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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目： **Physiology and Metabolism in Heavy Metal Toxicity and Tolerance of *Brassica* species: Roles of Different Exogenous Phytoprotectants**
Title of Dissertation (ブラシカ種の重金属毒性と耐性における生理と代謝：種々の植物保護剤の役割)

学位論文要約：
Dissertation Summary

Background of the study

The rapid growth of inhabitants in the world is putting an incredible pressure on the environment. At the same time, for meeting the demand of over increasing population, industrialization is expanding with a parallel rate, which causes serious environmental tribulations, including the generation and release of considerable amounts of toxic metals in the soil, water and air (Hasanuzzaman and Fujita 2012). Basically environment is contaminated with toxic metals by both natural and anthropogenic reasons. Natural contaminating sources are weathering of earth crust, volcanic eruption, forest fire etc., while manmade factors are metal based industries, power station, automobiles, metal smelting, fossil fuel combustion, mining, urbanization, municipal waste, different agrochemicals etc. (Hasanuzzaman and Fujita 2012; Emamverdian et al. 2015). Among the toxic elements heavy metals are the most important sorts of contaminant which lead to abiotic stresses and put health hazard to plants, animals and human. Comparison with other heavy metals, cadmium (Cd) and chromium (Cr) are gaining more attention, due to extensive exploitation in crop production. Excess Cd and Cr contamination in soil and water results in accumulation of these toxic elements in plants and subsequently into the food chain (Wang et al. 2013).

Figure 1: Common effect of heavy metals (Cd and Cr) in plants

Both metals severely shrink crop productivity by hampering their growth and development (Hasanuzzaman and Fujita 2012; Hasanuzzaman et al. 2017a). High reactivity, mobility and hydrophilic nature of Cd and Cr, assist their entry into the plant through the root system and translocation to aerial parts, which becomes a serious threat for living organism due to their neurotoxic, mutagenic and carcinogenic nature (Emsley 2001; Popova et al. 2009; Hasanuzzaman and Fujita 2012). Although, the pattern of damage in plant by Cd and Cr is almost similar, their actions at the molecular level are different. Cadmium is a non-redox-active metal, so, it is unable to generate reactive oxygen species (ROS) through Haber-Weiss reactions. But, inducing NADPH oxidase, upsetting electron transport chain, interacting with antioxidant system or disturbing metabolism of essential mineral nutrients, Cd can overproduce toxic ROS, including singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) (Hasanuzzaman et al. 2012a).

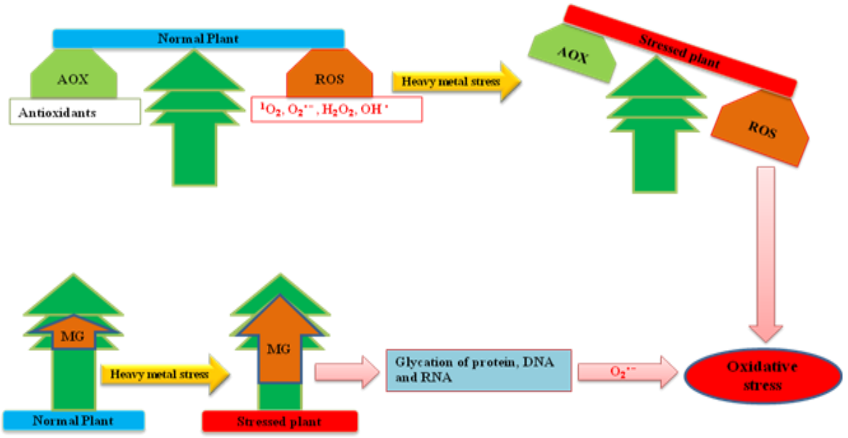

Figure 2: Reactive oxygen species (ROS) production under heavy metal (Cd and Cr) stress

On the other hand, Cr is a redox active metal, which directly produces ROS by reducing CO_2 fixation and photosynthesis through inhibiting electron transport and enzymes activity of Calvin cycle and ultimately affects growth and development of plant (Shanker et al. 2005; Sundaramoorthy et al. 2010; Zhang et al. 2010). Over generation of ROS are able to oxidize important cellular components of plants, such as lipids, proteins, nucleic acids, and finally lead to cell death (Dong et al. 2006; Qadir et al. 2004; Srivastava et al. 2004; Hasanuzzaman et al. 2012a). Additionally, methylglyoxal (MG), by-product of glycolysis, is a cytotoxic compound and present in a small amount in the plant cell under normal condition. However, metal toxicity increased its content in the cell. The production of MG varies in terms of stress types, intensity and plant genotypes. Heavy metal drastically amplify the MG level which also destroy cellular ultra-structure and cause mutation and even cell death by developing oxidative stress in plant cell (Nahar et al. 2016a,b).

