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Impact of copper nutrition on growth and biochemical parameters of the barley (*Hordeum vulgare* L.)

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Abstract
Barley is the important winter season cereal grown in stress environments specifically with limited moisture and saline soils. It is well known that under stress conditions plants try to combat the impact by various means. The production of osmolytes, such as proline could be one of the main strategies in this direction. Thus, impact of copper were evaluated in barley on germination, growth and some biochemical parameters such as chlorophyll, carbohydrate and proline content at 0.5, 1, 5 and 10 mg $CuSO_4.5H_2O$/litre concentrations. We found that as the copper concentration increases it results in decreased germination percentage, root length and chlorophyll content but there was no significance change was observed in shoot height. As copper also affects chlorophyll synthesis pathway, we noticed inhibition of this pathway resulted in drastic reduction in carbohydrates content. With the increase in the heavy metal stress, plant accumulated proline to mitigate the impact of copper stress. Similarly Relative Water Content (RWC) shows positive correlation with heavy metal concentration. It was concluded that besides parameters like germination percentage, root length and chlorophyll content, the proline accumulation could be the best indicator to access the heavy metal stress in barley plant.

Keywords: Chlorophyll content, germination percentage, RWC, proline

Introduction
Barley (*Hordeum vulgare* L.) is a winter season, annual grain crop, used as forage as well as, cover crop to improve soil fertility (Ghanbari *et al*., 2012) [14]. It ranks fifth among crops in grain production in the world after maize, wheat, rice and soybean (Ofosu-Anim and Leitch, 2009; Zeid, 2011; Soleymani and Shahrajabian, 2011) [7, 1]. It is important source of low-fat complex, carbohydrates for energy, relatively well-balanced protein minerals. The endosperm cell wall are rich in β–glucans that positively affect serum cholesterol and glucose level that can in turn impacts cardiovascular health and control diabetes respectively (Behall *et al.*, 2004) [8]. Barley is a hardy cereal, have the ability to grow in severe environments; it promotes generally stable yields and little labour is required (ICARDA, 2017). Due to sessile nature of plants, they undergo various challenges of biotic and abiotic stress conditions throughout their life cycle. Heavy metal contamination of soil is an example of abiotic stress. In this modern era of crop research, heavy metal toxicity/stress has become an inevitable constraint to crop productivity and quality. Heavy metal content in soil is increased from past few decades which could be result of various possible means such long term use of pesticides, fertilizers, dust from smelters, industrial waste, improper water management practices in agricultural land (Yang *et al*., 2005) [18]. As we know heavy metals occur naturally in earth's crust at different levels, but the main problem arises when they are released in excess amount into the environment due to natural or anthropogenic activities (Eapen and D'Souza, 2005;Kavamura and Esposito, 2010; Miransari, 2011) [16, 17, 12].

From previous studies it is clear that several macro and micro nutrients play crucial role in physiological and biochemical processes of plants such as chlorophyll biosynthesis, sugar metabolism, redox reactions in chloroplast and mitochondrion, and nitrogen fixation. For instance, copper (Cu) plays important role in CO_2 assimilation and ATP synthesis. It is also an essential component of various proteins such as plastocyanin and cytochrome. Exposure of plants at higher concentrations of copper plays cytotoxic role, which causes injury to plants ultimately leads to growth retardation and leaf chlorosis. Plants under excess copper conditions generates oxidative stress and ROS hence to avoid unevitable consequences plants cells have very tight control for uptake and utilization of heavy metals (Janicka-Russak *et al*., 2008; Dal Corso *et al*., 2013a) [10, 6].

Common toxic effects of heavy metals on plants are low biomass accumulation, inhibition of growth and photosynthesis, altered relative water content and nutrient assimilation and senescence, which leads to plant death. Plants grown in heavy metal contaminated soils result in accumulation of higher amount of heavy metals, ultimately leads to food chain contamination. This contaminated food chain is the primary route for entry of heavy metals into animal and human tissues, which give invitation to various types of cancers (Das P. *et al*., 1997, Knasmuller S. *et al*., 1998, McLaughlin *et al*., 1999) [4, 15, 11]. Persistence nature of heavy metals pose serious threat to human health, which is daunting challenge for researchers (Gisbert C. *et al*, 2003) [3], to mitigate the impact of heavy metals and meet the food demand for growing population simultaneously However to overcome the adverse impact of heavy metals on cereal crops in response to extensive land disposal of unregulated wastes as agricultural soil amendments have received very limited attention in India.

