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RESEARCH ARTICLE

EVALUATION OF HEAVY METAL TOLERANCE IN INDIAN MUSTARD (BRASSICA JUNCEA) SEEDLINGS

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ABSTRACT

As a part of the systematic study of heavy metals phytoremediation, hydroponically grown Indian mustard (*Brassica juncea*) seedlings were selected as model eukaryotic plant system for the characterization of Zn and Cd induced tolerance in phytoindicators. 10 cultivars of Indian mustard (*Brassica juncea*) were grown on MS media with varying levels of Zn and Cd separately as well as in combinations. 6 days old seedlings were harvested and dissected into different organs. Each organ was further analysed for its proline content. On the basis of their tolerance (in terms of morphological parameters and biochemical parameter proline content) towards heavy metals tested, PCR-7 was found to be the most tolerable *Brassica juncea* cultivar.

INTRODUCTION

Heavy-metal pollution of soils and waters caused by the mining and burning of fossil fuels is a major environmental problem, and exposure to these metals can be toxic to these living cells (Qian *et al*, 1999). Unlike organic pollutants, heavy metals cannot be degraded or biodegraded by microorganisms. One alternative biological approach to deal with this problem is phytoremediation i.e the use of trees and plants to detoxify chemical waste sites. Compared with other technologies, phytoremediation is less expensive (Cunnigham and Ow, 1996) and is particularly suitable for treatment of large volumes of substrates with low concentration of heavy metals.

Heavy metals and metalloids can be removed from polluted sites by phytoextraction, potent method of phytoremediation, which involves the accumulation of pollutants in plant biomass (Zayed *et al*, 1998). As a result, hyperaccumulators (plant species that accumulate extremely high concentrations of heavy metals in their shoots) become particularly useful. In addition, one can genetically engineer these species to improve their metal tolerance and metal-accumulating capacity. A suitable target species for this strategy is Indian mustard (*Brassica juncea*), which has a large biomass production and a relatively high trace element accumulation capacity. Most importantly, it can easily be genetically engineered (Zhu *et al*, 1999b).

Therefore, this paper incorporates the studies on heavy metals (Zn and Cd) phytoextraction capabilities of hydroponically grown Indian mustard (*Brassica juncea*) seedlings using artificial polluted media with the objectives: optimization of heavy metals requirement through seed germination assays, effect of metals treatment in terms of morphological parameters and free proline content in treated seedlings.

MATERIALS AND METHODS

Ten certified varieties (Rohini, BIO-902, PCR-7, SEJ-2, Kranti, Krishna, Maya, Pusa Bahar, Pusa Jagannath and Pusa Bold) of Indian mustard (*Brassica juncea*) seeds used in the present study were kindly provided by the National Research Centre on

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Indian mustard (*Brassica juncea*) seeds were properly washed with ethanol, bleaching powder and rinsed 5 times with sterilized water. After the imbibition the seeds were grown aseptically and hydroponically (using MS media) in the presence of different concentration of Zinc viz., 0, 2.5, 5.0, 10, 100, 250 and 1000mg/l and Cd viz., 0, 6, 12, 24, and 30mg/l. Seeds were also grown aseptically in presence of different concentrations of combined (cadmium + zinc) multiple metal treatment viz., 0, 30mg/l+2.5mg/l, 30mg/l + 250mg/l respectively. After that Erlenmeyer flasks were placed in cold for two days and then transferred to incubator at 24^0C, 70% RH under constant illumination (1600lux). After 6 days, the seedlings were harvested and dissected into different organs and 0.5g of each organ (Plant Tissue) was homogenized in 1.5ml (1:3) of 0.2M phosphate buffer (pH 7.2). Free proline extraction, and estimation was done as per the method of Bates *et al*. (1973). In brief, 200µl aliquot of phosphate extract was mixed with 800µl ninhydrin reagent (1% ninhydrin in a 60% acetic acid solution). The mixture was heated at 100^0C for 20min and then cooled on ice. 1ml toluene was added and sample was vigorously shaken for 15sec and stored in dark for 4hrs at room temperature. Absorbance of upper phase was taken at 520nm and free proline content was calculated using the standard graph.

RESULTS AND DISCUSSION

Bioremediation of heavy metal pollution remain a major challenge for environmental Biotechnologists. Phytoremediation, the use of trees and plants to detoxify chemical waste sites can be one of the best approaches. The first step towards a successful phytoremediation operation to a particular toxicant/pollutant is the selection for a suitable plant. We studied heavy metal induced changes in Indian mustard (*Brassica juncea*) seedlings because its role in removing heavy metals from contaminated sites is well documented (Bennett *et al*., 2001).