Get rid of from Cd and Cr toxicity, plants have developed numerous avoidance techniques to lessen the deleterious effects. Among these artifices, metal binding to the cell wall, decreasing metal transport across the cell membrane, active efflux, active excretion, compartmentalization, and metal chelation are very common (Hall 2002; Gratão et al. 2005). Though plants try to avoid metal toxicity, but with the increase of stress intensity they suffere from severe oxidative stress.

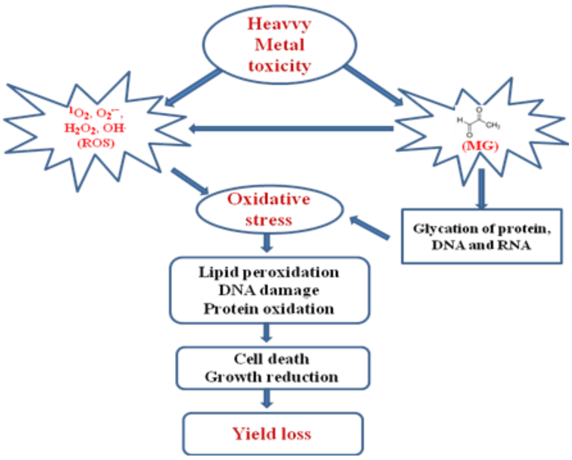

Figure 3: Consequence of heavy metal (Cd and Cr) toxicity

By genesis plant cells are equipped with non-enzymatic and enzymatic antioxidants to minimize oxidative stress, called antioxidant defense system. In this very system, ascorbic acid, AsA; glutathione, GSH; phenolic compounds; alkaloids; α-tocopherol and non-protein amino acids are non-enzymatic components; while enzymatic components are superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; and glutathione *S*-transferase, GST. Both the components work coordinately for scavenging ROS under stress condition (Gill and Tuteja 2010; Hasanuzzaman et al. 2012a).

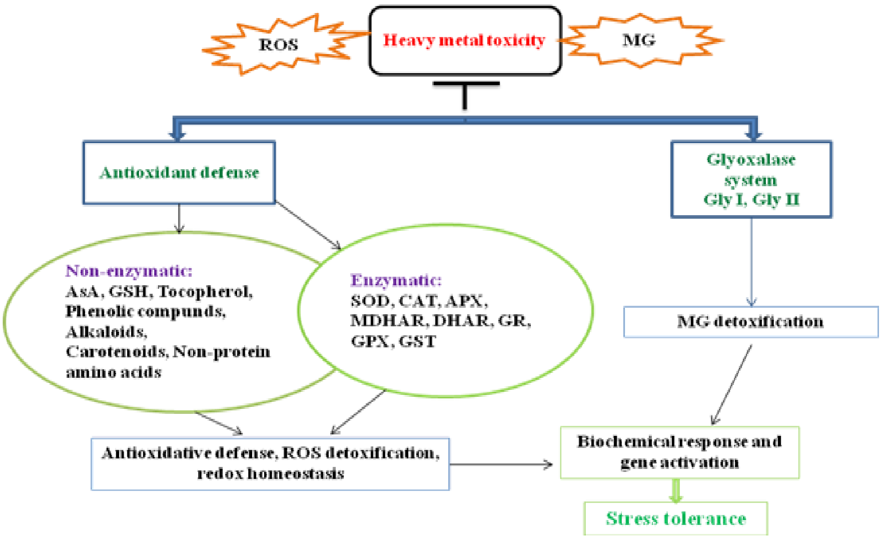

Figure 4: Antioxidant machinery for heavy metal stress (Cd and Cr) tolerance

On the other hand, for lessening the elevated level of MG from cell under stress condition, plant has a unique system called glyoxalase system. The enzymes of the glyoxalase system are glyoxalase I (Gly I) and glyoxalase II (Gly II). Theses enzymes play a key role for detoxification of toxic MG from plant cell and confer stress tolerance against MG-induced oxidative stress (Nahar et al. 2016a,b; Hasanuzzaman et al. 2017b).

