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ARTICLE

Effect of cadmium stress on seed germination, plant growth and hydrolyzing enzymes activities in mungbean seedlings

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ABSTRACT: Both protein and starch hydrolysis contribute to the effective mobilization of seed reserves to trigger seed germination. We studied the activities of hydrolytic enzymes in germinating seeds of mung bean (*Vigna radiata*) exposed to cadmium stress at 0, 25, 50, 75, 100 mg.L^{-1}. Exposure to Cd stress decreased the seed germination rate and early seedling growth traits, including root and shoot length and plant fresh and dry biomass compared to control. The adverse effects of Cd were more prominent in terms of shoot length than root length. Seedling α-amylase and protease activities exhibited significant reductions with increasing Cd dose. We also recorded Cd-induced reduction in total soluble proteins and sugars while the concentration of free amino acids exhibited an increase. Above all, a decrease in the hydrolysis process of reserve carbohydrates, proteins and impaired reserve translocation contributed to the inhibition of seedlings' germination and early growth traits.

Index terms: α-amylase, cadmium stress, hydrolyzing enzymes, protease.

RESUMO: Tanto a hidrólise de proteínas quanto de amido contribui para a mobilização eficaz das reservas para desencadear a germinação das sementes. Estudamos a atividade de enzimas hidrolíticas na germinação de sementes de feijão mungo (*Vigna radiata*) expostas ao estresse de cádmio a 0, 25, 50, 75, 100 mg.L^{-1}. A exposição ao estresse por Cd diminuiu a taxa de germinação das sementes e as características de crescimento inicial das plântulas, incluindo comprimento de raiz e parte aérea e biomassa fresca e seca da planta em comparação com o controle. Os efeitos adversos do Cd foram mais evidentes no comprimento do caule do que no comprimento da raiz. As atividades de α-amilase e protease de plântulas exibiram reduções significativas com o aumento da dose de Cd. Também observamos redução induzida por Cd em proteínas solúveis totais e açúcares, enquanto a concentração de aminoácidos livres exibiu um aumento. Em síntese, uma diminuição no processo de hidrólise de carboidratos de reserva, proteínas e translocação de reserva prejudicada contribuiu para a inibição da germinação das mudas e características de crescimento inicial.

Termos para indexação: α-amylase, estresse por cádmio, enzimas hidrolíticas, protease.

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INTRODUCTION

In cereals, endosperm development and its physiological roles have been well documented. Initially, the embryo scutellum synthesizes and secrete gibberellin, a germination-promoting hormone, into the aleurone layer of the endosperm. It ultimately enhances the synthesis of hydrolytic enzymes in the aleurone layer (Yan et al., 2014). Hydrolytic enzymes such as α-amylase, protease, and acid phosphatases accelerate the process of seed germination and seedlings' establishment. However, the presence of heavy metals leads to a reduction in the activities of these enzymes in barley seeds (Kalai et al., 2014; Sanal et al., 2014).

Cadmium (Cd) is well known to constrain seed germination rates via different mechanisms such as impairment of water uptake in seeds which ultimately limits the water availability for embryo development (Vijayaragavan et al., 2011). However, inadequate water supply is not the only constraint for the germination phase. Inhibition of starch mobilization is another process in endosperm affected by Cd, which causes impaired translocation of sugars towards the developing embryonic axis and ultimately results in starvation of the developing embryo (Kuriakose and Prasad, 2008). In *Sorghum bicolor* seeds, reduction in hydrolyzing enzyme activities such as in acid phosphatase, α-amylase, and protease has been reported under Cd stress which suggests a reduction in the storage mobilization process. Furthermore, a reduction of α-amylase activity has been observed, which reduced starch release from cotyledons (Kalai et al., 2016). He et al. (2010) further clarified that, as Ca is a vital component for α-amylase activity; therefore, substituting Ca with Cd ion could interrupt amylase functioning. Cd stress also induces the accumulation of malondialdehyde contents in some cereals such as *Oryza sativa* and *Triticum aestivum*, which indicates membrane damage (Qin et al., 2020). The leakage of amino acids and soluble sugars due to loss of membrane integrity is another indication of alteration in the remobilization process observed in seeds of *Vicia faba* and *Pisum sativum* (Rahoui et al., 2010b; Basahi, 2021).