Materials and Methods

Seeds of BH-946 a popular high yielding variety of barley (*Hordeum vulgare* L.) used in this study was kindly provided by ICAR-Indian Agricultural Research Institute, New Delhi situated at 77.15 longitude and 28.63 latitude.

Germination testing

Seeds were surface sterilized using 0.1% mercuric chloride solution for 2-3 min. and then washed with sterilized distilled water 3-4 times for 10 min each and placed on petri plates having autoclaved filter paper under control air-dried at an ambient temperature of 32°C in the laboratory. Seed germination screening was performed for six days at different copper concentration treatments in comparison to control on the whatmann filter paper No-1 in petri dishes with following copper treatment were as 0mg, 0.5 mg, 1mg, 5mg and10mg. These treated seeds are kept at 25 ºC in the dark and the germination percentage is calculated on sixth day. A pot experiment was performed to study the evaluate the impact of copper on barley plants. Sterilized seeds were sown at uniform depth (1 cm) in each pot containing autoclaved sand. The Hogland solution were aerated externally to prevent anaerobic conditions and kept at 25-30 ºC.

Morphological data

Fresh weight of five plants shoot / root was measured using standard methodology, a weighing balance of four digits after decimal was used in each case. Shoot / Root sample were oven dried at 60 ºC for 72 hrs. until a constant weight were obtained then weighed using a sensitive balance to determine dry weight per plantlet. Shoots and root length was measured by using scale. To calculate the turgid weight shoot and root were soaked for 4 hrs, then their weight is measured.

Biochemical analysis

Chlorophyll Estimation

The chlorophyll was estimated according to Arnon (1949) [2] tissue pieces were macerated with 80% acetone and pinch of carbonate was also added. The homogenate was centrifuged at 500 rpm for 10 min. the supernatant was made upto known volume with 80% acetone. OD of the supernatant was taken at 645 and 663 nm against 80% acetone as blank.

Total chlorophyll content (mg * g^{-1}) = 20.2 (A_{645}) + 8.02 (A_{663}) V/1000*W

Carbohydrate estimation

Anthrone (hedge, 1962) method was used to estimate the carbohydrate concentration. For this purpose, 1 ml sample of protein free carbohydrates from root, shoot, leaves were taken and then followed by addition of 4ml of anthrone. Absorbance was taken at 620 nm using D-Glucose as standard.

Proline estimation

Proline content was estimated using the method of Bates *et al*. (1973) [19]. Leaves, shoot, roots were ground in 1.5 ml of aqueous sulfosalicylic acid 3% (w/v) and proline was estimated by ninhydrin reagent. The ninhydrin reaction mixture was partitioned against toluene and absorbance of the toluene phase was read at 520 nm.

Relative water content (RWC)

Relative water content was measured on leaf samples after cutting the base of lamina and leaves were sealed in plastic bags and transferred to laboratory quickly fresh weight was determined by excision. Turgid weight were obtained after soaking leaves with distilled water in test tubes for 4h at room temperature (25 ^{0}C) and low light conditions after soaking determination of turgid weight was estimated. Dry weight was determined after oven drying at 60 ^{0}C for 72 hrs relative water content was calculated according to following:

RWC%= [FW-DW] / [TW-DW] *100

FW=fresh weight, TW=turgid weight, DW=dry weight

Statistical analysis

Resulted physiological and biochemical data were subjected to statistical analysis by using ANOVA. The results of one-way ANNOVA were found statistically significant.

Result and Discussion

In our study, heavy metals had significant effect on *Hordeum vulgare* L. by reduction of some growth parameters. This may be due to the fact that in saline soil, the water uptake slow, which lead to inhibition of plant growth. Metals also hinders plant metabolic functioning and may cause plant injury at various levels. Plant height was significantly reduced by increasing metals level and this may be due to concentration of soluble metals through their high osmotic pressure which might have an effect on plant growth by restricting the uptake of water by roots. Metals can affect plant growth, due to high concentration of metals in the soil solution it interferes with the absorption of essential nutritional ions by plant.

This study revealed that there were no significant effects among metal levels on number of leaves, but this character may get decreased at harvest. Results showed that metal had significant effect on fresh weight. High Cu concentration inhibits plant growth. Dry weight was not significantly affected by different copper concentrations because plant sensitivity to metals continuously changes during the growing season.

