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INFLUENCE OF COPPER ON EMBRYO VIABILITY AND DEVELOPMENT IN HYACINTH BEAN SEEDS

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SUMMARY

An experiment was conducted to study the role of Cu in biosynthesis and accumulation of seed reserves and impact of exogenous ABA application on Cu deficiency. Fertilized ovules of hyacinth bean (*Dolichos lablab* L.), cultured aseptically at 8 and 10 days after anthesis developed copper deficiency after 5 days in culture at 0.02 µM Cu resulting in reduction in seed reserve accumulation and loss in viability of embryos. The results suggest that decrease in the activities of acid invertase and Cu enzymes, ascorbate oxidase and polyphenol oxidase and induction of some Cu stress proteins resembling HSP_{70} affected the seed development in hyacinth bean at deficient Cu. Exogenous ABA application (0.1 mM) enhanced the level of some reserves but was unable to reverse the Cu deficiency effects.

Key words: **Abscisic acid, copper deficiency, embryo viability, hyacinth bean, seed reserves**

INTRODUCTION

The requirement of copper for the development of reproductive parts is generally higher than vegetative parts (Nautiyal *et al.* 1999a). Seed yield in strawberry clover (*Trifolium fragiferum*) was found to be more sensitive to sub-optimal copper supply than vegetative dry matter yield (McFarlane 1989). The ovaries and anthers containing developing pollens are normally very high in copper demand (Knight *et al.* 1973). Cu is necessary for production of viable pollen and seed set, even in a less Cu tolerant plant, *Mimulus guttatus* (Harper *et al.* 1998), its deficiency causes delayed flowering and reduction in number of flowers in *Chrysanthemum* (Davies *et al.* 1978). Copper deficiency in wheat retarded the development of ears, anthers and pollen grains (Agarwala *et al.* 1980) and also decreased the grain number without decreasing the weight of individual grain (Hill *et al.* 1979). However, application of Cu increased the grain protein content and protein yield in wheat (Magomedalier *et al.* 1993).

Exogenous ABA inhibited the precocious germination of immature embryos in wheat, cotton, soybean and corn cultured *in vitro* (Kermode 1995) while triggered the accumulation of storage reserves and overcome the problem of poor germination in maturing somatic embryos of tea (Sharma *et al.* 2004). ABA has also been identified as a signaling element regulating gene expression during seed development (Finkelstein *et al.* 2002).

The present study focuses on the growth, ultra-structural and biochemical changes in developing hyacinth bean seeds in relation to copper supply *in vitro* during the active period of seed filling. The developing seeds were cultured in modified Monnier's liquid medium at a deficient and an adequate Cu level, without or with exogenous supply of ABA. Cu deficiency responses were observed on growth, embryo viability, tissue Cu concentration, biosynthesis and accumulation of seed reserves such as carbohydrates, nitrogen fractions, starch, phenols and protein contents along with some

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enzyme activities and micro-structural details of cultured seeds.

MATERIALS AND METHODS

Developing seeds of hyacinth bean (*Dolichos lablab* L.), excised from pods at 8 and 10 days post anthesis (DPA), were cultured aseptically in modified Monnier's (1978) liquid medium with 5% sucrose at two levels each of Cu and ABA. The stock solutions of macronutrients were prepared separately from Analytical Reagent (AR) grade salts and purified by phosphate carbonate absorption method followed by extraction of Cu as dithiozonate at pH 6.0 (Hewitt, 1966). Copper was supplied as $CuSO_4$ at two levels, low (0.02 µM) and adequate (0.2 µM), without or with ABA (0.1 mM). All glass wares and culture media were sterilized in an autoclave at 103 kPa and 120°C for 25 min.

Ten to fifteen developing seeds were transferred to petridishes and placed in BOD incubator at 25±1°C and 75 Wm^{-2} for 16 h photoperiod.

The dimension of the harvested seeds was recorded after 5 d in culture. Seeds were rinsed with distilled water, blotted dry, weighed for fresh weight and oven dried at 70 °C for 48 h for determination of dry weight and tissue Cu concentration.

