BIOLOGIA PLANTARUM 50 (2): 307-310, 2006

BRIEF COMMUNICATION

Phytotoxic effects of Cu, Zn, Cd and Pb on *in vitro* regeneration and concomitant protein changes in *Holarrhena antidysenterica*

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Abstract

The nodal explants of *in vitro* shoots of *Holarrhena antidysenterica* L. were cultured on Murashige and Skoog's (MS) medium augmented with 15 μM N^6-benzyladenine (BA) alone (control) or supplemented with different concentrations (1, 5, 10 and 20 mg dm^{-3}) of $CdCl_2$, $CuSO_4$, $Pb(NO_3)_2$ and $ZnSO_4$. The maximum morphogenic response in terms of average shoot number (4.95 ± 0.17) was seen in control. $ZnSO_4$ proved to be less inhibitory in comparison to $CuSO_4$, $Pb(NO_3)_2$ and $CdCl_2$. None of the explants cultured on $CdCl_2$ containing medium induced multiple shoots. Maximum protein content [3.80 ± 0.04 mg g^{-1}(f.m.)] was observed in control and slightly less [3.50 ± 0.02 mg g^{-1}(f.m.)] in tissues exposed to 1 mg dm^{-3} of $CuSO_4$ and minimum [1.00 ± 0.02 mg g^{-1}(f.m.)] in Zn treated (20 mg dm^{-3}) explants. SDS-PAGE analysis of the treated tissues revealed that two new polypeptides (29 and 20 kDa) in response to Cu and Zn treatment, respectively, have been synthesized.

Additional key words: N^6-benzyladenine, nodal explants, multiple shoots, Murashige and Skoog's medium, protein profile.

Heavy metals hamper the growth of plants by disturbing many biochemical, physiological and metabolic processes. They trigger changes in the transcript levels of numerous genes coding for proteins, thought to have protective functions against damage caused by stress (Nishizono *et al*. 1989, Steffens 1990, Zhou and Goldsbrough 1994, 1995, Didierjean *et al*. 1996, Murphy *et al*. 1997).

Holarrhena antidysenterica, commonly known as inderjab, is a medicinally valuable small tree reaching up to 12 m tall. Its decreased population at several habitats has been a matter of great concern for scientists. Attempts have therefore been made to develop tissue culture technique for its micropropagation (Raha and Roy 2001, 2003, Kumar *et al*. 2005). In continuation of the earlier work, the study has been extended to evaluate the effect of heavy metals on its growth and concomitant biochemical changes in protein contents.

The nodal segments excised from *in vitro* shoots of *Holarrhena antidysenterica* L. were used as the experimental material. The explants were cultured on Murashige and Skoog (1962; MS) medium supplemented with N^6-benzyladenine (BA; *Sigma-Aldrich*, St. Louis, USA] and individual heavy metal salts like $CuSO_4$, $CdCl_2$, $Pb(NO_3)_2$, $ZnSO_4$ (*Qualigens*, Mumbai, India). The media were supplemented with 3 % (m/v) sucrose (*DCM*, Daurala, India) as carbon source and gelled with 0.8 % (m/v) agar (*Qualigens*). The pH of the media was adjusted to 5.8 using 0.1 M NaOH or HCl before autoclaving and 20 cm^3 of molten medium was poured in each rimless tube (2.5 × 1.5 cm; *Borosil*, Mumbai, India) with non-absorbant cotton wrapped in two layered muslin cloth. Media were sterilized by autoclaving at 1.05 kg cm^{-2} for 15 min. After autoclaving the media were allowed to set as slants. Cultures were incubated in the culture room at 25 ± 2 °C under continuous light of 45 - 46 W m^{-2} emitted by cool day white fluorescent tubes (40 W, *Philips*, Calcutta, India) and the relative humidity was

Received 6 August 2004, *accepted* 29 June 2005.

Abbreviations: BA - N^6-benzyladenine; PAGE - polyacrylamide gel electrophoresis; SDS - sodiumdodecylsulphate.

Acknowledgements: The authors are grateful to the University Grants Commission, New Delhi, India for financial assistance provided in the form of a project sanctioned to Dr. Veena Agrawal.

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55 - 65 %. Twenty four replicates for each treatment were taken and average number of shoots per explant has been represented as mean values along with standard error.

In order to study the protein profiles under different stress conditions, the protein extraction of different tissues was carried out at 4 °C. Frozen shoots were homogenized in a pestle and mortar with Zivy's buffer (pH 8.5; Zivy *et al.* 1983). The homogenates were centrifuged at 12 000 *g* for 30 min and the supernatant was kept and stored in separate aliquots at -20 °C. The protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (*Sigma*) as standard. Aliquots of the denatured shoot samples were used in sodium-dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) together with the protein markers (*Sigma*) having molecular mass ranging 29 - 116 kDa.

The nodal explants, reared on regenerative medium (MS + 15 µM BA) differentiated an average of 4.95 ± 0.17 shoots with an average shoot length of 1.76 ± 0.07 cm. Addition of different concentrations (1, 5, 10 or 20 mg dm^{-3}) of $CdCl_2$, $CuSO_4$, $Pb(NO_3)_2$ and $ZnSO_4$ to the aforesaid regenerative medium drastically decreased the response in terms of multiple shoot production as well as growth.

