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Full Length Article

Exogenous Application of Ethylenediaminetetraacetic Acid and Oxalic Acid Improve the Seed Germination and Enzymes Activities of Sunflower (*Helianthus annuus*) Under Cadmium Stress

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Abstract

The study was conducted to examine the deleterious effects of cadmium (Cd) on metabolic enzymes and their end products during seed germination and the effect of ethylenediaminetetraacetic acid (EDTA) and oxalic acid (OA) in easing the Cd toxicity in two sunflower hybrids (Hysun-33 and FH-533). To accomplish this, a pot experiment was carried out in small sand-filled plastic pots under normal environment temperature (28±2ºC). Seeds of both sunflower hybrids were exposed to Cd levels (250 and 450 mg/kg) alone and in combination of optimized level of chelating agents EDTA and OA (each at the rate of 1g/kg) at the time of sowing. The enzymes activities of two germinating hybrids were recorded over time from 24 to 120 h after sowing. When Cd was applied alone it reduced the germination rate, enzymes activities and total soluble sugars contents. Mobilization of stored proteins and amino acids were also suppressed in germinating seeds of both hybrids. However, application of EDTA and OA in combination with Cd improved the germination rate of seeds by surpassing the biochemical metabolism and enhanced the activities of α-amylase and protease revealing the ameliorating effect to Cd toxicity. Results revealed that minimum values of all the attributes are obtained at 450 mg/kg of Cd level, while chelators treatments proved effective in relieving Cd stress of which OA had 10% more pronounced germination percentage than EDTA and 30% more as compared to control. Interactive studies showed that both the chelators detoxify the 250 mg/kg of Cd more as compared to 450 mg/kg of Cd. Results clearly indicated that sunflower hybrid Hysun-33 showed greater activities of two hydrolyzing enzymes and efficient mobilization of reserved sugars and proteins than hybrid FH-533 under Cd stress, so it can be grown on Cd contaminated soils for higher plant productivity. Thereafter EDTA and OA could be supportive to improve the seed germination security in Cd polluted soil.

Keywords: *Helianthus annuus*; Cd stress; Chelator; α-amylase; protease

Introduction

Industry flourishes analogous to urbanization but augmented industrialization produced industrial waste especially metallic elements which are harmful for the environment (Wei and Yang, 2010; Yaylalı-Abanuz, 2011; Mireles *et al*., 2012). Among metallic elements Cd is a potential pollutant which penetrates in agricultural soil through irrigation with industrial effluents, application of sewage sludge, pesticides and commercial fertilizers (Wu *et al*., 2004; Kidd *et al*., 2007; Papafilippaki *et al*., 2007; Grant and Sheppard, 2008; Cotuk *et al*., 2010) and contaminate the soil resulting in its amassing in different plant parts (Cheng *et al*., 2006). Plants accumulate the Cd in edible parts as being primary producer of the food chain and thus serve as a main source of intake in animals and humans (Wahid and Ghani, 2008; Pinot *et al*., 2000; Lopez-Millan *et al*., 2009).

Incorporation of the Cd takes place in plants through nonspecific pathways. Owing to inordinate solubility and toxicity of Cd; it exerts deleterious effects on growth leading to plant death (Pinto *et al*., 2004). Higher Cd concentration generates oxidative stress indirectly by reticence of the photoactivation process in chloroplasts resulting in free radicals formation that may impair the tissues of plants (Heyno *et al*., 2008). It also effects the gas exchange parameters, diminish the chlorophyll contents and disrupts the plant water relations (Singh and Tewari, 2003; Vijayaragavan *et al*., 2011). The literature presents that higher Cd levels adversely affect the metabolic activities of germinating seeds resulting in impaired germination and reduced plant growth. α-amylase enzyme is responsible for conversion of starch into sugars (Junyu *et al*., 2008) and protease is responsible for conversion of proteins into amino acids (Kranner and Colville, 2011) Cd inactivates the

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enzymes by reacting with their SH- group (Ramon *et al*., 2003) therefore, both are susceptible to Cd stress.

