in response to ABA supply (5 mg/l).

Compared to normal Cu, the starch content decreased both at low and excess Cu after 8 days culture of wheat kernels excised at the pre-storage phase.The enhancement in the level of sugars in cultured kernels from that of before culture (Fig.2) shows the uptake of sugars from the medium to meet the requirement for biosynthesis of reserves. Maximum increase in sugars at normal Cu shows that the supply of 0.1µM Cu as sufficient for the reserve accumulation and normal growth of wheat kernels cultured*in vitro*.The observed decrease in the starch content at low Cu in wheat caryopsis (Fig.1; Table II) is similar to the reports on Cu deficient anthers of wheat [1] and barley [11] and *in vitro* cultured hyacinth bean seeds [28].This might be due to impaired supply of sugars to the growing sink in Cu deficiency as has been observed in wheat [2, 23]. The supply of ABA increased the starch content at low and normal Cu levels in wheat kernels might be due to induction in starch synthase activity. Compared to normal Cu, decrease in protein and non-protein nitrogen content in wheat kernels at low Cu (Fig.3;TableII) shows the involvement of Cu in nitrogen uptake and biosynthesis of reserve proteins. The results are in accord with the previous studies carried out on seed development in rice plants grown in sand culture [29] and hyacinth bean seeds cultured *in vitro*[28].

The increased level of total phenols in cultured wheat kernels at low Cu (Fig.1) is attributed to the reduction in polyphenol oxidase activity in Cu deficiency [12, 36].The observed increase in peroxidase activity in Cu deficient wheat kernels(Fig.4) is in accord with the reports of Nautiyal*et al*.[30] in sunflower leaves and Nautiyal and Awasthi[28]in hyacinth bean seeds.This could also be due to low Cu availability for Cu/Zn SOD resulting in induction of oxidative stress [22].

The increase in tissue Cu content was observed only at excess Cu in cultured wheat kernels from that of before culture (Fig.1). This shows higher inherent Cu concentration in the kernels before culture. Cu concentration in cultured wheat kernels increased with an increase inCu supply. The values of tissue Cu concentration obtained here are in accord with the previous reports on wheat and other cereal crops [29, 33]. Supply of excess Cu inhibited the growth of the wheat kernels by decreasing their size, fresh and dry weight (TableI). Poor grain development at excess Cu further substantiates the earlier reports of decreased yield and quality of seeds in excess Cu [29, 30].

Compared to normal Cu, excess Cu in wheat kernels reduced the sugars and starch content and increased the protein and non-protein nitrogen content(Fig. 1 to 3; TableII). The observed decrease in sugar content in wheat caryopsis at excess Cu is in accord with the reports of Nautiyal*et al*. [29] in rice grains. The supply of ABA seems to have increased the sugar content in wheat kernels but could not resume the lowered starch content at excess Cu.

The cultured wheat kernels in absence of ABA showed considerable increase inthe tissue Cu, both protein and non-protein nitrogen content at excess Cu (Fig. 1 and 3; Table II) which were otherwise decreased to the level equivalent to normal Cu in presence of ABA. The results show ABA induced inhibition in uptake and utilization of nitrogen especially at excess Cu.

C. Enzyme activity

Excess Cu decreased the peroxidase activity in wheat kernels with a simultaneous increase in the soluble protein content in presence of ABA(Fig. 4; Table II). The increase in the specific activity of peroxidase in absence of ABA might have resulted in peroxidative damage of the membranes at excess Cu [6, 36].

D. SDS-PAGE

The SDS-PAGE profile of total proteins (Fig. 5) shows the expression of all seed proteins in cultured wheat kernels as compared to that of before culture. Compared to normal Cu, at low and excess Cu, the expression of proteins of MW 89, 80, and 39 kDa was less marked in the absence of ABA but not in its presence. It appears that the synthesis of these proteins is hampered due to disturbance in the synthesis of amino acids in Cu stress [24]. No change in other proteins of MW 61, 50, 32, 25 and 22 kDa was observed by variation in Cu or ABA supply.

Table I Growth Parameters, Fresh and Dry Weight (± SE, n=3) of Wheat Kernels Before and After Culture at Variable Cu and ABA.