Embryos were dissected out from freshly harvested seeds and incubated in a solution of 0.1% (w/v) triphenyl tetrazolium chloride (TTC) for 2h in dark at 40°C. The vital part of the embryos developed pink or red color after staining.

For micro structure studies, fresh hand-cut sections of embryo and cotyledons of cultured seeds were fixed for 12h in a fixative solution containing 1.5% (w/v) glutaraldehyde in 1.0 M sodium phosphate (pH 7.2). The samples were dehydrated through a graded series of ethanol for 5 to 10 min. Thereafter, the samples were coated with gold and scanning electron micrographs were taken at different magnifications. Determination of carbohydrate and nitrogen fractions were made in developing seeds before and after 5d in culture. The freshly harvested samples were fixed in 80% (v/v) boiling ethanol and ground at room temperature. Sugars, phenols and non-protein nitrogen contents were estimated in the alcohol soluble fraction while that of starch and protein nitrogen in the alcohol insoluble fraction as described earlier (Nautiyal *et al.* 2005). For tissue analysis, oven dried samples were digested in nitric and perchloric acid mixture (10:1). Cu concentration was estimated in clear digests by atomic absorption spectrophotometery.

The crude enzyme extracts were prepared in suitable extracting media by grinding the freshly harvested material in an ice bath. The supernatant after centrifugation of the extract at 4°C at 5000 x g for 20 min was used for enzyme assay (Nautiyal *et al.* 1999a, b). Polyphenol oxidase (PPO) was extracted in 0.1 M phosphate buffer pH 6.0 containing 1% (w/v) polyvinyl pyrrolidone. The assay mixture (Shinshi and Noguchi 1975) contained 200 µmoles phosphate buffer pH 6.0 and enzyme extract in 3 ml. The reaction was initiated by addition of 0.5 ml 2 % (w/v) DL β-3, 4-dihydroxy-phenylalanine and was allowed to proceed for 30 minutes at room temperature and stopped by addition of 0.5 ml 0.25 M lead acetate. The mixture was centrifuged and the absorbance of the supernatant was measured at 470 nm on a Spectrochem MKII spectrophotometer. One enzyme unit of PPO is defined as an increase in absorbance of 0.001 under conditions of the assay. The ascorbate oxidase was extracted in 0.1 M phosphate buffer pH 7.4 containing 0.1% (w/v) Bovine serum albumin. The assay mixture for ascorbate oxidase (Racker 1952) contained 0.2 M citrate phosphate buffer pH 5.6 and enzyme extract in 2.9ml. The reaction was started by addition of 0.1ml 0.05% (w/v) ascorbic acid in neutralized solution of 1% (w/v) EDTA (1:1) and changes in absorbance were measured at 270 nm using spectrophotometer.

The enzyme acid invertase was extracted in 0.1 M tris-HCl buffer (pH 5.4). The reaction mixture (Hatch and Glasziou 1963) consisted of 10 µmoles sucrose and 40 µmoles sodium acetate buffer pH 5.4 and enzyme extract in 0.5 ml. The reaction proceeded for 30 minutes at room temperature and stopped by addition of 0.5 ml saturated solution of lead acetate and centrifuged at 1000 x g for 10 minute at room temperature. The inverted sugars formed were determined in a suitable aliquot of the supernatant colorimetrically as described earlier

(Nautiyal *et al.* 2005). Peroxidase was extracted in 0.1 M phosphate buffer pH 7.0. The assay mixture (Luck 1963) consisted of 500 µmoles phosphate-buffer pH 7.0, 10 µg H_2O_2, 50 mg p-phenylenediamine and crude extract of enzyme in 8 ml. The reaction started by addition of the enzyme extract and stopped by addition of 2 ml 5 N H_2SO_4. The increase in absorbance was recorded at 485 nm using spectrophotometer. Total proteins from finely powdered materials were extracted in extraction buffer containing 50 mM tris-HCl buffer pH 6.8, 2% (w/v) SDS, 5% (v/v) ß-mercaptoethanol, 10% (v/v) glycerol and 20 mM $MgCl_2$. The total protein content of the extract was estimated according to the method of Lowry *et al.* (1951). SDS-PAGE of the crude extract was performed on 12 % acrylamide gels (Laemmli 1970) in a vertical mini gel electrophoresis unit and the gels were stained with Coommassie brilliant blue R-250. The molecular weight markers were run simultaneously for determination of molecular weight of the protein bands (Nielsen and Reynolds 1978).