The Chelators, organic or inorganic agents are being widely used for metals mobilization to rehabilitate the metal contaminated soils by plants (Madrid *et al*., 2003; Chen *et al*., 2014), they form the water soluble complexes with metal ions and desorb them from various soil components (Sun, 2009). Although the chelators effectiveness is well reported for phytoremediation, but their effect on plants growth and development and various physiological and biochemical processes has not been inspected in depth. EDTA, a well-known synthetic chelator, is one of the successful and admired chemical regents because of its strong affinity for Cd (Saifullah *et al*., 2009). It is a powerful, recoverable and comparatively biostable chelator which has the ability to remediate the metal affected soils (Meers *et al*., 2005). EDTA promotes the Cd solubility as it has definite affinity and binding ability with Cd, but it did not enhances the Cd uptake in plants and consequently ameliorate its toxic effect (Romkens *et al*., 2002). However, most synthetic chelators like EDTA and EDDS form complexes with metals which have high stability and contaminate the groundwater (Zeremski-Skoric *et al*., 2010). Low-molecular-weight organic acids (LMWOA) like oxalic acid (OA) and citric acid (CA) may prove environmentally compatible alternatives for synthetic chelators because they are the part of root exudates thus acting as natural chelators (Hsiao *et al*., 2007). Natural chelators affect the sorption and desorption of Cd as they have strong tendencies to bind with metals (Nigam *et al*., 2001). They form complexes with Cd ranging from low to moderate constancy (Evangelou *et al*., 2006) and present a benefit over synthetic chelators because of their high biodegradation rate in soil (Quartacci *et al*., 2005).

Worldwide sunflower is one of the major promising edible oil seed crops (Taran *et al*., 2013). During 2011 the area under sunflower cultivation in Pakistan was 877 hectares, which fulfilled the 179 tons oil demand in the country (Govt. of Pakistan, 2012). Sunflower being a most competent deep rooted crop with fast growing rate (Prasad, 2004) high biomass production (Zhuang *et al.*, 2005) and metal tolerance potential is being cultivated on heavily metal contaminated areas (Jadia and Fulekar, 2008). Keeping in view the deleterious effects of Cd on seed germination this investigation was conducted under the hypothesis that "addition of EDTA and OA in the Cd contaminated growth medium improves seed germination of sunflower by enhancing α-amylase and protease activities and their resulting products".

Materials and Methods

The experiment was conducted under natural environmental conditions of light and temperature (28±2ºC) in the wire house of Dept. of Botany, University of Agriculture, Faisalabad. Seeds of sunflower hybrids Hysun-33 and FH-533 were obtained from Ayub Agricultural Research Institute (AARI). Experiment was laid down in a completely randomized design having three replicates with factorial arrangement. Twenty seeds were sown in each plastic pot containing 1/2 kg of acid and distilled water washed sand. From preliminary screening experiments three levels of Cd (control, 250 and 450 mg/kg) and two levels of EDTA and OA (control and 1 g/kg) were selected for sunflower, a promising crop for environment industry having appreciable tolerance level against metals (Sadiq, 2014). These treatments were applied once separately and in combination on both hybrids at the time of sowing. The seeds were allowed to grow for one week under natural conditions. Germination was recorded after 120 h of seed sowing. The α-amylase and protease activities and sugars, proteins and amino acids contents were determined in the germinating seeds after 24, 48, 72, 96 and 120 h of sowing. The activity of α-amylase enzyme was determined by using the method of (Chrispeels and Varner, 1967), while the protease activity followed the method of (Ainouz, 1970). Sugars were estimated according to the method of (Riazi *et al*., 1985), total soluble proteins by following the protocol as described by (Lowry *et al*., 1951) and total free amino acids by the procedure of (Hamilton and Van-Slyke, 1943).

Statistical Analysis

The data was statistically analyzed using COSTAT computer package (CoHort Software, 2003, Monterey, California). Analysis of variance (ANOVA) was performed and least-significant difference (LSD) test was used for comparison of means (Steel and Torrie, 1986).