Ascorbate and GSH are imperative non-enzymatic antioxidants, which play an important function in modulating cellular redox potential in plants under abiotic stress (Mahmood et al. 2010; Hasanuzzaman et al. 2012a). Ascorbate, a water-soluble antioxidant, directly scavenges ROS including $O_2^{\bullet-}$and $OH^{\bullet}$ by reacting with them (Gill and Tuteja 2010). Monodehydroascorbate reductase and DHAR, two important antioxidant enzymes of the AsA-GSH cycle, are involved in AsA renaissance at the cellular level. Therefore, the amount of AsA largely depends on the activities of MDHAR and DHAR (Gill and Tuteja 2010; Hasanuzzaman et al. 2012a; Nahar et al. 2016a,b). Glutathione works as a substrate of GPX and decreases ROS by preventing oxidation of protein (Noctor et al. 2002; Hasanuzzaman et al. 2012b). It also has roles in the stress signal, adaptation, and defense mechanism of plants (Noctor et al. 2002). The regeneration of GSH highly depends on the activity of GR (Gill and Tuteja 2010).

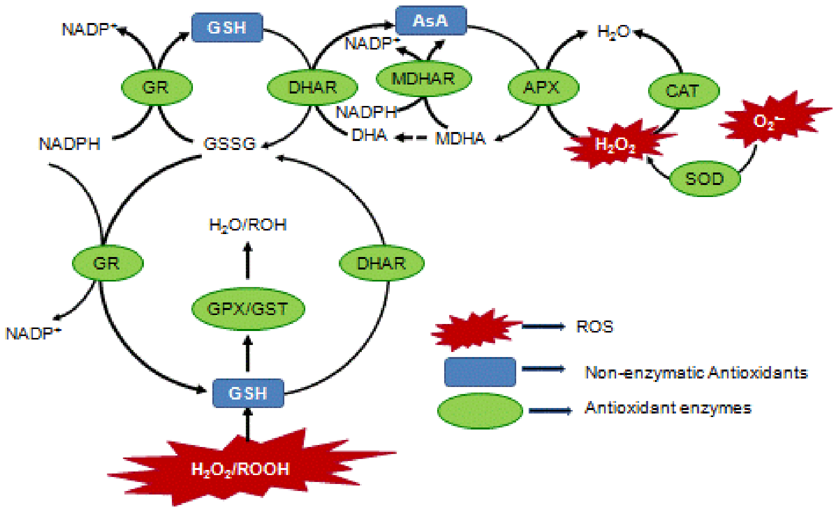

Figure 5: Antioxidant defense system for ROS detoxification

Superoxide dismutase provides the first line defense against abiotic stress by scavenging ROS ($O_2^{\bullet-}$), converting $O_2^{\bullet-}$to H_2O_2. Hydrogen peroxide is then detoxified by CAT and APX (Miller et al. 2008; Hasanuzzaman et al. 2012a; Gill et al. 2015). Therefore, upregulation of SOD and CAT is important in stress tolerance. Superoxide dimutase and CAT are highly susceptible to heavy metal stress resulting in a decline in their catalytic actions (Panda and Choudhury 2005).

The enzymes of the glyoxalase system can detoxify MG effectively through two-step reactions. In the first step, MG is converted to S-D-lactoyl glutathione (SLG) by using the Gly I, where GSH acts as co-factor, and in the second step, SLG is converted to D-lactate by the action of Gly II, where GSH is recycled back (Mustafiz et al. 2010; Hasanuzzaman et al. 2017b).

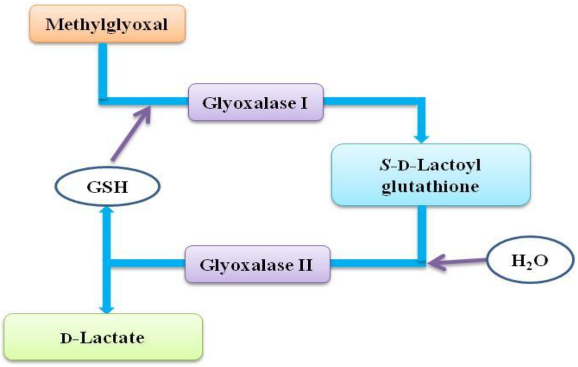

Figure 6: Glyoxalse system for MG detoxification

Efficient and coordinated function of antioxidant defense and glyoxalase systems against ROS and MG under metal stress condition determines the tolerance capability of plants. However, the efficiency of antioxidant defense and glyoxalase system varies greatly with the plant genotypes and stress intensity. Under mild stress condition plant can survive by upregulating the antioxidant defense and glyoxalase systems but under severe stress the systems become disrupted, even totally collapsed.