The above experiment was carried out in randomized block design and repeated thrice and standard error of mean (±SE; n=3) was determined. The data were subjected to two-way ANOVA and significance of the values was determined by post-hoc Newman-Keuls (q) test.

RESULTS AND DISCUSSION

Growth parameter of hyacinth bean (*Dolichos lablab* L.) seeds cultured at 8 and 10 DPA did not reveal significant effect of copper deficiency after 5 d in culture (Table 1). However, the biochemical parameters showed remarkable changes in the levels of reserve materials in cultured seeds in response to both Cu and ABA supply. Except for development of brown pigment in the medium, no visible signs of Cu deficiency were observed in hyacinth bean seeds after 5 d in culture. The reduction in the size of embryo (Figs. 1 and 2) with a marked decrease in Cu concentration and activities of PPO and ascorbate oxidase (Fig. 3 and 4) indicated that the seeds experienced a short span of Cu deficiency in culture. The SEM of cotyledon and embryonic axis at low Cu showed compactly arranged cotyledon cells with deeper in folding of their lateral walls (Fig. 1). At deficient Cu the cells appeared almost empty due to reduced accumulation of seed reserves. The embryos developed at deficient Cu (0.02 µM) on staining with TTC showed loss in viability (Fig. 2). In presence of ABA, at adequate Cu (0.2 µM)

Table 1. Growth parameters of hyacinth bean seeds cultured at variable Cu and ABA concentration. Values are mean ±SE (n=3). For each variable values with uncommon letters are significantly different according to Newman-Keuls test ($P \leq 0.05$). DPA = days post anthesis

DPA	Before culture	After culture Cu concentration (µM)			
		0.2 -ABA	0.02 +ABA	0.2 -ABA	0.02 +ABA
		Length (mm)			
8	6.0±0.05a	7.0±0.11a	6.8±0.10a	7.5±0.12a	7.0±0.10a
10	8.0±0.09b	8.5±0.10b	8.0±0.08ab	9.0±0.09b	8.5±0.07b
		Width (mm)			
8	1.9 ±0.02a	3.0±0.02a	2.5±0.04a	3.5±0.03b	2.5±0.04a
10	2.7±0.01a	3.0±0.01a	2.8±0.02a	3.5±0.03b	3.0±0.04a
		Fresh weight (mg ovule^{-1})			
8	10.0±0.20a	11.0±0.18a	10.5±0.12a	11.3±0.19a	10.9±0.12a
10	34.0±0.22b	35.0±0.24b	34.0±0.21b	34.0±0.23b	33.5±0.24b
		Dry weight (mg ovule^{-1})			
8	1.0±0.02a	1.2±0.05a	1.20±0.02a	1.36±0.04a	1.40±0.06a
10	3.7±0.05b	3.8±0.04b	3.70±0.06b	3.85±0.04b	3.83±0.06b