Results

Germination Rate (%)

Cd exerted significant ($p<0.05$) effect on germination rate of two sunflower hybrids. The maximum germination rate was observed in seedlings under control conditions followed by both chelators i.e., OA and EDTA, combination of both chelators with lower dose of Cd i.e., 250 mg Cd/kg + 1 g OA/kg and 250 mg Cd/kg + 1 g EDTA/kg, combined application of both chelators with higher Cd level i.e. 450 mg Cd/kg + 1 g OA/kg and 450 mg Cd/kg + 1 g EDTA/kg and then lower and higher levels of Cd (Fig. 1a, b). Both hybrids followed the similar trend but FH-533 experienced more reduction in germination rate under all treatments than Hysun-33.

Alpha Amylase and Sugars

Imposition of Cd had pronounced effect on α-amylase activity. Generally the enzyme activity increased with the passage of time i.e., 24, 48, 72, 96 and 120 h after seed sowing. While in term of treatments the maximum activity

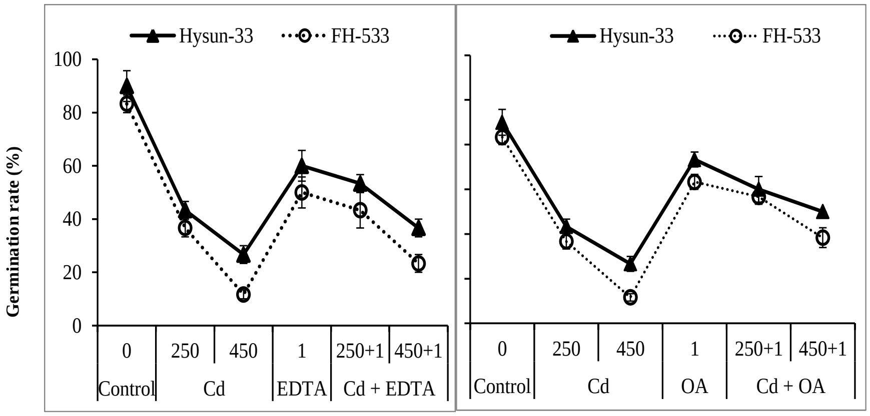

Fig. 1: Germination rate of two sunflower hybrids under Cd (mg/kg), EDTA (g/kg) and OA (g/kg) application. Error bars are shown

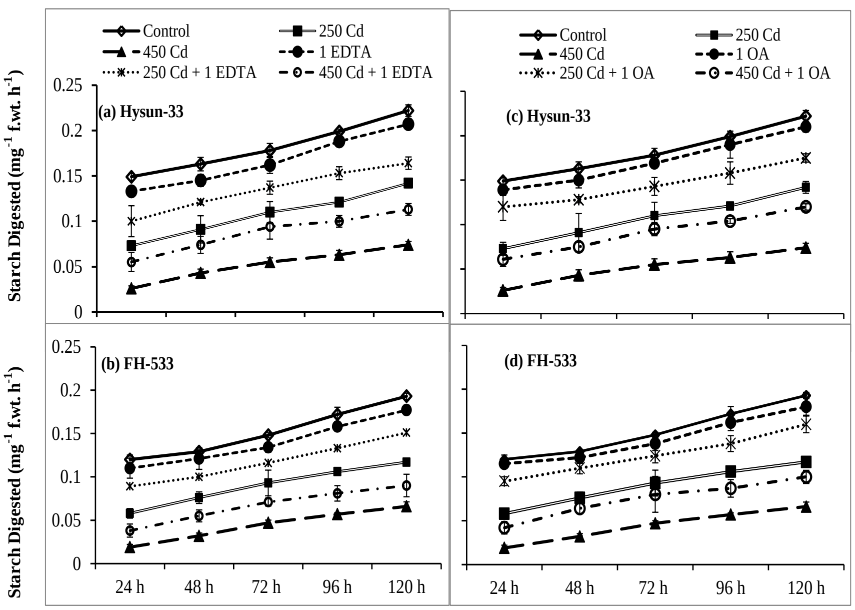

Fig. 2: Effect of Cd (mg/kg) on α-amylase activity of two sunflower hybrids under the influence of EDTA (g/kg) (a, b) and OA (g/kg) (c, d). Error bars are shown