Mungbean (*Vigna radiata* L.) is an important legume species that contributes a special part to the human diet. Some available reports showed the sensitivity of *V. radiata* towards Cd stress, as Cd application decreases the root and shoot growth, leaf area, and dry biomass content of mungbean as well as cause genotoxicity and oxidative stress in mungbean seedlings (Hassan et al., 2021). Further, Cd stress disturbs the water status in leaves, stomatal regulation, nitrogen-fixing ability, and abscisic acid content in mungbean (Ghani, 2010). Therefore, in this study, we investigated the effect of cadmium stress on seed germination, biomass, metabolites, and activities of some hydrolyzing enzymes such as α-amylase and protease were investigated.

MATERIALS AND METHODS

Plant material and stress treatments

The experiment was carried out in small plastic pots (12 cm diameter) containing sand. The pots were placed in the wire house of the Nuclear Institute for Agriculture and Biology Faisalabad, during winter 2018 under natural conditions. Ten seeds of the mungbean (*Vigna radiata* L.) cultivar (NM-11) were sown in each pot, and different concentrations of Cd (0, 25, 50, 75, and 100 $mg.L^{-1}$) were applied using anhydrous $CdCl_2$. Each treatment has three replications. The five germinating seeds were removed after 24, 48, 72, 96, and 120 h of Cd treatments from each pot to determine enzyme activities, reducing, non-reducing sugars, total soluble protein, and total free amino acids. While the rest of the seedlings were harvested after two weeks of treatment for the determination of growth attributes such as root lengths (cm), shoot lengths (cm), fresh weight/plant (g), and dry weight/plant (g) of root and shoot.

Determination of α-amylase activity

The seedlings material (1 g), frozen in liquid nitrogen, was homogenized in 10 mL phosphate buffer (pH 7.2), extracted with cold 1% NaCl, and centrifuged at 4000 rpm for 30 minutes. The supernatant was used to determine the enzyme activity according to the method reported by Chrispeels and Varner (1967). The enzyme activity was calculated as mg of starch hydrolyzed g^{-1}.fresh weight.h^{-1}.

Determination of protease activity

Protease activity was determined as described by Ainouz (1970). For this purpose, 1 g seedlings were homogenized and extracted with chilled 1% NaCl in 0.2 M phosphate buffer (pH 7.5), centrifuged at 12000 rpm for 30 minutes. 1 mL of supernatant and 5 mL of 1% casein solution were incubated at 50 °C in 0.2 M sodium phosphate buffer (pH 6.0). For termination of the reaction, 1 mL of 40% TCA was added after 60 minutes (Ainouz, 1970). The proteolytic activity was measured at 570 nm in TCA soluble fraction after reaction with the Folin Phenol reagent (Lowry et al., 1951).

Estimation of reducing and non-reducing sugars

For the estimation of sugars, one gram of seedlings was ground in 10 mL of 80% ethanol, filtered, and used for estimation of total sugars, reducing sugars, and non-reducing sugars. Total soluble sugars were estimated by the phenol-sulphuric acid method using glucose as standard (Dubois et al., 1956). Reducing sugars were estimated by 3,5-dinitrosalicylic acid method (DNSA) using glucose as standard (Miller, 1959) with a slight modification (Krivorotova and Sereikaite, 2014). DNSA reagent was prepared by dissolving 1 g of DNSA and 30 g of sodium-potassium tartaric acid in 80 mL of 0.5 N NaOH at 45 °C. After dissolution, the solution was cooled down to room temperature and diluted to 100 mL with the help of distilled water. For the measurement, 1 mL of plant extract was mixed with 2 mL of DNSA reagent in a test tube and kept at 95 °C for 5 min. After cooling, 7 mL of distilled water was added to the solution and the absorbance was measured at 540 nm using a UV-VIS spectrophotometer. The reducing sugar content was calculated from the calibration curve of D-glucose, and the results were expressed as mg D-glucose equivalent per gram fresh weight. Non-reducing sugars were expressed as difference between total sugars and reducing sugars.