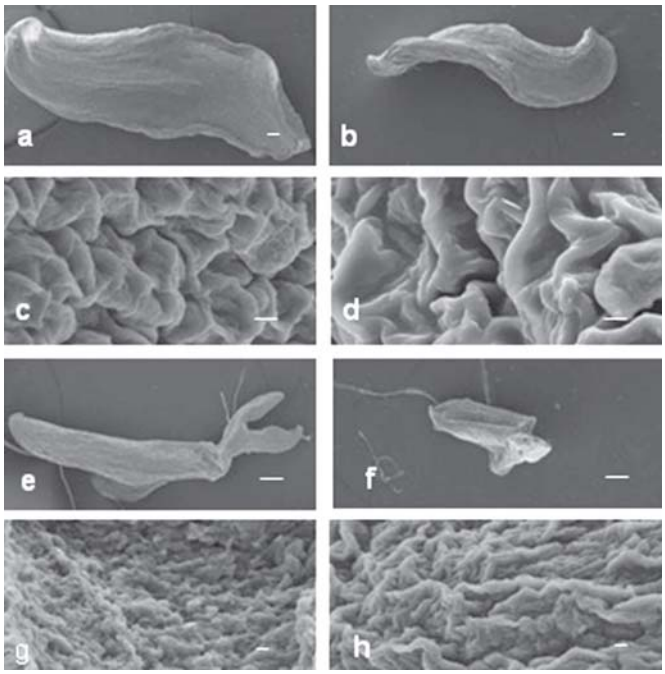

Fig. 1. Scanned electron micrographs of cultured hyacinth bean seeds at variable Cu. Sections of cotyledons at 0.2 (a,c) and 0.02 (b,d) ìM Cu; embryos at 0.2 (e,g) and 0.02 (f,h) ìM Cu. Scale bar = 200 (a,b), 100 (e,f) and 2(c,d; g,h) ìm.

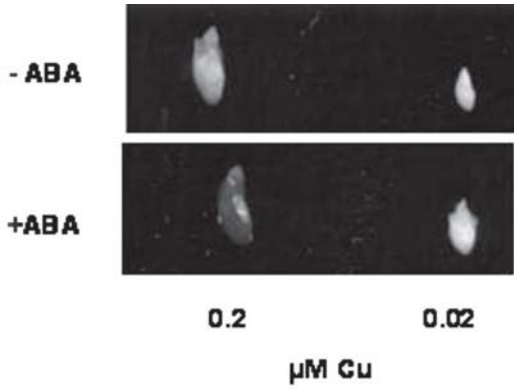

Fig. 2. TTC staining of embryos from hyacinth bean seeds cultured at variable Cu and ABA concentration.

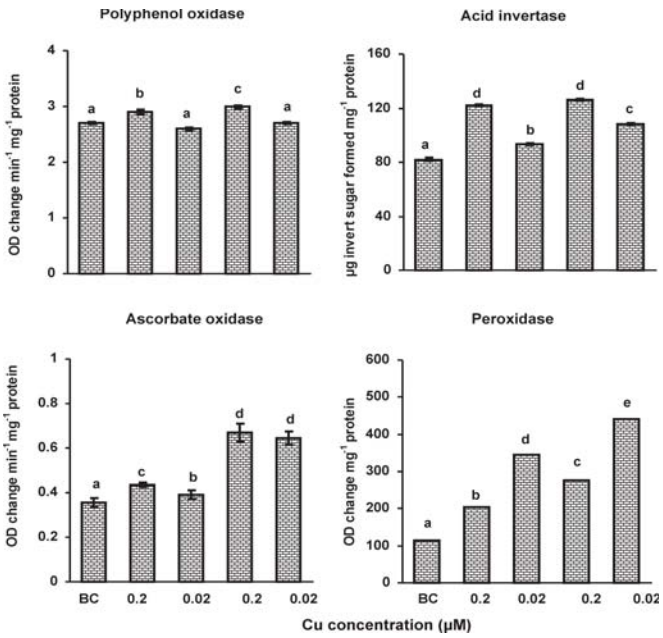

Fig. 3. Specific activities of enzymes in hyacinth bean seeds at variable Cu and ABA concentration. Bars with same letters are not statistically significant (p<0.05). Vertical lines represent ±SE (n = 3); BC= before culture.

the embryos showed better staining pattern and seeds had higher Cu concentration and enhanced activities of the two Cu containing enzymes and lower phenol content. While at deficient Cu, tissue Cu concentration and Cu enzymes increased in presence of ABA but the embryos were non-viable which might be due to induction of oxidative stress as evident by enhanced POD activity and accumulation of phenols (Figs. 2 - 4).

The accumulation of phenols in copper deficiency has resulted in development of brown color in the medium due to their subsequent oxidation to quinones on exposure to light and air. The accumulation of phenols in Cu deficiency might be due to decrease in the activities of Cu-containing enzymes, PPO and ascorbate oxidase following a decrease in Cu concentration in seeds as has been reported in other plant parts (Judel 1972; Robson *et al.* 1981). Phenols are known as inhibitors of the development of reproductive parts and could be the reason behind loss in viability of embryos in copper deficiency. Earlier, failure in seed setting in Cu deficiency was attributed to poor development of pollen grains, pollen viability and shortage of supply of photo-assimilates to the growing sinks (Nautiyal *et al.* 1999a, b).