Copper supply	**Before culture**	**After culture**	
(µM)		**-ABA**	**+ABA (0.1 mM)**
		Length : mm	
0.01		5.7±0.06	5.7±0.12
0.1	3.9±0.06	7.4±0.03	6.5±0.18
0.5		6.3±0.09	6.7±0.38
		Cu^{**} ; ABA^{ns} ; $LSD_{0.05} = 0.81$	
		Breadth : mm	
0.01		3.2±0.29	3.3±0.01
0.1	2.9±0.06	3.9±0.10	4.0±0.15
0.5		3.0±0.01	3.3±0.09
		Cu^{**} ; ABA^{ns} ; $LSD_{0.05} = 0.36$	
		Thickness : mm	

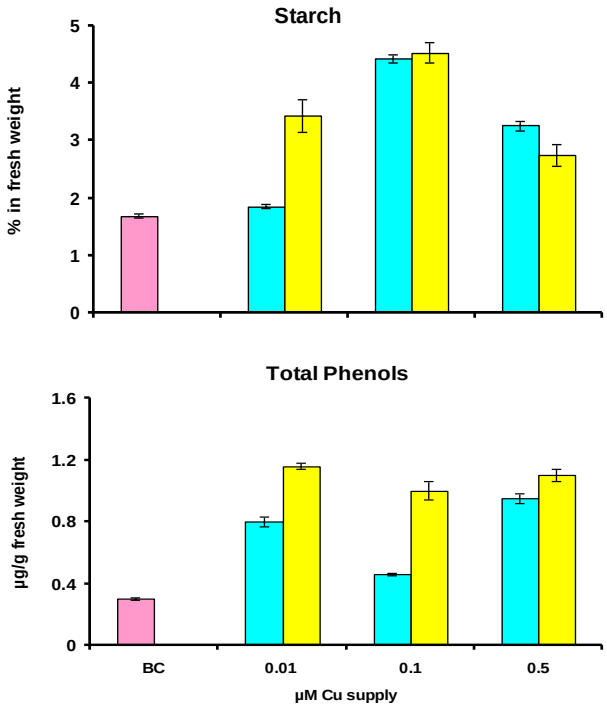
0.01		1.9±0.12	2.1±0.06
0.1	1.0±0.12	3.0±0.10	2.3±0.15
0.5		2.3±0.06	2.0±0.12
		Cu^{**} ; ABA^{**} ; $LSD_{0.05} = 0.73$	
		Fresh weight : mg / kernel	
0.01		36±0.7	29±0.1
0.1	14±0.6	33±0.1	29±0.1
0.5		31±0.1	28±0.6
		Cu^{ns} ; ABA^{*} ; $LSD_{0.05} = 1.0$	
		Dry weight : mg / kernel	
0.01		13±0.1	10±0.9
0.1	8±0.6	14±0.1	15±0.1
0.5		13±0.1	10±0.1
		Cu^{*} ; ABA^{*} ; $LSD_{0.05} = 2.8$	

*, **, *** significant at P = 0.05; P = 0.01 and P = 0.001 respectively.
^{ns} – Not significant at P = 0.05.

Table II Significance Levels of Variance ratios in ANOVA for the Effect of Variable Cu and ABA Supply on Starch, Phenols, Tissue Cu, Sugar and Nitrogen Fractions and Activity of Peroxidase in Wheat Kernels Cultured *in vitro*.

S.No.	Parameters	Variations		$LSD_{0.05}$
		Cu levels	ABA supply	
1.	Starch	**	ns	1.06
2.	Total Phenols	**	**	0.21
3.	Tissue Cu	**	*	3.0
4.	Sugars			
	Non reducing sugars	ns	ns	0.08
	Reducing sugars	*	ns	0.10
	Total sugars	***	**	0.02
5.	Nitrogen			
	Non Protein nitrogen	**	***	0.03
	Protein nitrogen	**	**	0.13
	Total nitrogen	*	**	0.28
6.	Peroxidase activity			
	Fresh Weight Basis	**	ns	0.18
	mg Protein basis	**	ns	0.26
	Soluble Protein	ns	ns	0.36

*, **, *** Significant at P=0.05, P = 0.01 and 0.001 respectively
ns- Non Significant at P=0.05



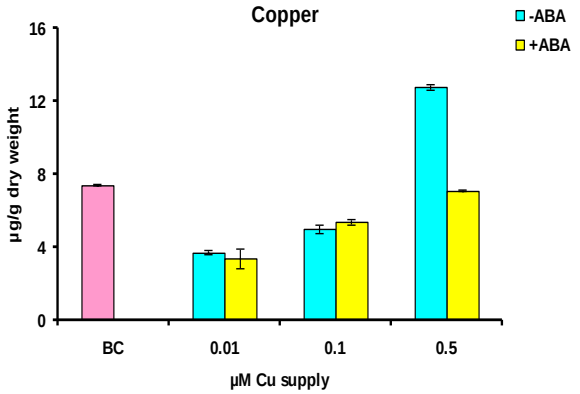


Fig. 1Starch, total phenols and tissue copper concentration in wheat kernels before and after culture at variable copper and ABA.Vertical lines represent ± S.E. BC = before culture.