was recorded in plants under controlled condition (0 mg Cd/kg + 0 g EDTA/kg) and minimum activity was observed in plants treated with 450 mg Cd/kg, while other treatments laid in between them i.e., 1 g EDTA/kg, 250 mg Cd/kg + 1 g EDTA/kg, 250 mg Cd/kg, 450 mg Cd/kg + 1g EDTA/g (Fig. 2a, b). OA application in the presence and absence of Cd also followed the same trend but it proved more effective in ameliorating the toxic effects of Cd on alpha-amylase activity of germinating seeds than EDTA (Fig. 2b, c). Hysun-33 had greater activity of alpha-amylase as compared to FH-533. The reduction in α-amylase activity appeared to have straight inhibitory influence on starch hydrolysis, resulting in reduced sugars formation. Overall the concentration of sugars persistently increased in both hybrids over time, which was highest at 120 h after sowing (Fig. 3a, b, c, d).

Protease, Proteins and Amino Acids

The application of Cd expressively inhibited the protease activity hence the stored proteins conversion into amino acids. The maximum enzyme activity in term of treatment was recorded in control plants followed by 1 g EDTA/kg, 250 mg Cd/kg + 1 g EDTA/kg, 250 mg Cd/kg, 450 mg Cd/kg + 1 g EDTA/kg and 450 mg Cd/kg treated plants (Fig. 4a, b). Same is the case with OA application i.e., control was followed by 1 g OA/kg, OA application in combination with metal exerted less suppressing effects on protease activity than separate application of Cd (Fig. 4c, d). Protease activity increased with the passage of time under the influence of all treatments in selected sunflower hybrids. Generally after 48 h of treatment application, the proteolytic

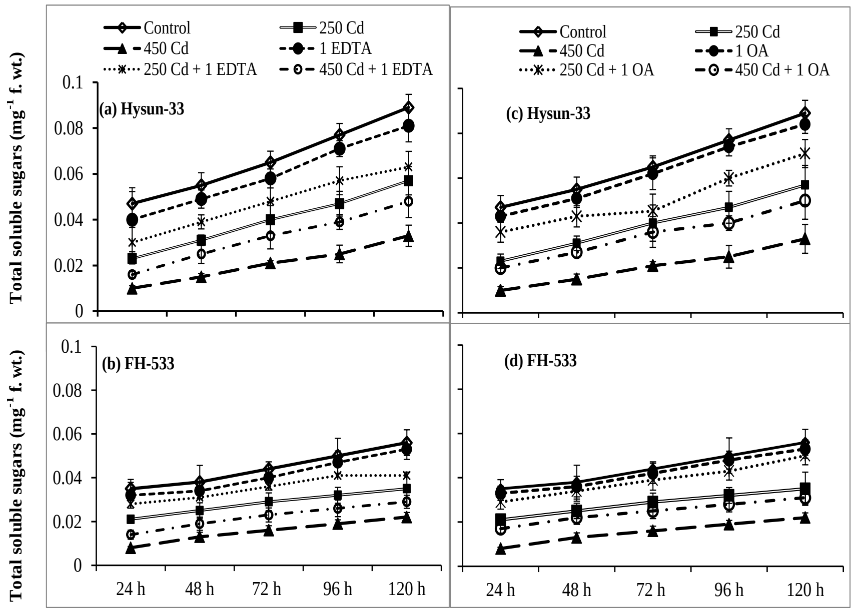

Fig. 3: Effect of Cd (mg/kg) on total soluble sugars of two sunflower hybrids under the influence of EDTA (g/kg) (a, b) and OA (g/kg) (c, d). Error bars are shown