So, using exogenous protectants such as plant hormones, organic acids, signaling molecules, and trace elements is now common in research and is anticipated to enhance abiotic stress tolerance including that of metal toxicity. Use of chelator showed their activity either chelating the metal in growing media and reduces metal uptake by plant or conversely increasing phytoremediation with conferring stress tolerance. Organic acid is gaining global interest because it plays dual role in plants: phytoremediation of toxic metals (by increasing the solubility of metals) and enhancement of abiotic stress tolerance (by upregulating different antioxidant enzymes) (Ehsan et al. 2014; Zaheer et al. 2015). Different plant genotypes show increased metal accumulation and tolerance against metal exposure after using diverse organic acids (Afshan et al. 2015; Ali et al. 2015; Hawrylak-Nowak et al. 2015; Zaheer et al. 2015). In addition, some amino acids are also showing their efficiency to mediate different kind of abiotic stress (El-Samad et al. 2011). Amino acid in plants varies from acting as osmolyte, regulation of ion transport, modulating stomatal conductance, and detoxification of heavy metals. It also affects synthesis and activity of some enzymes, gene expression, and redox-homeostasis (Rai 2002).

Plants of Brassicaceae family are known as hyper-accumulators of heavy metals, as they can easily accumulate heavy metals (Prasad and Freitas 2003). So, in terms of the biochemical and physiological performance inquisition under heavy metal (Cd and Cr) stress and/or as a candidate of phytoremidiation, *Brassica* species are promising.

Ethylenediaminetetraacetic acid, EDTA (metal chelator) and citric acid, CA (organic acid) against Cd toxicity were used in the present research. On the other hand, γ-aminobutyric acid, GABA (amino acid) and maleic acid, MA (organic acid) was evaluated under Cr stress. In a few previous studies, most of the above mentioned protectants were used as an agent of phytoremediation and/or mediator of plant stress tolerance, but the coordinated action of antioxidant defense and glyoxalase systems of plants under Cd stress is not investigated yet. Even, studies of GABA and maleic acid in *B. juncea* seedlings under Cr toxicity are the first to report.

Considering the abovementioned strategies, several studies were carried out-

i. To identify the relative tolerance and accumulation capacity of *Brassica* species under heavy metal (Cd) stress
ii. To investigate the physiological and biochemical response of *Brassica* species under Cd and Cr stress
iii. To investigate the regulatory role of different phytoprotectants (organic acid, amino acid and chelator) under heavy metal stress in terms of stress tolerance and enhancing phytoremediation
iv. To investigate coordinated interaction between the antioxidant defense mechanism and glyoxalase system in conferring heavy metal stress tolerance in *Brassica* species

To accomplish these objectives, several experiments were conducted and the findings were described in five different titles as follows:

1. Relative tolerance of different species of *Brassica* to cadmium toxicity: Coordinated role of antioxidant defense and glyoxalase systems
2. Ethylenediaminetetraacetic acid (EDTA) reduces cadmium toxicity in mustard (*Brassica juncea* L.) by enhancing metal chelation and the antioxidant defense and glyoxalase systems
3. Insights into citric acid-induced cadmium tolerance in *Brassica juncea* L.: coordinated functions of metal chelation, antioxidant defense and glyoxalase systems
4. γ-aminobutyric acid (GABA) confers chromium stress tolerance in *Brassica juncea* L. by modulating the antioxidant defense and glyoxalase systems
5. Maleic acid assisted improvement of metal chelation and antioxidant metabolism confers chromium tolerance in *Brassica juncea* L.