The increased concentration of non-reducing sugars at deficient Cu in presence of ABA (Table 2) could be due to lowered invertase activity (Fig. 3) hampering the mitotic activity of the cells (Weber *et al.* 1996). This could be also due to the non-utilization of sugars for the

Table 2. Sugars, starch and nitrogen concentration in hyacinth bean seeds cultured at variable Cu and ABA concentration. Values are mean ±SE (n=3). For each variable values with uncommon letters are significantly different according to Newman-Keuls test ($p \leq 0.05$). DPA = days post anthesis

DPA	Before culture	After culture Cu concentration (µM)			
		0.2 -ABA	0.02 - ABA	0.2 + ABA	0.02 +ABA
		(mg g^{-1} fresh wt) Reducing sugars			
8	0.23±0.04a	0.45±0.05b	0.28±0.04a	0.68±0.06c	0.32±0.02a
10	0.34±0.05a	0.40±0.05ab	0.36±0.03a	0.80±0.04c	0.39±0.03a
		Non-reducing sugars			
8	0.75±0.07a	1.25±0.14b	0.76±0.08a	1.14±0.07a	1.19±0.04a
10	0.94±0.05a	1.42±0.24b	0.94±0.20a	1.11±0.20a	1.19±0.18a
		Total sugars			
8	0.98±0.14a	1.70±0.19a	1.04±0.07a	1.82±0.13a	1.51±0.08a
10	1.28±0.07a	1.82±0.13a	1.30±0.15a	1.91±0.18a	1.58±0.05a
		Starch			
8	6.74±0.09a	9.85±0.29c	9.27±0.38c	8.52±0.13c	5.96±0.15a
10	8.91±0.20b	12.60±0.29d	12.05±0.30d	12.90± 0.30d	11.11± 0.18d
		Protein nitrogen			
8	3.39±0.16a	3.84±0.10a	3.18±0.12a	5.84±0.20c	4.93±0.25b
10	3.26±0.15a	4.29±0.16a	3.83±0.20a	7.19±0.30e	6.27±0.16d
		Non- protein nitrogen			
8	1.88±0.08b	1.90±0.10b	1.88±0.12b	2.83±0.08c	0.87±0.08a
10	1.76±0.09b	1.82±0.12b	1.80±0.14a	2.76±0.19c	0.81±0.08a
		Total nitrogen			
8	5.27±0.12a	5.74±0.21a	5.06±0.18a	8.67±0.19b	5.80±0.18a
10	5.02±0.10a	6.11±0.15a	5.63±0.22a	9.55±0.25b	7.08±0.19a

biosynthesis of starch, more prominently in ovules cultured at the early growth stage (Table 2). The severity of the Cu deficiency effects on post-fertilization growth of ovules shows higher Cu requirement for seed setting (Knight *et al.* 1973). Higher Cu requirement for seed development is also reflected as the decrease in the level of total sugars and starch in seeds after 5 d of culture at deficient Cu (Table 2). The decrease in total sugars and starch contents in cultured hyacinth bean seeds at deficient copper are similar to reports on wheat (Brown and Clark 1977; Mizuno *et al.* 1983) and rice (Nautiyal *et al.* 1999a).

In cultured hyacinth bean seeds, the results on both protein and non-protein nitrogen fractions (Table 2) show biosynthesis of reserve proteins in seeds cultured at the later developmental stage (10 DPA) which is also evident in the SDS-PAGE of total proteins (Fig. 5). Both nitrogen fractions decreased in seeds at deficient copper but the non-protein nitrogen content was more severely affected in presence of exogenous ABA (Table 2). The synthesis of proteins during seed maturation or late embryogenesis depends on a regular replenishment of amino acids, the building blocks of proteins. It appears that the seeds were not able to synthesize amino acids in Cu deficient conditions due to disturbance in carbohydrate metabolism (Mizuno *et al.* 1983). The SDS-PAGE profile of cultured seeds shows the expression of some specific Cu deficiency stress proteins of MW 58, 53 and 50 kDa (Fig. 5) more prominently in