Proteins and free amino acids analysis

Total soluble proteins were determined using the method of Lowry et al. (1951). Seedlings (1.0 g) were ground in 10 mL of 0.2 M phosphate buffer (pH 7.0) and filtered via nylon cloth. The plant filtrate was precipitated with 10% TCA in equal volume, centrifuged at 10,000 rpm for 5 minutes. The protein was assessed after resuspension of the pellet with 0.1 mol NaOH dm^{-3}. Total proteins (mg/g) were calculated using the standard curve of Bovine Serum Albumin (BSA) solution (Bradford, 1976). Total free amino acids were determined by following the ninhydrin method described by Hamilton et al (1943). For this purpose, 1 mL plant extract was mixed with 1 mL of 10% and 1 mL of 2% ninhydrin solutions. The OD was read at 570 nm using a spectrophotometer and values of amino acids (mg/g fresh weight) were calculated using the standard curve of Leucine solution.

Statistical analysis

The data were statistically analyzed using the analysis of variance (ANOVA). The Fisher’s Least Significant Difference test at a 5% probability level was used to assess the differences among significant means. The associations between variables were determined by computing Pearson correlation coefficients using the XLSTAT add-in of Microsoft Excel.

RESULTS AND DISCUSSION

Effect of cadmium stress on germination and morphological attributes

Cadmium stress significantly decreased all the growth (germination rate, root and shoot length, fresh mass, dry mass) parameters (Table 1). It was observed that the highest supplementation of Cd (100 mg.L^{-1}) caused maximum damage to plants and decreased the germination percentage (27%), shoot length (63%), and root length (27%) as compared to control (0 mg.L^{-1}) plants. Further, 100 mg Cd.L^{-1} application displayed a significant decrease in shoot and

Table 1. The effect of Cd and time of application of Cd on the germination rate and morphological traits of germinating seedlings of *Vigna radiata.*

$CdCl_2$	Germination rate (%)	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
0 mg.L^{-1}	93.0 a	8.53 a	11.65 a	0.63 a	0.36 a	0.46 a	0.09 a
25 mg.L^{-1}	79.0 ab	4.02 b	8.82 ab	0.63 a	0.33 ab	0.30 b	0.06 b
50 mg.L^{-1}	75.0 b	3.64 c	9.43 b	0.55 b	0.29 b	0.28 b	0.05 b
75 mg.L^{-1}	70.0 c	3.55 c	9.92 c	0.47 b	0.18 c	0.25 b	0.02 c
100 mg.L^{-1}	67.1 c	3.09 d	8.39 c	0.45 c	0.14 c	0.22 b	0.01 d

root fresh mass by 28% and 61%, respectively, compared to non-treated plants. Root and shoot dry biomass also exhibited the same pattern and showed a 2-fold and 9-fold decline, respectively, under Cd treatment (100 mg.L^{-1}) than control. The reduction in germination rates can be described by a decrease in metabolic and physiological activities such as a reduced movement of water and reserves towards the embryonic axis (Vijayaragavan et al., 2011). Further, Rahoui et al. (2010a) reported delays in germination when the seeds of pea and faba beans were contaminated with Cd due to membrane damage and solutes leakage.