Experiment-1

Relative tolerance of different species of *Brassica* to cadmium toxicity: Coordinated role of antioxidant defense and glyoxalase systems

Plant materials and stress treatments

Uniform sized seeds of three *Brassica* species (*Brassica campestris* L. cv. BARI Sharisha-9, *Brassica napus* L. cv. BARI Sharisha-13 and *Brassica juncea* L. cv. BARI Sharisha-16 were selected and surface sterilized with 70% ethanol followed by washing several times with sterile distilled water. Then sterilized seeds were sown in perti dishes (9 cm) lined with six layers of filter paper moistened with 10 ml of distilled water for germination and kept for 2 days in germinator. Each petri dish contained 60 germinated seedlings and those were grown under controlled conditions (light, 350 µmol photon m^{-1} s^{-2}; temperature, 25±2°C; relative humidity, 65–70%) in growth chamber; 5,000-fold diluted Hyponex solution (Hyponex, Japan) was applied as nutrient every day according to necessity. Cadmium was added to the nutrient medium as $CdCl_2$ in two concentrations: 0.25 mM and 0.5 mM on ten days old seedlings. Control seedlings were grown in Hyponex solution only. After three days of Cd treatment, leaves and roots were harvested and used for studying various morphological and physiological parameters. The experiment was conducted following Completely Randomized Design (CRD) with nine treatments and it was repeated three times under the same condition.

Measurements of biological and physiological parameters

Fresh leaf samples were used to determine the different physiological and biological parameters including fresh weight (FW), dry weight (DW), leaf relative water content (RWC), chlorophyll (chl) content, proline (Pro) content, lipid peroxidation (malondialdehyde, MDA content), H_2O_2 content, histochemical detection of ROS (H_2O_2 and $O_2^{\bullet -}$), lipoxygenase (LOX) activity, MG content, contents of non-enzymatic antioxidants (AsA and GSH content), activity of enzymatic antioxidants (APX, MDHAR, DHAR, GR, SOD, CAT, GPX) and glyoxalase system enzymes (Gly I and Gly II) following standard methods. Similarly dry leaf and root samples were used to determine metal (Cd) content.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and the mean differences were compared by the Fisher's LSD test using XLSTAT v. 2015 software (Addinsoft 2015) from three replicates. Differences at $P \leq 0.05$ were considered significant.

Results and summary

Among the *Brassica* species studied, *B. juncea* accumulated the highest amount of Cd in a dose-dependent manner, and in every case, the Cd content was higher in the roots than the shoots. Cadmium stress reduced seedlings biomass, leaf RWC, and chl content, whereas, Pro, MDA, and H_2O_2 content and LOX activity increased in all species. Under Cd stress, AsA content reduction was lower and GSH content increase was higher in *B. juncea* compared with other species. Monodehydroascorbate reductase, GR, and SOD activities increased significantly in *B. juncea* under Cd stress compared with the other species. Catalase activity did not decrease in *B. juncea* due to Cd stress, compared with the other species. Dehydroascorbate reductase activity decreased with both levels of Cd stress in all species except for *B. juncea* under 0.25 mM $CdCl_2$ stress. Glyoxalase system components performed better in *B. juncea* than the other species under Cd stress. Methylglyoxal increased substantially under both levels of Cd stress, but MG content was lower in *B. juncea* compared with the others. Considering the antioxidant defense and glyoxalase systems performance *B. juncea* is relatively tolerant species to Cd toxicity though it accumulated highest Cd.

Experiment-2

Ethylenediaminetetraacetic acid (EDTA) reduces cadmium toxicity in mustard (*Brassica juncea* L.) by enhancing metal chelation and the antioxidant defense and glyoxalase systems

Plant materials and stress treatments

Uniform-sized seeds of mustard (*Brassica juncea* L. cv. BARI Sharisha-11) were collected for the experiment. Seedlings were grown from the seeds under similar condition as Experiment-1. Ten-day-old mustard seedlings were then treated by EDTA (0.5 mM) and Cd (0.5 mM and 1.0 mM $CdCl_2$) separately and in combination. We considered 0.5 mM and 1.0 mM $CdCl_2$ as mild and severe stress, respectively. Control plants were grown in Hyponex solution only. After 3 d of treatment, samples were harvested and used for the study of various morphological and physiological parameters. The experiment was conducted following a CRD with six treatments and repeated three times.

Measurements of biological and physiological parameters

Different biological and physiological parameters were determined like as Experiment-1. Additionally, biological concentration factor (BCF), biological accumulation coefficient (BAC), translocation factor (TF) and phytochelatin (PC) content were measured according to standard methods.