The explants cultured on $CdCl_2$ containing medium revealed that though, the bud break was seen on all its concentrations but none of them (buds) developed into proper shoot. The shoot growth was severely inhibited at higher concentrations of $CdCl_2$ (10 mg dm^{-3}) and at 20 mg dm^{-3}, even the leaf turned abnormal leading to browning of explants.

$CuSO_4$, when used in different concentrations, also inhibited growth and production of multiple shoots in the explants. Its low concentration (l mg dm^{-3}), however, favoured organogenesis where a maximum of 87 % explants differentiated an average of 2.24 ± 0.18 shoots with an average length of 0.94 ± 0.07 cm (Table 1). Beyond this, a decline in shoot number was seen and at 20 mg dm^{-3}, the average shoot number was 1.33 ± 0.15 (Table 1). Though, the induction of multiple shoot buds was seen at its higher contents but the growth was very poor.

$Pb(NO_3)_2$ caused an appreciable decline in morphogenesis. The decline in response (80 - 54 %) corresponded with the increasing concentration of $Pb(NO_3)_2$ from 1 to 20 mg dm^{-3}, respectively (Table 1). The average shoot number declined from 1.91 ± 0.23 at 1 mg dm^{-3} to 1.41 ± 0.25 of $Pb(NO_3)_2$ at 20 mg dm^{-3} (Table 1).

In contrast to above, $ZnSO_4$ proved less injurious to shoot growth compared with other metals. Nearly, 100 % cultures developed shoots on 1 and 5 mg dm^{-3} $ZnSO_4$ (Table 1), however, the average shoot number varied from 4.00 ± 0.19 at 1 mg dm^{-3} to 2.33 ± 0.09 at 5 mg dm^{-3} $ZnSO_4$ but the shoot length (1.25 ± 0.12 and 1.16 ± 0.1) was more or less similar (Table 1). Axillary buds were seen to be healthy at this salt. Thus, $ZnSO_4$ proved to be less inhibitory in comparison to $CuSO_4$, $Pb(NO_3)_2$ and $CdCl_2$.

Table 1. Morphogenic response of nodal explant from *in vitro H. antidysenterica* shoots cultured on MS +15 µM BA supplemented with various concentrations of different metals (Cd, Cu, Pb and Zn) for 30 d (* - means of 24 replicates ± SE, ** - means of 3 replicates ± SE).

Metals	[mg dm^{-3}]	Explants developing shoots [%]	Number of shoots [$explant^{-1}$]*	Shoot length [cm]*	Total soluble proteins [mg g^{-1}(f.m.)]**
Control		79	4.95 ± 0.17	1.76 ± 0.07	3.80 ± 0.04
$CdCl_2$	1	66	1.00 ± 0.19	0.94 ± 0.17	2.55 ± 0.02
	5	-	--	--	2.37 ± 0.01
	10	-	--	--	1.95 ± 0.01
	20	-	--	--	1.60 ± 0.03
$CuSO_4$	1	87	2.24 ± 0.18	0.94 ± 0.07	3.50 ± 0.02
	5	83	1.75 ± 0.17	0.68 ± 0.08	2.90 ± 0.04
	10	83	1.50 ± 0.15	0.55 ± 0.12	2.17 ± 0.02
	20	75	1.33 ± 0.15	0.40 ± 0.06	1.10 ± 0.04
$Pb(NO_3)_2$	1	82	1.91 ± 0.23	0.77 ± 0.12	2.40 ± 0.03
	5	74	1.61 ± 0.23	0.73 ± 0.07	2.30 ± 0.03
	10	82	1.58 ± 0.21	0.67 ± 0.05	2.00 ± 0.03
	20	54	1.41 ± 0.25	0.65 ± 0.07	1.37 ± 0.04
$ZnSO_4$	1	100	4.00 ± 0.19	1.25 ± 0.12	2.37 ± 0.03
	5	100	2.33 ± 0.09	1.22 ± 0.08	1.75 ± 0.04
	10	95	2.00 ± 0.12	1.14 ± 0.08	1.45 ± 0.04
	20	83	1.66 ± 0.15	1.16 ± 0.10	1.00 ± 0.02

From the above observations, Cd was clearly proved to be most toxic both for shoot induction as well as growth in comparison to other metals. This may be due to the fact that Cd is more mobile than Pb within the plant tissues (Khan and Frankland 1983). Browning of tissues at higher concentrations may be due to the damage of photosynthetic apparatus, such as disrupted chloroplast structure and dilation of the thylakoid membranes (Quzoumidou *et al*. 1997). Our findings corroborate with those of Ali *et al*. (1998) where the regenerants of *Bacopa monniera* L. did not survive beyond 50 µM Cd. Analysis of total proteins also supported this observation as amount of protein got declined on increasing concentrations of $CdCl_2$ (Table 1). The inhibitory effect of Cu may be due to Cu-induced Fe deficiency leading to chlorosis and suppression of shoot growth. Subsequently, all the ultrastructural changes occurred due to the toxic effects of high concentration of copper, *i.e.* poorly developed internal membrane system consisting of thylakoids arranged parallel to each other with only a few, rudimentary grana.