Optimization of heavy metal requirement

Optimum, supraoptimum and toxic levels of Zn and Cd for young seedlings of *Brassica juncea* varieties were determined through seed germination assays. On the basis of germination percentage and length of versatile organ hypocotyls (Table 1a & 1b) the heavy metal requirement/tolerance regimes for different competent varieties of *Brassica juncea* seedlings may be presented as below:

Competent variety	**Heavy metal**	**Characteristic regimes (µg/ml)**		
		Optimum	Supraoptimum	Toxic
BIO-902		2.5	10	250
PCR-7		2.5	10	250
Kranti		2.5	10	250
Pusa Bahar	Zn	2.5	10	250
Pusa Bold		2.5	10	250
PCR-7		…	…	30
SEJ-2		…	…	30
Krishna	Cd	…	…	30
Maya		…	…	30

Table 1a Response of *Brassica juncea* cultivars to heavy metal Zn in terms of germination percentage and hypocotyl length

Zn treatment (µg/ml)	Germination percentage and hypocotyl length of different cultivars									
	BIO-902		PCR-07		Kranti		Pusa Bahar		Pusa Bold	
	%	cm	%	cm	%	cm	%	cm	%	cm
Control	60	2	60	0.67	60	2.5	60	2.83	60	1.5
2.5	100	2.6	60	1.5	80	3.75	80	2.67	40	1.75
5.0	80	0.5	60	0.5	100	0.86	80	0.4	20	0.75
10	60	0.5	100	1.7	60	1.6	80	1.5	40	0.83
100	40	1.0	40	1.0	80	0.75	80	0.87	20	0.4
250	60	0.4	40	0.4	60	0.25	40	0.27	40	0.2
1000	80	-	66	0.0	0.0	0.0	60	-	40	0.2

Table 1b Response of *Brassica juncea* cultivars to heavy metal Cd in terms of germination percentage and hypocotyl length

Cd treatment µg/ml	**Germination percentage and hypocotyl length of different cultivars**							
	PCR-07		SEJ-02		Krishna		Maya	
	%	cm	%	cm	%	cm	%	cm
Control	100	5.8	60	1.0	100	2.9	60	2.67
6	80	5.5	60	1.5	60	1.17	60	2.33
12	60	0.5	80	1.12	60	0.83	60	0.55
24	80	0.42	40	0.25	40	0.70	80	0.65
30	80	0.40	20	0.5	60	0.66	60	0.83

As indicated, 5 varieties of *Brassica juncea* (BIO-902, PCR-7, Kranti, Pusa Bahar, Pusa Bold) were found competent for Zn tolerance and 4 varieties (PCR-7, SEJ-2, Krishna, Maya) for Cd tolerance. Out of these, PCR-7 was found competent for both Zn and Cd treatments.

Optimum, supraoptimum and toxicity of Zn in *Brassica juncea* varieties reflects its dual nature i.e. micronutrient on one hand and toxic environmental factor on the other. Cd is non essential element in metabolic process in plants or animals and it can accumulate to levels that are toxic or lethal to the organisms (Lock and Janseen, 2001). In this study, four competent varieties of *Brassica juncea* showed growth response even at Cd toxicity and supports the findings of Su and Wong (2004). This might be due to their metabolic adjustment towards more synthesis of phytochelatins (PCs) and stress related proteins by such seedlings as compared to other varieties (Cobett and Goldsbrough, 2000). PCR-7 competency towards both Zn and Cd elements reflects its suitability for the successful phytoremediation operation to these heavy metals.

Multiple metal tolerances

The common variety PCR-7 was further tested for combined tolerance of Zn and Cd. The response in terms of same physiological parameters at various combinations of two heavy metals was determined as per the Table 2. When the data of Table 2 are compared with PCR-7 data of table 1a & 1b, it is clearly evident that Zn enhanced 4 times tolerance competency of PCR-7 towards Cd toxicity in terms of growth and development of the seedlings via promoting adventitious roots. Such multiple metal tolerance of PCR-7 towards Zn and Cd also reflects the metabolic integrity of the seedlings under heavy metal toxicity.