Shoot and root lengths are important elements for any plant to transport nutrients and water. The results included in Table 1 show that both shoot and root length of mungbean seedlings were adversely affected by Cd treatment compared to non-stressed plants. However, data showed that shoot length (52%-63% decrease) was affected more than root growth (14% to 24% decrease) under the Cd stress. Intriguingly, Cd stress at 25 mg.L^{-1} decreased the root length by 24%, while a 19% decrease in root length was observed at 50 mg.L^{-1} and 75 mg.L^{-1} Cd treatments. Thus, it shows that root length showing some degree of tolerance to Cd treatments. Due to the mobile nature, Cd enters into plants through roots and is further translocated towards shoots via different transporters of xylem and phloem in an ionic form. Both apoplastic and symplastic transport pathways are used for the entry of Cd into the xylem, then it is loaded to vessel elements or tracheids of the stele and ultimately translocated to the shoot (Dong et al., 2019). It indicates that the sensitivity of growth parameters to Cd stress varied with species, genotypes, and cultivars. Further, a significant decline in fresh and dry biomass of mungbean seedlings was observed under increasing Cd concentrations. These growth losses might be due to impairment of PSII activity, inhibition of cell division as well as direct or indirect inhibition of different physiological processes like photosynthesis, respiration, and plant-water relation, which ultimately renders the activity of cell and leads to poor growth and plant biomass as reported by (Rahoui et al., 2010a; Vijayaragavan et al., 2011; Irfan et al., 2014).

Effect of cadmium stress on α-amylase activity and sugar contents

Cadmium stress had a pronounced effect on the activity of α-amylase and other associated changes (sugar contents) in mungbean (Figure 1a). Cadmium contamination significantly reduced the α-amylase activity of mungbean seeds during the germination process. The maximum reduction in α-amylase was observed at 100 mg Cd.L^{-1}. The α-amylase activity was decreased by 75, 419, 122, 204, and 121% as compared to their control at 24, 48, 72, 96, and 120 h respectively. At 25, 50, and 75 mg Cd.L^{-1} stress the maximum reduction in α-amylase activity was observed at 96 h after stress, while at 100 mg Cd.L^{-1} was at 48 h of stress. The time duration influenced the α-amylase activity and the highest activity was observed at 120 hours while the lowest at 24 hours under control (0 mg.L^{-1}) at different concentrations of Cd stress. The statistical analyses indicated that the Cd treatment and time duration interaction was non-significant.

The α-amylase synthesized de novo during the germination process and its activity plays a major role in the starch degradation process during the germination process in cereal grains. We observed a significant reduction in α-amylase activity in Cd-treated mungbean seedlings. Similarly, Kuriakose and Prasad (2008) also reported a considerable decreased in α-amylase activity in embryonic axes and seeds of *Sorghum bicolor* under Cd (0.5 mM) stress. However, α-amylase activity showed an increasing pattern with time and showed maximum values after 120 h. Similar effects of Cd stress on seedling growth and seed amylase activities were reported in rice (He et al., 2008). Reduction in α-amylase activity disturbs the process of starch breakdown which ultimately decreases germination and growth (Kaur et al., 2001). Secondly, amylase activity requires calcium ions, therefore reduce α-amylase activity might be due to the low availability of calcium ions under stress conditions (Liao et al., 2019).

Furthermore, the contents of reducing and non-reducing sugars also followed the trend of a-amylase activity (Figures 1b and c). The levels of sugars increased gradually with time but sugar concentrations declined under increased concentrations of Cd stress. Specifically, under the lower concentration of Cd stress (25 mg.L^{-1}), reducing sugar contents increased by 17%, 20%, and 29% after 72, 96, and 120 h, respectively, whereas, non-reducing sugar levels increased by 8% and 9% after 96 and 120 h, respectively, as compared to their respective controls. The statistical analyses indicated that the Cd treatment and time duration interaction for both reducing and non-reducing sugars was significant. As