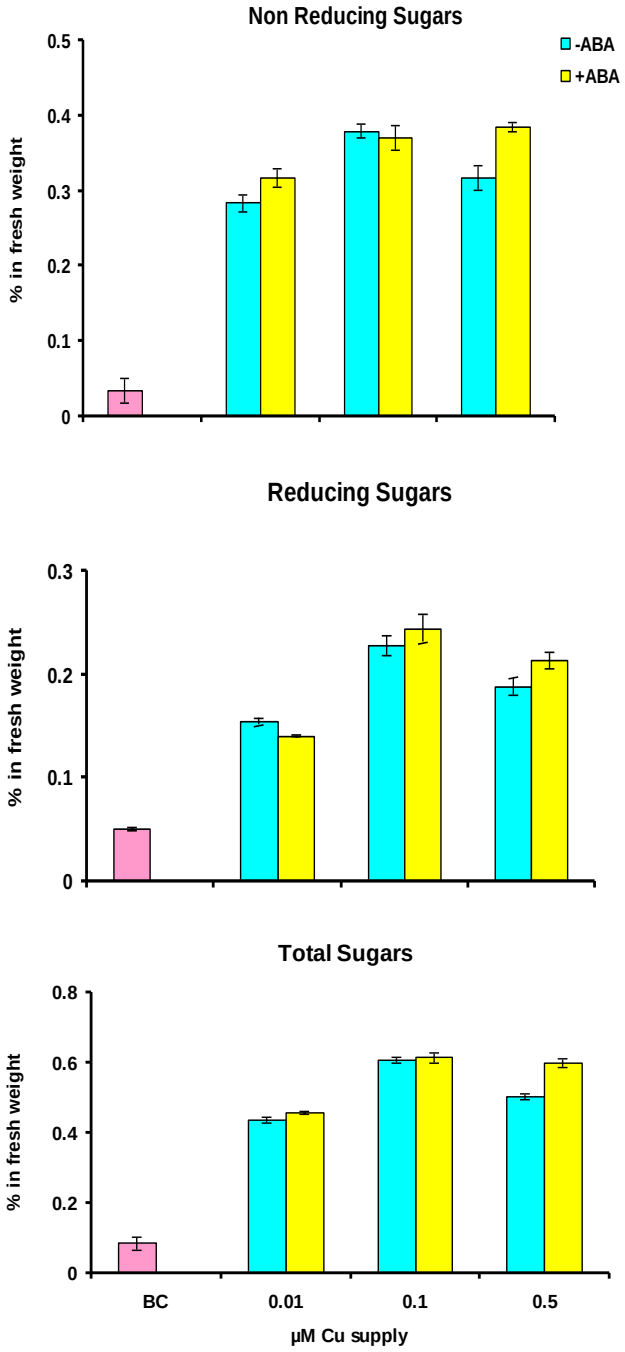


Fig. 2 Sugar content in wheat kernels before and after culture at variable copper and ABA.Vertical lines represent ± S.E.BC = before culture.