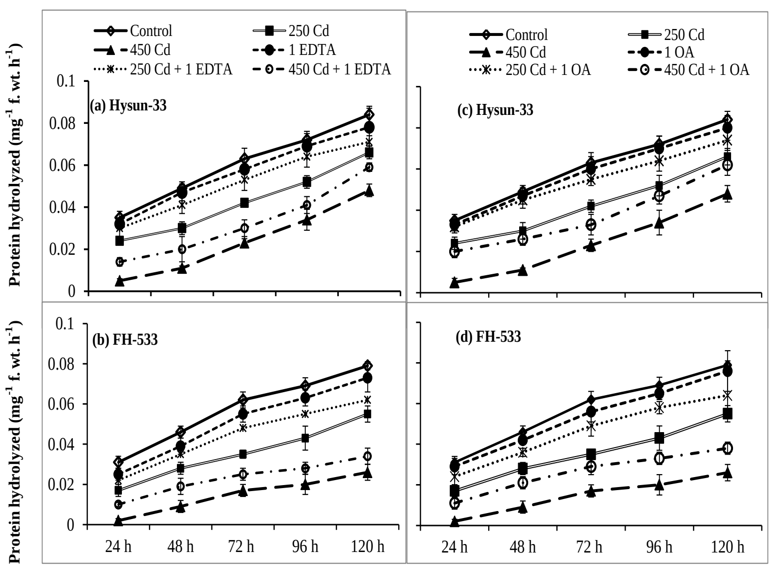

Fig. 4: Effect of Cd (mg/kg) on protease activity of two sunflower hybrids under the influence of EDTA (g/kg) (a, b) and OA (g/kg) (c, d). Error bars are shown

activity displayed a steep increase and at 120th h reached the maximum value. Seedlings of sensitive hybrid FH-533 contained more protein contents than tolerant Hysun-33 under all tested treatments during all time intervals. The lower protease activity in germinating seeds of FH-533 was evident from its higher protein contents (Fig. 5b, d). The amino acids contents increased the activity of protease in both hybrids under all treatments (Fig. 6a, b, c, d). The amino acids contents also increased over the passage of time and reached the highest value at 120th h.

Discussion

Germination ability of seed is a useful parameter for the decision of tolerance level as it is the first interface for material exchange between plant development and soil environment (Rahoui *et al*., 2010). Some previous studies have proved that higher Cd levels imposed adverse effect on seed germination resulted in retarded plant growth (Shafiq *et al*., 2008; Aydinalp and Marinova, 2009; Zhang *et al*., 2012) as in alfalfa seeds (Peralta *et al*., 2001). Cd

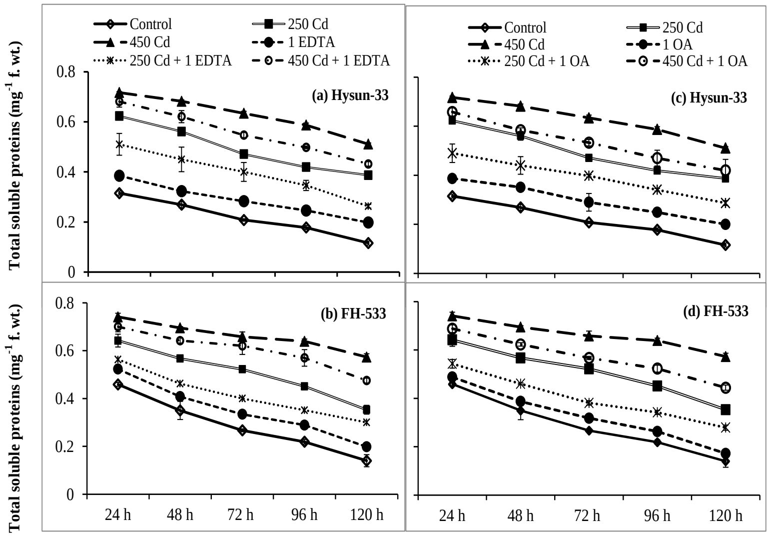

Fig. 5: Effect of Cd (mg/kg) on total soluble proteins of two sunflower hybrids under the influence of EDTA (g/kg) (a, b) and OA (g/kg) (c, d). Error bars are shown