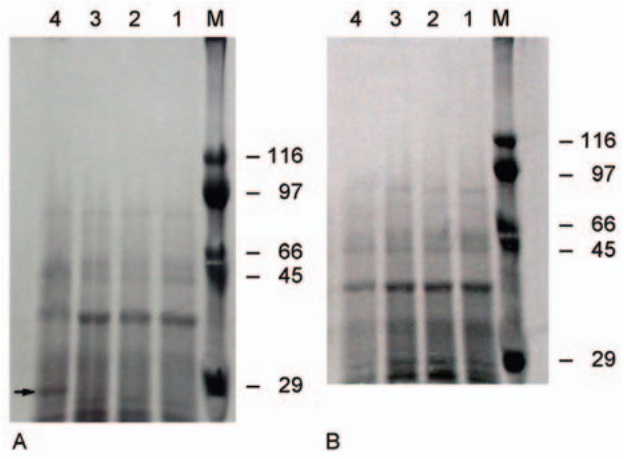

Fig. 1. SDS-PAGE analysis of explants bearing shoots reared on $CuSO_4$ containing medium (*A*) and $ZnSO_4$ containing medium (*B*). *Lanes* M, 1, 2, 3 and 4 reveal markers, 1, 5, 10 and 20 mg dm^{-3}, respectively.

Quantitative and qualitative analysis of the total soluble proteins of the treated *vis-a-vis* controlled tissue showed very significant differences. The quantities of total soluble proteins got declined with the increasing concentration of all the metals (Table 1). In the explants treated with $CdCl_2$, the total soluble proteins, decreased from 2.55 ± 0.02 mg g^{-1}(f.m.) at 1 mg dm^{-3} to 1.60 ± 0.03 mg g^{-1}(f.m.) at 20 mg dm^{-3} (Table 1). However, for $Pb(NO_3)_2$, there was a relatively less variations in the amount of proteins. It dropped from 2.40 ± 0.03 mg g^{-1}(f.m.) at 1 mg dm^{-3} to 1.37 ± 0.04 mg g^{-1}(f.m.) at 20 mg dm^{-3} (Table 1). Contrary to the above, drastic decline was seen in $ZnSO_4$ treated explants, where the amount of proteins dropped from 2.37 ± 0.03 mg g^{-1}(f.m.) to 1.00 ± 0.02 mg g^{-1}(f.m.) when exposed to 1 mg dm^{-3} and 20 mg dm^{-3}, respectively (Table 1). Thus, a maximum amount [3.50 ± 0.02 mg g^{-1}(f.m.)] of proteins was found in the tissues grown on 1 mg dm^{-3} $CuSO_4$ (Table 1).

Another notable change was seen in the synthesis of some new polypeptides in the tissues exposed to different metal stresses (Figs. 1*A*,*B*; data of Cd and Pb not given). The SDS-PAGE analysis of the metal treated tissues showed 8 new polypeptides. Incidentally at all the concentration of Cu tried, 40 kDa polypeptide was observed (Fig. 1*A*). On the other hand, alterations in contents of certain specific polypeptides were also found to be unique subsequent to exposures to the given abiotic stress (Figs. 1 *A*,*B*). For instance, 29 kDa and 26 kDa polypeptide were induced in response to both Cu and Zn stress of 20 mg dm^{-3} and 1 mg dm^{-3}, respectively, but the enhancement was too low to be interpreted as stress-inducible proteins.

It is already known that higher plants not only respond to heavy metal treatment by the synthesis of phytochelatins or related peptides, but also by the synthesis of stress related proteins with a molecular mass ranging from 10 to 70 kDa (Didierjean *et al*. 1996). It is perhaps this hypothesis which might have worked in this system of *H. antidysenterica* which induced the synthesis of polypeptide in this range (29 and 26 kDa; Fig. 1A,*B*). It is likely that some of these new proteins (40, 29 and 26 kDa) might have helped in *H. antidysenterica* to encounter stressful environments. In the earlier work, several proteins such as (LEA, HSP, RAB) isolated from higher plants have already been shown to play a very significant role in abiotic stresses (Singla and Grover 1994, Magnard *et al*. 1996, Hvoslef-Eide and Croke 1997, Sundar *et al*. 2003). In more recent study, boiling stable protein of 66 kDa in *Sesbania sesban* var. *bicolor* have been found to be induced under desiccation on ABA treatment (Purty *et al*. 2005). Thus the new proteins 26 and 29 kDa subsequent to metal stress might have helped for encountering their inhibitory effects. Further work, on sequencing of these proteins is in progress. Results thus obtained should be useful for improving the plants by incorporating the relevant stress tolerant genes in the system.

Concluding the above mentioned results it is emphasized that the rate of inhibition in morphogenesis was in the order of Cd>Pb>Cu>Zn.

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