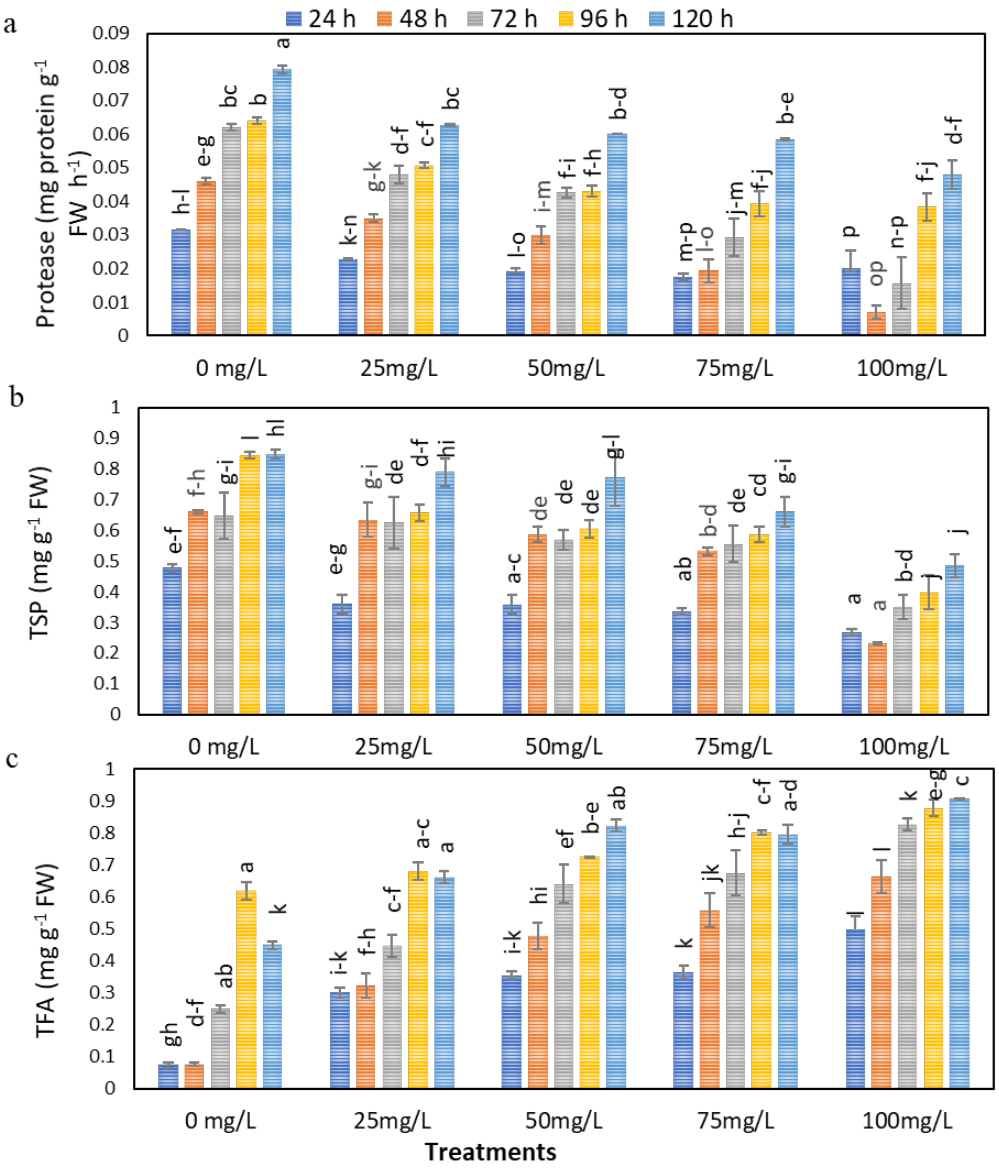

Figure 1. The effect of Cd and time of application of Cd on the a) protease activity, b) total soluble protein, and c) total free amino acid contents of germinating seedlings of *Vigna radiata*. Each bar indicates means of 3 replicates ± standard error. Different alphabets on bars show non-significant differences among treatments.