Statistical analysis

All the values of measured parameters are the means of three replications. One way analysis of variance (ANOVA) was undertaken using XLSTAT v. 2016 software (Addinsoft 2016) and the mean differences were compared by Fisher's LSD test. Differences at $P \leq 0.05$ were considered as significant.

Results and summary

Without EDTA, both the roots and shoots accumulated Cd in a dose-dependent manner, which disrupted antioxidant defense and led to increased generation of H_2O_2 and $O_2^{\bullet -}$ with a concomitant increase in LOX activity and lipid peroxidation. In Cd-treated plant MG increased markedly. Cadmium stress also reduced growth, chl content, and leaf RWC but increased the Pro content. Moreover, Cd-stressed seedlings supplemented with EDTA upregulated the components of AsA-GSH pool with the increased activities of APX, MDHAR, DHAR, GR, GPX, SOD, and CAT. Glyoxalase I activity increased while Gly II activity decreased under both levels of Cd stress. However, applying EDTA to the Cd-stressed seedlings further increased Gly I activity with no changes in Gly II activity. It reduced the Cd content in the roots and shoots; decreased H_2O_2, $O_2^{\bullet -}$ and MG levels; diminished lipid peroxidation and increased chl, Pro and RWC in the leaves, which resulted in better growth. The results showed that adding EDTA to the Cd-treated plants rendered the mustard seedlings to tolerate Cd-induced oxidative damage by limiting Cd uptake and improving their antioxidant defense and glyoxalase systems.

Experiment-3

Insights into citric acid-induced cadmium tolerance in *Brassica juncea* L.: coordinated functions of metal chelation, antioxidant defense and glyoxalase systems

Plant materials and stress treatments

Healthy uniform mustard (*Brassica juncea* L. cv. BARI Sharisha-11) seeds were used in this experiment and seedlings were grown following similar approach described in Experiment-1. Mustard seedlings (12 d old) were exposed to citric acid (CA, 0.5 mM and 1.0 mM) and cadmium (Cd, 0.5 mM and 1.0 mM $CdCl_2$) separately and in combination. We considered 0.5 mM as mild stress and 1.0 mM $CdCl_2$ as severe stress. Control seedlings were grown in Hyponex solution only. After 3 d of treatment, the leaves and roots were harvested and used to study various growth and physiological parameters following a CRD with nine treatments and repeated three times under similar conditions.

Measurements of biological and physiological parameters

In addition with biological and physiological parameters determined in Experiment-1 and Experiment-2, histochemical determination of lipid peroxidation in root was performed following standard method.

Statistical analysis

Data were statistically analyzed similar with Experiment-2.

Results and summary

Cadmium accumulation in the roots and shoots of the mustard seedlings increased in a dose-dependent manner and was higher in the roots. Increasing the Cd concentration led to reduced growth, biomass, water status, and chl content resulting from increased oxidative damage (elevated MDA content, H_2O_2 level, $O_2^{\bullet-}$ generation, LOX activity, and MG content) and downregulating of the major enzymes of the antioxidant defense and glyoxalase systems. Under Cd stress, both doses of CA improved the growth of the plants by enhancing leaf RWC and chl content; reducing oxidative damage; enhancing the pool of AsA and GSH and the activities of the antioxidant enzymes (APX, MDHAR, DHAR, GR, GPX, SOD, CAT); improving the performance of the glyoxalase system (Gly I and Gly II activities); and increasing the PC content. Exogenous CA also increased the root and shoot Cd content and Cd translocation from the roots to the shoots in a dose-dependent manner. Our findings suggest that CA plays a dual role in mustard seedlings by increasing phytoremediation and enhancing stress tolerance through upregulating the antioxidant defense and glyoxalase systems.

Experiment-4

γ-aminobutyric acid (GABA) confers chromium stress tolerance in *Brassica juncea* L. by modulating the antioxidant defense and glyoxalase systems

Plant materials and stress treatments

Uniform seeds of mustard (*Brassica juncea* L. cv. BARI Sharisha-11) were selected and seedlings were grown from the seeds under similar condition as Experiment-1. Eight-day-old mustard seedlings were exposed to GABA (0.125 mM) and Cr (0.15 mM and 0.3 mM K_2CrO_4) separately and in combination. The concentrations of 0.15 mM and 0.3 mM K_2CrO_4 were considered as mild stress and severe stress, respectively. The control seedlings were grown in Hyponex solution only. After five days of Cr treatment, the leaves and roots were harvested and used to study various morphological and physiological parameters. The experiment was conducted following a CRD with six treatments and was repeated three times under the same conditions.