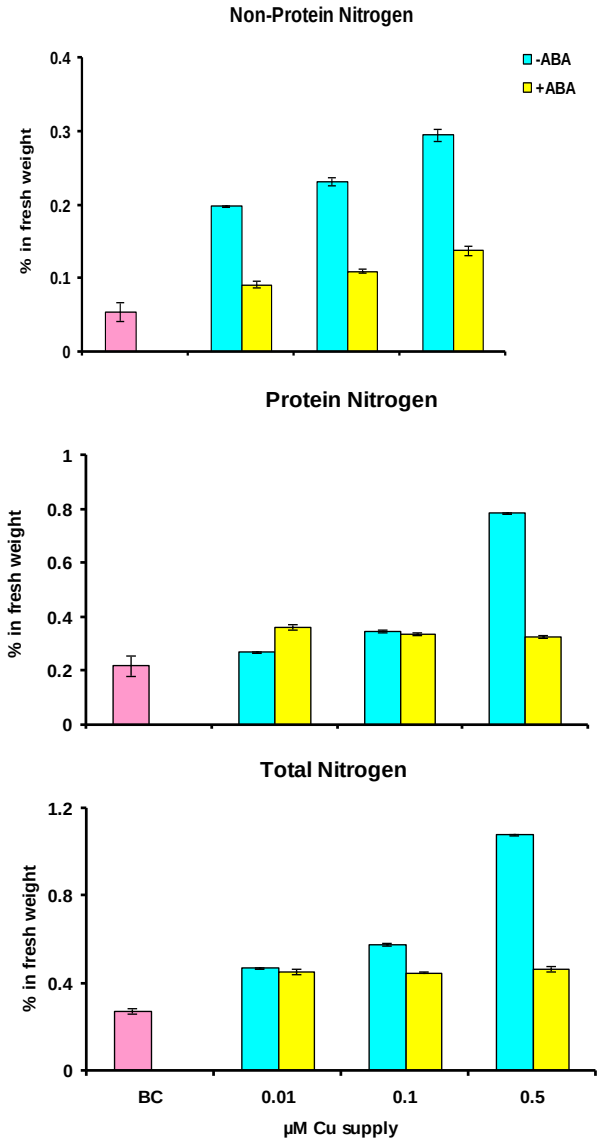
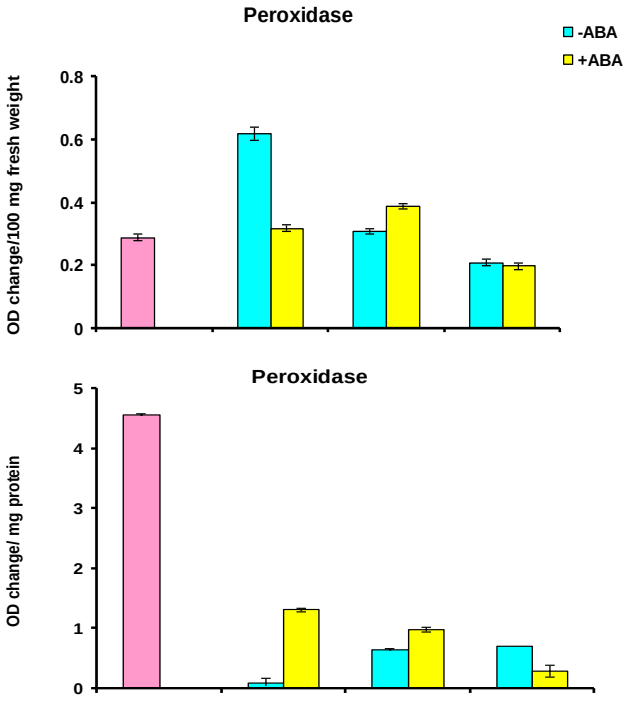


Fig. 3 Nitrogen content in wheat kernels before and after culture at variable copper and ABA.Vertical lines represent ± S.E.BC = before culture



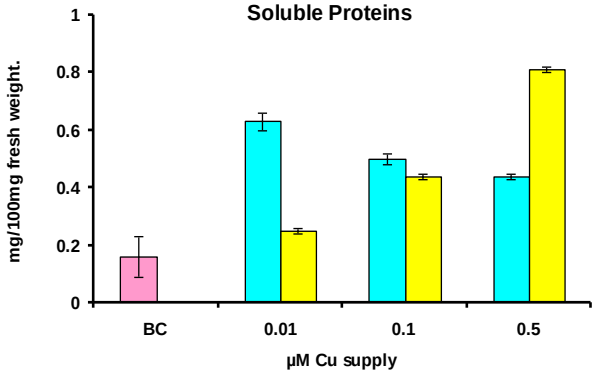


Fig. 4 Peroxidase activity and soluble protein content in wheat kernels before and after culture at variable copper and ABA.Vertical lines represent ± S.E. BC = before culture.

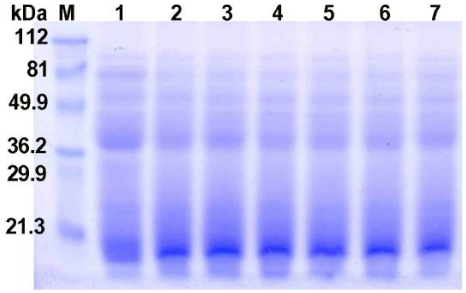


Fig.5 SDS - PAGE profile of total proteins in wheat kernels in response to variable Cu and ABA supply.

Lane M, molecular weight markers; Lane 1,kernels before culture;Kernels after cultureat 0.01 µm Cu (lanes 2 & 5); 0.1 µm Cu (lanes 3 & 6); 0.5 µm Cu (lanes 4 & 7); without ABA (lanes 2 to 4) and with 0.1 mM ABA (lanes 5 to 7).

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