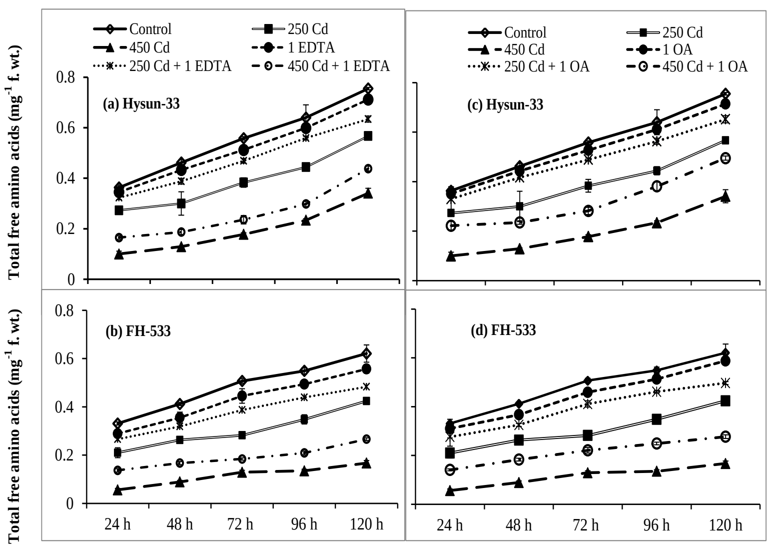

Fig. 6: Effect of Cd (mg/kg) on total free amino acids of two sunflower hybrids under the influence of EDTA (g/kg) (a, b) and OA (g/kg) (c, d). Error bars are shown