Measurements of biological and physiological parameters

Biological and physiological parameters were determined according to previously mentioned experiments.

Statistical analysis

Data were analyzed statistically following the same procedure as Experiment-2.

Results and summary

The roots and shoots of the seedlings accumulated Cr in a dose-dependent manner, which led to an increase in oxidative damage [lipid peroxidation, H_2O_2 content, $O_2^{\bullet-}$ generation, LOX activity], MG content, and disrupted antioxidant defense and glyoxalase systems. Chromium stress also reduced growth, leaf RWC, and chl content but increased PC and Pro content. Furthermore, supplementing the Cr-treated seedlings with GABA reduced Cr uptake and upregulated the non-enzymatic antioxidants (AsA, GSH) and the activities of the enzymatic antioxidants including APX, MDHAR, DHAR, GR, GPX, SOD, CAT, Gly I, and Gly II, and finally reduced oxidative damage. Adding GABA also increased leaf RWC and chl content, decreased Pro and PC content, and restored plant growth. These findings shed light on the effect of GABA in improving the physiological mechanisms of mustard seedlings in response to Cr stress.

Experiment-5

Maleic acid assisted improvement of metal chelation and antioxidant metabolism confers chromium tolerance in *Brassica juncea* L.

Plant materials and stress treatments

Uniform mustard (*Brassica juncea* L. cv. BARI Sharisha 11) seeds were collected for the experiment and seedlings were grown following similar approach described in Experiment-1. Mustard seedlings (eight days old) were treated with MA (0.25 mM) and Cr (0.15 mM and 0.3 mM K_2CrO_4) independently and in combination, where 0.15 mM and 0.3 mM K_2CrO_4 considered as mild stress and severe stress, respectively. Seedlings grown in Hyponex solution only were used as control. After five days of treatment, the leaves and roots were collected and used to examine morphological and physiological parameters. The investigation followed a CRD with six treatments and was replicated three times under similar condition.

Measurements of biological and physiological parameters

Biological and physiological parameters were determined according to previously mentioned experiments.

Statistical analysis

Data were analyzed statistically resembling as Experiment-2.

Results and summary

Under Cr stress, plants accumulated Cr in both the roots and shoots in a dose-dependent manner, where the roots showed higher accumulation. Chromium stress reduced the growth and biomass of the mustard plants by reducing water status and photosynthetic pigments, and increased oxidative damage due to generation of toxic ROS and MG. Chromium stress also interfered with the function of the antioxidant defense and glyoxalase systems. However, using MA in the Cr-stressed plants further increased Cr uptake in the roots, but it slightly reduced the translocation of Cr from the roots to the shoots at a lower dose of Cr and significantly at a higher dose. Moreover, MA also increased the other non protein thiols (NPTs) containing phytochelatin (PC) content of the seedlings, which reduced Cr toxicity. Supplementing the stressed plants with MA upregulated the non-enzymatic antioxidants (AsA, GSH); the activities of the enzymatic antioxidants including APX, MDHAR, DHAR, GR, GPX, SOD, and CAT; and the enzymes of the glyoxalase system including Gly I and Gly II; and finally reduced oxidative damage and increased the chl content and water status as well the growth and biomass of the plants. Our findings suggested two potential uses of MA: first, enhancing phytoremediation, principally phytostabilization and second, working as an exogenous protectant to enhance Cr tolerance.

Conclusion

Brassica juncea L. is a well-known hyper-accumulator of heavy metal, which was established again in our experiment. Cadmium and Cr induced oxidative stress in *B. juncea* by reducing the activity of photosynthesis, increasing ROS and MG, hampering the water status, and disrupting the antioxidant defense and glyoxalase systems. Eventually, the growth and biomass of the plants were greatly affected because of the Cd and Cr. However, exogenous application of different phytoprotectants in the growing media with Cd and Cr noticeably alleviated the detrimental effect through upregulating the antioxidant defense and glyoxalase enzymes. Among the phytoprotectants, organic acid played potential role against Cd and Cr stress as they increased phytoremediation as well as counteracted Cd and Cr toxicity.

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