Morphological data of plants were collected from five plants of all stress condition of experiment is discussed in the following sections.

Germination percentage

The number of seed required in field environment is determined by germination percentage. In this study we found the impact of copper on seed germination of barley. Highest percentage of germination was found at control conditions, as we increase the copper concentration there is significant reduction in germination percentage as depicted in figure. This reduction in germination may be due several means such as degradation of β-amylase and acid phosphatase which leads to decreased seed germination (Bansal *et al*. 2002) [13].

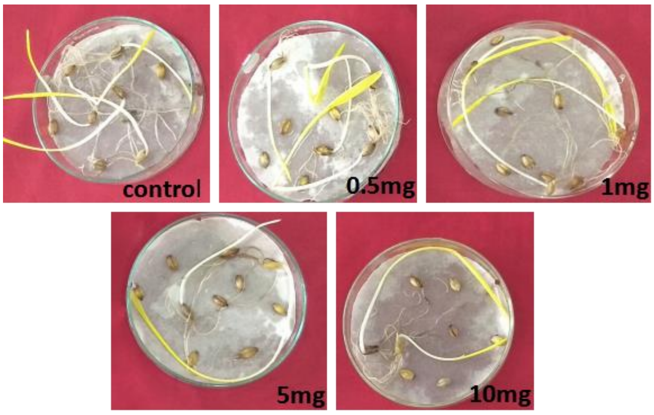


Fig 1: Seed germination in barley under different copper stress levels of $CuSO_4.5H_2O$.

Germination percentage is highest at control level conditions. But as we increased the copper concentration there is significant impact on seed germination but after certain amount germination percentage is not that much affected.

Morphological evaluation of the *Hordeum vulgare* L. seedlings after 5 days

Hordeum vulgare L. grown in Hogland medium for 5 days. Size of the root and shoot diminished as a $CuSO_4.5H_2O$ concentration. is increased. The absorbed $CuSO_4.5H_2O$ was localized to a larger extent in roots than in shoots because root is the primary organ which helps the selective uptake of elements.

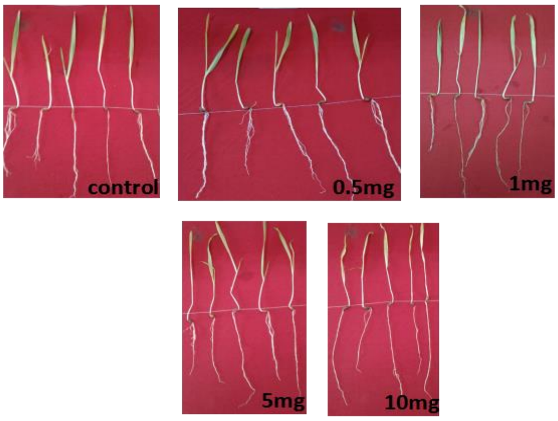


Fig 2: Plantlets of barley under different concentrations of $CuSO_4.5H_2O$ after 5 days.

The growth of plantlets was observed after 5 days, shoots are very weak as compared to the control. Higher concentration of $CuSO_4.5H_2O$ results in plant shoots length reduction. Lower concentrations of $CuSO_4.5H_2O$ showed slightly changes in colour of leaves changes due to higher concentrations of $CuSO_4.5H_2O$.

Table 1: Morphological parameters and relative water content (RWC) of the barley after 5 days of germination under different levels of copper concentrations.

$CuSO_4.5H_2O$conc (mg/l)	Germination (%)	Shoot height (cm)	Root length (cm)	Shoot weight/plant (mg)	Root weight/10 plants (mg)	RWC (%)
0	89±.9	16.9±1.4	13.6±1.7	19.06±0.50	5.97±0.36	64±2.6
0.5	81±1.3	16.7±2.0	13.1±1.5	19.26±0.30	5.62±0.29	84±1.8
1.0	67±1.8	15.2±1.9	10.8±2.1	17.91±0.35	4.90±0.25	71±.3.2
5.0	35±1.2	15.5±2.1	8.9±1.2	18.66±0.40	4.52±0.16	93±1.5
10	32±2.1	15.8±1.7	8.1±0.9	18.13±0.60	4.33±0.17	90±2.5
CD (P=0.05)	0.000196	0.016891	0.041	0.049744	0.000005	0.0000276

Shoot height increased at 0mg concentration as compare to the other concentrations. At 1mg concentration significant reduction have been seen as compared to the other concentrations. The data mentioned in Table 1 revealed the decrement/increment of shoot weight. Shoot weight shows remarkable decrease at higher concentrations. Root length shows the inverse correlation at different Cu concentrations.