interference changes the permeability of cell membrane which lessened absorption and transport of water as well as reduced the stress tolerance potential during germination (Russak *et al*., 2008). The results included in Fig. 1 (a, b) show that Cd induced significant decline in germination rate of both sunflower hybrids i.e., Hysun-33 and FH-533. However, addition of chelating agents in Cd containing growth medium assisted in removing the toxic effect of Cd on seed germination. Chelating substances prevent the entry of metal ions across the seed coat by engulfing them and forming stable EDTA-Cd and OA-Cd complexes (Mejre and Bulow, 2001; Mohanty and Patra, 2011). Imbibition of seed activates hydrolytic enzymes during the germination process (Sreenivasulu *et al*., 2008) and these are inactivated or suppressed by the metal stress as in Fig 2 (a, b, c, d). Hydrolytic enzymes make the availability of reserve materials that deliver fuel for respiration and various anabolic reactions in the form of metabolites. Enzymes of starch and protein digestion i.e., α, β-amylase and protease are accountable for breakdown and mobilization of these major reserve materials of seeds (Alencar *et al*., 2012). The maximum retarded activity of enzymes was observed when Cd was applied @ 450 mg/kg while both the chelators especially OA helped in relieving the toxic effects of Cd on both hydrolytic enzymes. These results coincide with work on chickpea reported by Mondal *et al*. (2013). The major starch hydrolysing enzymes of seeds endosperm are α-amylase, starch phosphorylase and α-glucosidase, (Zhu *et al*., 1998). Among them, α-amylase is most crucial one, as it is responsible for breakdown of α-(1–4) linkages of starch and converts it to either amylopectin or amylose by random hydrolysis of polymer. Thus various micromolecules having low molecular weight and short chains known as dextrins are generated from these macromolecules. Reduction of α-amylase activity in both germinating hybrid seeds under Cd stress occurred as compared to control i.e., untreated seeds. However, with passage of time the overall α-amylase activity was augmented, indicating that Cd stress directly affected the enzyme activity available to fulfil the least requirements of developing shoot apices in phase of early growth (Fig. 2a, b, c, d). Impaired carbohydrates mobilization due to disturbed α-amylase activity under Cd was reported in oregano (Farashah *et al*., 2011), pea (Mihoub *et al*., 2005; Smiri, 2011), wheat (Amirjani, 2012) and bean seeds (Sfaxi-Bousbih *et al*., 2010). Under Cd stress the concentration of total soluble sugars increased with incubation time. However, the elevation in sugars concentration was significantly lower than control for all treatments of metal either with or without chelators (Fig. 3a, b, c, d). Veer (1989) reported same results for sugar contents of seeds germinating under Cd stress. However, Loreti *et al*. (2003) reported that maintaining high sugar contents by cereal grains at germination time was the result of minor effect of stress on activity of α-amylase. Contrary, the present results indicated the activation of hydrolytic activity of enzyme in germinating seeds of both sunflower hybrids which was meaningfully delayed under different treatments of Cd. Enzymes such as α and β amylase, starch phosphorylase and starch invertase are responsible for carbohydrate metabolism in seeds (Yang *et al.,* 2001; Ende *et al*., 2002). Presence of high concentrations of various metals in soil either essential or non-essential causes a significant perturbation in carbohydrates (Jha and Dubey, 2005). As a result of this direct influence on activity of metabolic enzymes of germinating seeds, the level of vital biomolecules including sugars, proteins, amino acids and nucleotides is altered (Subedi and Bhattarai, 2003). Another enzyme, protease is thought to participate in various physiological as well as metabolic process of plants related to protein turnover. This, as a result regulate various stress responses such as senescence, defence responses, abiotic stress and programmed cell death (Fontanini and Jones, 2002; Segarra *et al*., 2002; Roberts *et al*., 2003; Coffeen and Wolpert, 2004). For example, Balestrasse *et al*. (2003) reported suppressed protease activity in Cd stressed nodules of soybean roots. Some previous findings also proved reduced activity of protease in duckweed and rice when treated with high doses of Cd and Pb respectively (Sethy and Ghosh, 2013). Some reports have shown that interaction of metal ions with S, O and N ligands of enzymes active groups is the main cause of inhibition of enzyme activities (Bavi *et al.*, 2011). Fig. 4 (a, b, c, d) showed that protease activity was markedly affected under all Cd treatments i.e., with and without chelators. A notable decrease in protein contents was observed with the increase in seedlings age. Protein contents were also decreased with the increasing concentration of Cd in growth medium, which was less for treatments having chelating agents either OA or EDTA (Fig. 5a, b, c, d). Zeid (2001) also reported high protein contents under metal stress, while chelating agents helped in reducing the toxic effects of metals on proteins and their hydrolysing enzyme by limiting the metal availability to germinating seeds. Germinating seeds obtain amino acids by the hydrolysis of stored proteins. The protein hydrolysis and succeeding amino acids conversion in germinating seeds are accurately maintained to fulfil the requirements of new proteins biosynthesis and some other biomolecules as well, including apices growth controlling enzymes (Bewley, 2001). Total free amino acids levels were elevated gradually with passage of time, while declined with different Cd treatments in growth medium (Fig. 6a, b, c, d). High Cd levels disturbed the stored protein hydrolysis and resulted in reduced amino acids pool (Mihoub *et al*., 2005; Rahoui *et al*., 2010). The results presented here indicated that although chelators played their role in relieving Cd stress but significant decrease in protein hydrolysis and its conversion into amino acids under Cd stress was the direct outcome of Cd induced suppression of protease activity. Activation of ribonucleolytic and proteolytic enzymes is also essential for seedling development (Rahman *et al*., 2008). They participate in seed germination process by regulating RNA turnover and mobilization of food from storage tissues in seeds (Wang *et al*., 2007). RNases and proteases activity is essential for breakdown of protein, their recycling and amino acids mobilization towards the growing embryo in seeds (Palma *et al*., 2002; Yamauchi, 2003). Disturbed activity of both these enzymes under metal stress resulted in altered protein and amino acids levels in germinating seeds (Maheshwari and Dubey, 2008). The two chelators- natural (Oxalic acid) and synthetic (EDTA) relieve the stress on α-amylase and protease activity by subdue the availability of Cd ions in growth medium, consequently the activity of both the enzymes was not severely affected (Zeid, 2001).

Conclusion

It is concluded that the seed germination is noticeably reduced by Cd toxicity due to reduced activities of key hydrolyzing enzymes α-amylase and protease. EDTA and OA application alone caused slight reduction in seed germination and on enzymes activity as compared to control exhibit the protective effects of both chelators against Cd noxiousness that might be credited to the reticence/curtailment of Cd uptake in seeds. The EDTA and OA addition in Cd contaminated growth medium upgraded these parameters proving the stress ameliorating potential of chelators to Cd toxicity. However OA proved more effective than EDTA, therefore, its application is suggested for Cd contaminated mediums/soils.

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