α-amylase activity was not fully inhibited under Cd stress conditions, therefore α-amylase might have continued to degrade starch into soluble sugars (reducing & non-reducing) which further accumulated in the germinating seedlings due to impaired translocation. Our results strengthen this point as Cd stress decreased the levels of both reducing and non-reducing sugars significantly, but an increasing trend was observed with an increase in time duration and maximum values of soluble sugars were observed at 24 h. Kuriakose and Prasad (2008) also reported that Cd toxicity in germinating seedlings of *Sorghum bicolor* impaired hydrolyzing enzyme activities and inserted a negative effect on the translocation of hydrolysis products towards the growing embryonic axis.

Effect of cadmium stress on protease activity

As depicted in Figure 2a, the proteolytic activity of mungbean seedlings significantly increased with time and the highest was observed at 120 h after sowing under all cadmium treatments but declined by increasing concentrations of Cd stress. The highest protease activity was observed in seedlings grown under normal conditions (0 mg.L^{-1}). Whereas,

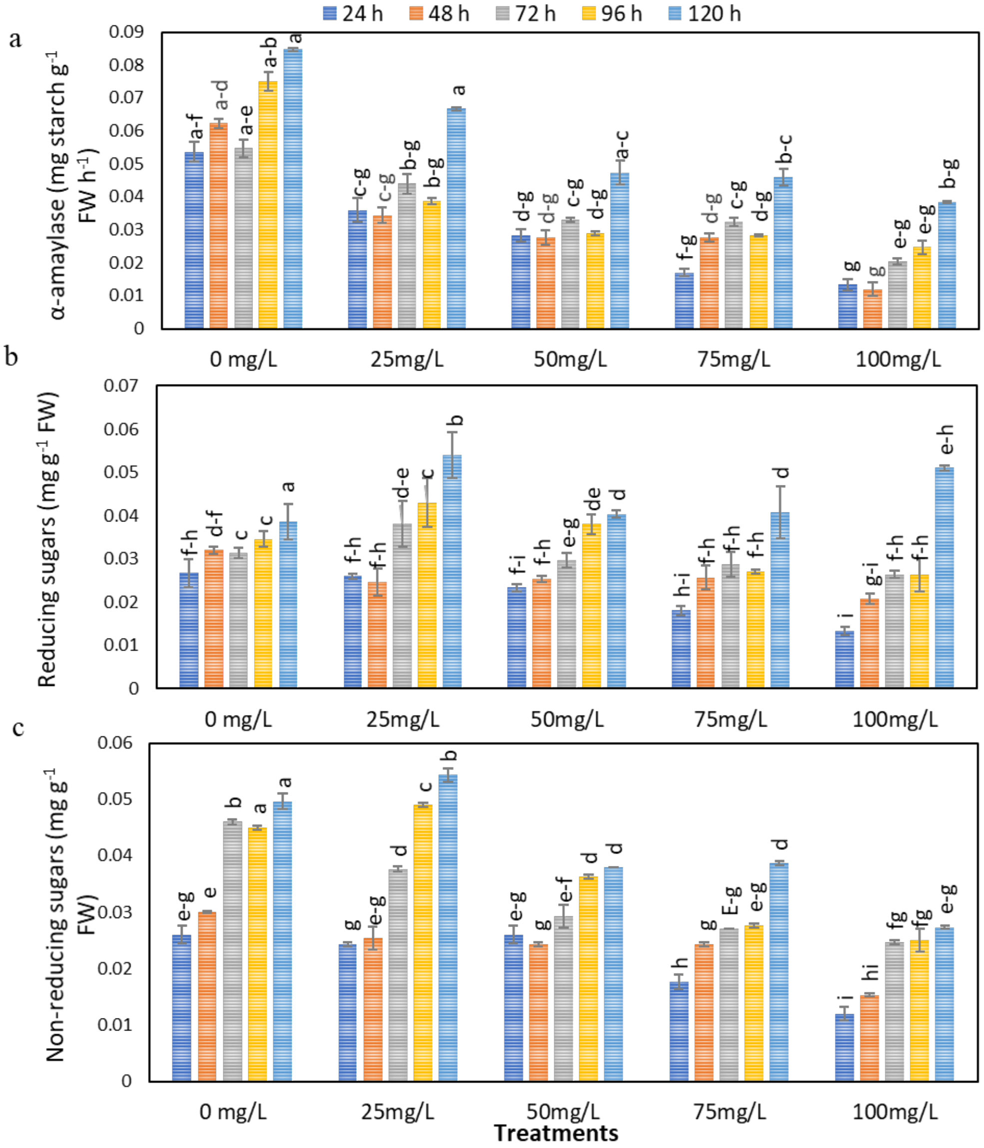

Figure 2. The effect of Cd and time of application of Cd on the a) amylase activity, b) reducing sugars, and c) total non-reducing sugars of germinating seedlings of *Vigna radiata*. Each bar indicate means of 3 replicates ± standard error. Different alphabets on bars showed non-significant difference among treatments.

maximum decline (84%) was observed under 100 mg.L^{-1} Cd stress after 48 has compared to control. It was also noted that at 25 mg.L^{-1} and 50 mg.L^{-1} Cd treatment, a sharp decrease in protease activity was observed at the start of germination, such as 29% and 38% decline under 25 mg.L^{-1} and 50 mg.L^{-1} Cd treatment respectively at 24 h as well as 23% and 34% decline at 48 h. However, after 48 h the protease activity started to appear stable.

Soluble protein content

Total soluble protein content was significantly influenced by Cd contamination (Figure 2b). The maximum decline (65%) in protein contents was observed under 100 mg.L^{-1} Cd exposure for 48 h. On the contrary, higher values of soluble proteins were observed in mungbean seedlings grown under control conditions (0 mg.L^{-1}). Furthermore, the total soluble protein content was significantly influenced by the duration of germination as protein contents increased with the passage of time and observed