Biochemical analysis

RWC% (Relative Water content) of the plants at different concentrations also mentioned in Table 1. The RWC% percentage of the plantlets increased as the concentration of the copper increases. RWC% of the 0 mg 64.61 which is low when we compare with different $CuSo_4$. $5H_2O$ concentrations. These results shows that the RWC% of the plantlets increase as the concentration of the copper increase.

Copper effect on chlorophyll content

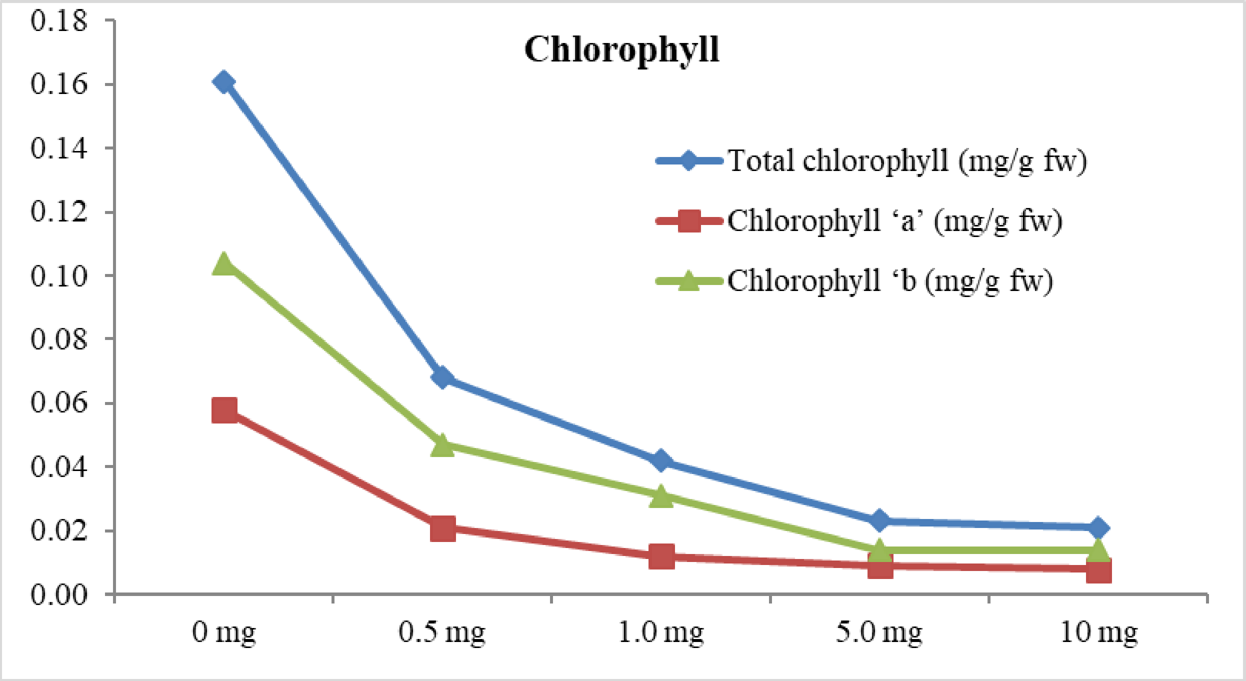
Different types of pigments are present in plants, chlorophyll is the most abundant among all. Most important function of pigments is to capture light for photosynthesis purpose. From previous studies it is found that rate of photosynthesis is directly related to productivity. Data given in table indicates the impact of copper on pigment compositions. With the increase in copper concentration, chlorophyll content goes down drastically.