Table 2 Multiple metal tolerance of *Brassica juncea* (PCR-07) cultivars

Combined Treatment µg/ml	Response of PCR-07	
	Germination (%)	Hypocotyl length (cm)
Control	80	5.0
Zn (Optimum) + Cd (Toxic)	70	2.0
Zn (Toxic) + Cd (Toxic)	65	0.5

Proline content

Indian mustard (*Brassica juncea*) seedlings treated with Zn and Cd separately as well as in combinations were harvested after 6 days and were dissected into different organs for the estimation of their proline contents. The results are presented in the form of Table 3a, 3b and Table 4. From the Table 3a, it is evident that Zn didn't cause any significant change in the proline content of the different organs which reflects the metabolic stability (tolerance/superiority over other varieties) of PCR-7 towards Zn treatment. Interestingly, PCR-7 showed ~ 50% increase in proline content due to Cd treatment both in leaves and roots along with ~ 125% increase in hypocotyls (Table 4, 3b) which suggests the translocation of proline from its major synthetic site chloroplast containing photosynthetic leaves to the non photosynthetic roots via photosynthetic hypocotyls. Such translocation of proline from leaves to roots was further proved by the data presented in Table 4 where nearly 2 and 3 times increase can be seen in the proline contents at Cd (toxicity) alone and multiple metal toxicity respectively.

Table 3a Organwise pattern of proline in *Brassica juncea* seedlings subjected to Zn treatment.

Brassica juncea variety	Zn Treatment µg/ml	Proline content (µg/g fresh tissue weight)		
		Root	Hypocotyl	Leaf
BIO-902	Control	16.32	25.51	15.67
	Optimum	12.67	17.47	14.59
	Toxic	17.41	32.22	25.44
PCR-07	Control	17.41	14.62	17.92
	Optimum	15.61	13.41	13.76
	Toxic	18.43	16.64	20.48
Kranti	Control	18.43	14.91	37.53
	Optimum	15.87	12.48	36.8
	Toxic	24.80	24.41	40.64
Pusa Bahar	Control	22.08	12.99	4.8
	Optimum	15.23	12.12	25.44
	Toxic	22.81	13.69	20.16
Pusa Bold	Control	19.96	10.78	20.48
	Optimum	18.36	9.6	17.47
	Toxic	22.72	16.19	25.69

Table 3b Organwise pattern of proline in *Brassica juncea* seedlings subjected to Cd treatment.

Brassica juncea variety	Cd Treatment µg/ml	Proline content (µg/g fresh tissue weight)		
		Root	Hypocotyl	Leaf
PCR-07	Control	14.27	12.48	16.06
	Toxic	20.57	28.8	27.52
SEJ-02	Control	23.04	10.88	15.29
	Toxic	38.08	12.57	17.98
Krishna	Control	12.60	25.15	16.06
	Toxic	25.76	35.2	29.12
Maya	Control	16.38	9.66	14.01
	Toxic	17.92	20.89	16.44

In higher plants, proline accumulates under stress and shows an association with stress adaptation. The accumulation of proline under abiotic stress condition accounts for few millimolar concentration depending on the species and the extent of stress condition (Delauney and Verma, 1993; Bohnert *et al*, 1998). In this study, the increased proline levels in photosynthetic organs (leaves and hypocotyls) of heavy metals (Zn and Cd) treated seedlings simply reflect the presence of synthetic site of proline i.e. chloroplasts in these organs.

Table 4 Organwise pattern of proline in *Brassica juncea* (PCR-07) seedlings subjected to multiple metal treatment

Combined Treatment µg/ml	Proline content (µg/g fresh tissue weight)		
	Root	Hypocotyl	Leaf
Control	11.77	14.41	12.92
Zn (Optimum) + Cd (Toxic)	21.34	26.08	27.68
Zn (Toxic) + Cd (Toxic)	34.4	28.89	33.92

The increased level of proline in roots of seedlings in response to Zn and Cd toxicity reflects its nature as stress amino acid (Singh and Tewari, 2003; Guo *et al*, 2004) and might be due to its translocation from photosynthetic organs. This is in consistent with the findings of Singh *et al*, (2004) where they suggest proline synthesis in foliar parts of the plant and pointed out that proline can be adopted as reliable metal stress indicator.

CONCLUSION

On the basis of their tolerance (in terms of morphological parameters and biochemical parameter proline content) towards heavy metals tested, PCR-7 was found to be the most tolerable *Brassica juncea* cultivar and suggested to be used for phytoremediation.

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