maximum after 120 h of sowing. It is also noted that under 25 mg.L^{-1} and 50 mg.L^{-1} Cd treatments, the protein content of seedlings increased rapidly after 96 h such as 17% and 15% increase was noted as compared to protein content at 96 h. The metabolism of seed protein via proteolytic enzymes is a critical step during seed germination. It has been reported previously that activities of proteolytic enzymes mainly increase during earlier days of germination while protease inhibitors disappeared in this duration (Ali and Alozeiri, 2017). In this regard, reduced activities of hydrolyzing enzymes further hinder the mobilization of stored products. Different proteases are involved in developmental processes, senescence, and mobilization of reserve proteins (Kuriakose and Prasad, 2008). In this study, we observed a decrease in protease activity under increasing Cd concentration; however, an increasing trend was observed with the time of application of Cd stress as maximum protease activity was noted at 120 h under all Cd concentrations.

Effect of cadmium stress on total free amino acids

The results presented in Figure 2c revealed that the total free amino acid (TFA) contents displayed a significant increase upon exposure to Cd stress, whereas the most pronounced effect was observed at 100 mg.L^{-1} with 69%, 29%, and 46% increase after 72, 96, and 120 h respectively, as compared to control. Similarly, TFA content also displayed a gradual increase with time. The catalysis of seed proteins further releases free amino acids, which support the biosynthesis of other proteins in the seed endosperm and developing embryo that eventually leads towards germination. Our results also showed a similar trend as the protein levels decreased under Cd treatment while total free amino acids exhibited a significant increase with time after application of Cd stress. Vassilev and Lidon (2011) reported an increase in some specific amino acids such as aspartic acid, asparagine, proline, leucine, isoleucine, methionine, and valine in two barley cultivars under Cd stress conditions as compared to control. It might be because some amino acids play a significant role under heavy metal stress conditions, such as proline (Hayat et al., 2012), histidine, and cysteine (Ghori et al., 2019), so the higher accumulation of free amino acids in germinating seedlings is an indication of plant adaptation to stress conditions by stabilizing osmoregulation, metal chelation and signal transduction pathways.