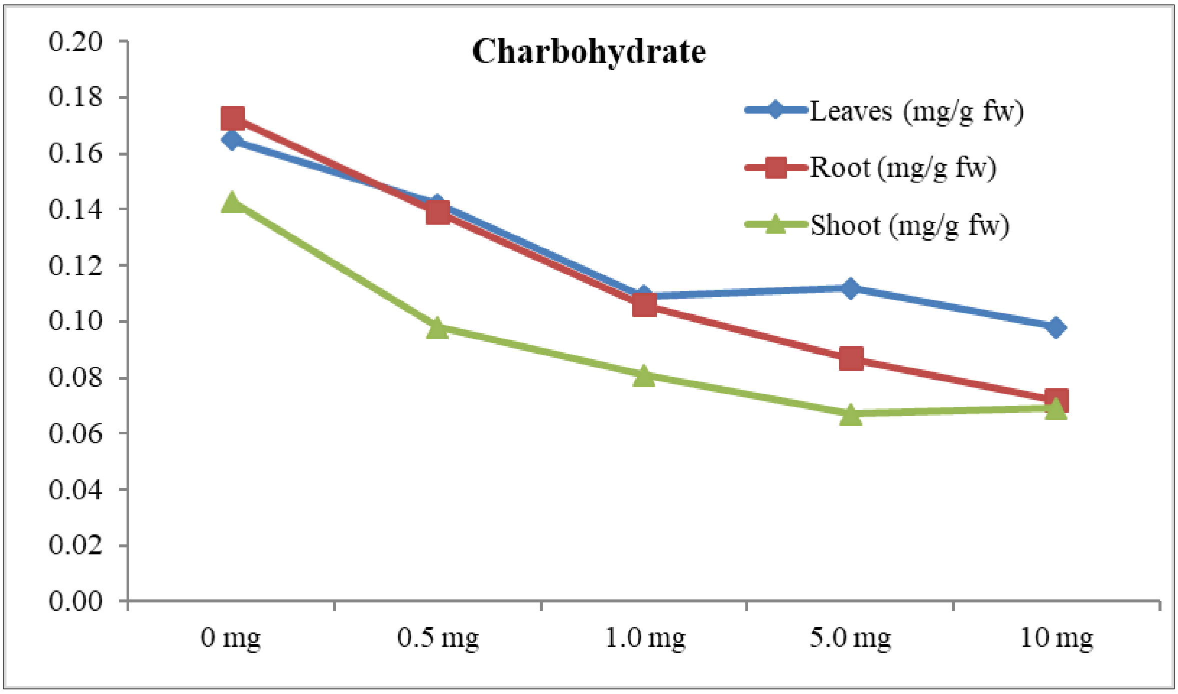
Table 2: Chlorophyll, carbohydrate and proline content of the various plant parts of barley after 5 days of germination under different levels of copper concentrations.

Chlorophyll content (mg/g of fresh weight)			
$CuSO_4.5H_2O$ concentration (mg/l)	**Total chlorophyll (mg/g fw)**	**Chlorophyll 'a' (mg/g fw)**	**Chlorophyll 'b (mg/g fw)**
0	0.161	0.058	0.104
0.5	0.068	0.021	0.047
1.0	0.042	0.012	0.031
5.0	0.023	0.009	0.014
10	0.021	0.008	0.014
CD (P=0.05)	0.034	0.00005	0.000045
Carbohydrate content (mg/g of fresh weight)			
$CuSO_4.5H_2O$ concentration (mg/l)	**Leaves (mg/g fw)**	**Root (mg/g fw)**	**Shoot (mg/g fw)**
0	0.165	0.173	0.143
0.5	0.142	0.139	0.098
1.0	0.109	0.106	0.081
5.0	0.112	0.087	0.067
10	0.098	0.072	0.069
CD (P=0.05)	0.0007	0.00005	0.000934
Proline content (mg/g of fresh weight)			
$CuSO_4.5H_2O$ concentration (mg/l)	**Leaves (mg/g fw)**	**Root (mg/g fw)**	**Shoot (mg/g fw)**
0	0.039	0.043	0.041
0.5	0.051	0.058	0.056
1.0	0.067	0.071	0.069
5.0	0.078	0.091	0.084
10	0.079	0.089	0.087
CD (P=0.05)	0.002106	0.00008	0.000032

Copper effect on carbohydrate content

Carbohydrate content shows significant reduction with increase in copper concentration in all three samples leaves, root and shoot as depicted in given table. This massive reduction in carbohydrate content may be due to inhibition of chlorophyll biosynthesis pathway (Kupper *et al*. 1998) [9].