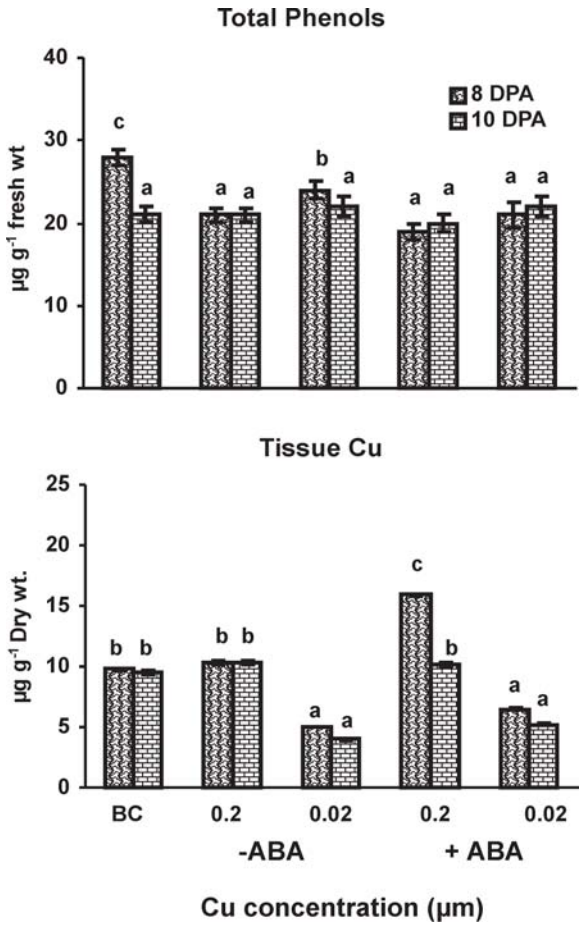

Fig. 4. Total phenols and tissue Cu concentration in hyacinth bean seeds at variable Cu and ABA concentration. Bars with same letters are not statistically significant (p<0.05). Vertical lines represent ±SE (n = 3); BC= before culture.

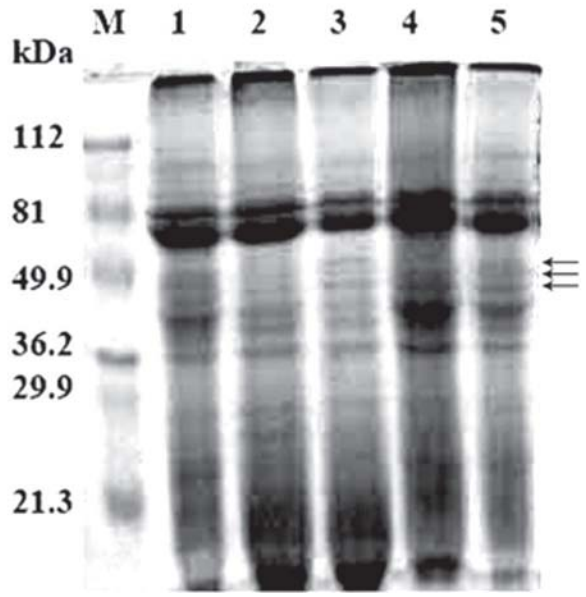

Fig. 5. SDS-PAGE profile of total crude proteins in hyacinth bean seeds at 10 DPA. Lane M, molecular weight markers; lane 1, Seeds before culture; lane 2, sufficient Cu -ABA; lane 3, deficient Cu -ABA, lane 4, sufficient Cu +ABA and lane 5, deficient Cu +ABA.

presence of ABA. These proteins resembled with HSP_{70} and their appearance was inversely related to Cu concentration (Fig. 4) and paralleled with the copper stress response. The results further substantiate the involvement of ABA, a signaling element, in induction of the expression of some genes encoding stress proteins (Finkelstein *et al.* 2002).

It is concluded that in developing seeds of hyacinth bean, a short span of Cu deficiency adversely disturbed the accumulation of reserves and embryo viability. The exogenous ABA supply enhanced the level of some of the reserves but could not reverse the Cu deficiency effects.

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