Pearson's Correlation Coefficients

The correlation coefficients indicated a positive relation among growth traits, *i.e.*, shoot length, root length, fresh weight, and dry weight of shoots and roots (Figure 3). The activity of α-amylase was positively related, while protease activity was negatively related to all the growth traits. The activity of non-reducing sugar was positively related to fresh biomass while negatively related to protease and total soluble protein.

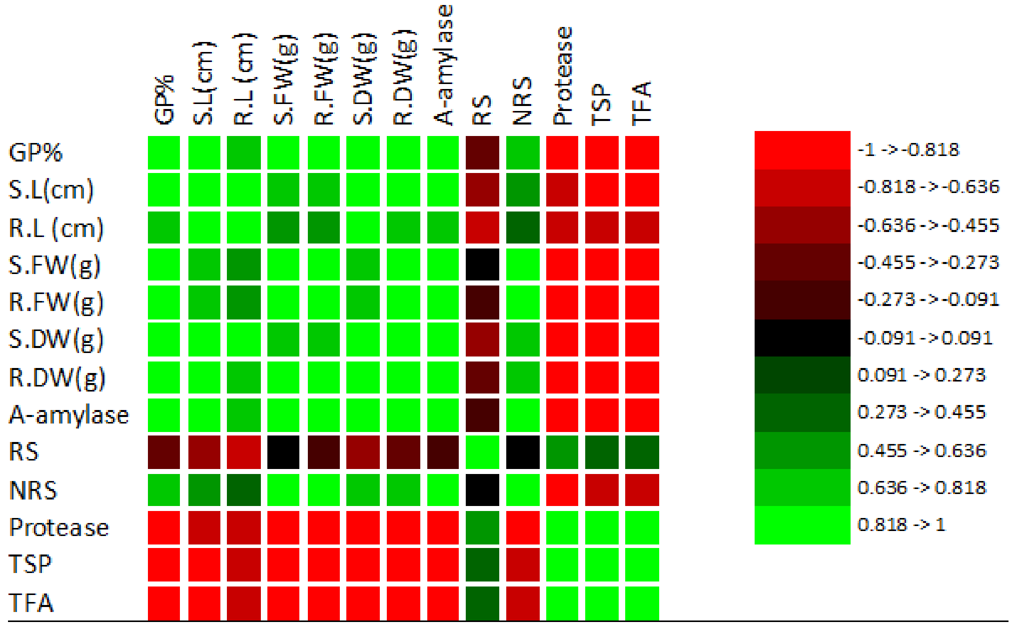

Figure 3. Pearson correlation between different variables of germinating seedlings of *Vigna radiata*. GP: germination percentage; RS: reducing sugars; NRS: non-reducing sugars; TSP: total soluble proteins; TFA: total free amino acids.

CONCLUSIONS

Cadmium stress decreased all the germination parameters and activities of hydrolytic enzymes. The highest Cd level (100 mg.L^{-1}) caused a decrease in germination percentage (27%), shoot length (63%), root length (27%), shoot fresh biomass (28%), root fresh biomass (61%), root dry biomass (2-fold), and shoot dry biomass (9-fold) compared to non-treated plants. In addition, total soluble sugars and protein levels declined, while free amino acid level increased under Cd stress. But the activities of starch hydrolyzing enzyme, total reducing and non-reducing sugar levels, total soluble protein contents, and free amino acids displayed a significant increase with time, but increase was less under Cd stress. These results showed that Cd inhibits hydrolytic enzymes and mobilization and translocation of hydrolyzed sugars to embryonic axis which ultimately inhibited seedling growth.

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