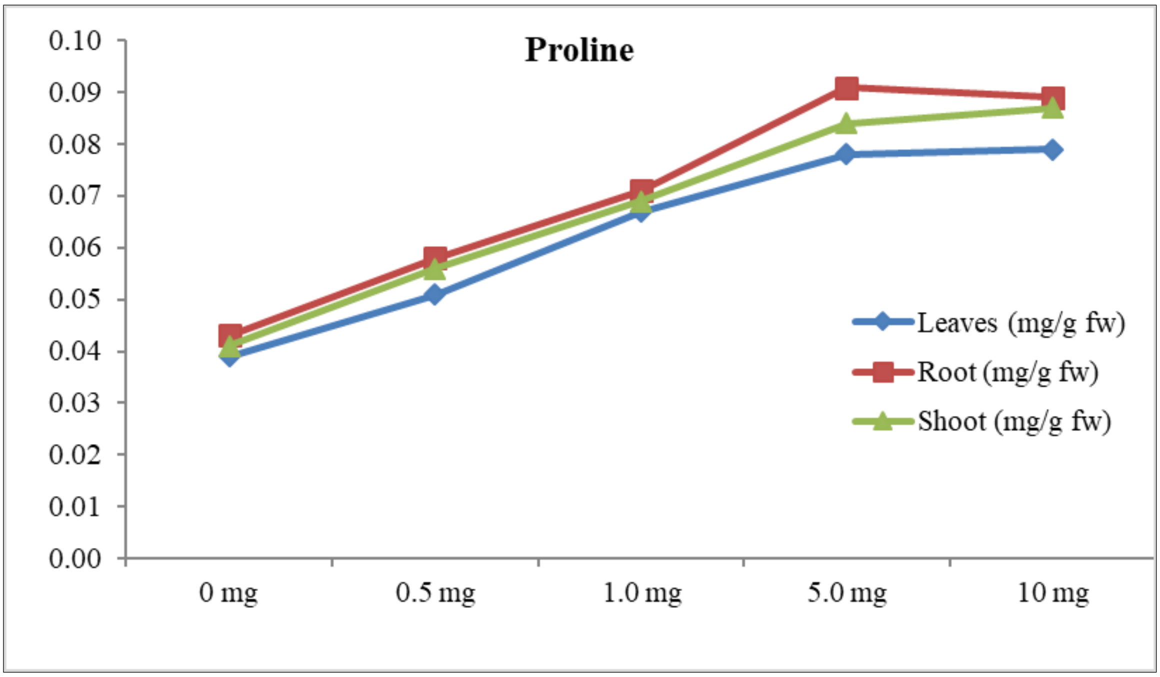


Fig 3: Effect of various copper concentrations on the chlorophyll, carbohydrate and proline content in the various pant parts of the barley.

Copper effect on proline content

In this study inhibitory effect of Cu on proline accumulation were determined as depicted in Table 2. As we increase the amount of copper, rapid accumulation of proline content were found in all three organs i.e leaves, root and shoots. Proline accumulation may serve as compatible solute to maintain the osmotic balance between the cytoplasm and vacuoles (Flowers *et al*. 1977) [5]. Result obtained from this study revealed that copper shows toxic effect on seed germination percentage and seedling growth of barley. With the increase in the copper concentration leads to reduction in germination percentage as compared to control conditions. Similarly root and shoot inhibition takes place with increase in copper concentration when we compare with control. Massive reduction in carbohydrate takes place in copper treated plants which may be due to inhibition of chlorophyll biosynthesis pathway, ultimately leads to carbohydrate reduction.

Looking at present scenario, use of chemical, fertilizers and promotion of industries leads to deterioration of soil quality because with their usage, accumulation of heavy metals is increasing at massive rate. To maintain and improve the soil health stringent control measure should be taken in consideration. This is the utmost requirement for sustainable agriculture practices. Phytoremediation technique can help in this massive task. Many plants have the ability to uptake or neutralize the toxic effect of heavy metals by various means. Hence by the study of their exact mechanism of detoxification with the help of genomics, transcriptomics, proteomics and metabolomics. By finding the responsible genes for detoxification can be applied in stress susceptible varieties.

Results obtained from this study can help to investigate the heavy metal major targets in chlorophyll biosynthesis, carbohydrates accumulation and by engineering the plants in such a way that plant able to accumulate higher amount of osmolytes such as proline. Hence the use of biotechnological advances in phytoremediation can help to improve the heavy metal contaminated soils.

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