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Attenuation of cadmium induced oxidative stress in cucumber seedlings by modulating the photosynthesis and antioxidant machinery through foliar applied glutamic acid

Summeya Munawar[1], Muhammad Awais Ghani[1], Basharat Ali[2], Muhammad Azam[1], Muhammad Zahid Rashid[3], Romana Anjum[4], Muhammad Sarwar[2], Tanveer Ahmad[5], Anam Noor[6], Qumer Iqbal[7], Kaiser Latif Cheema[8], Muhammad Muzammil Jahangir[1], Jahanzeb Ahmad[1], Muhammad Mehran Abbas[1]*

[1]*Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan*
[2]*Department of Agronomy, University of Agriculture, Faisalabad, Pakistan*
[3]*Horticultural Research Institute, Ayub Agricultural Research Institute Faisalabad, Pakistan*
[4]*Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan*
[5]*Department of Horticulture, MNS University of Agriculture, Multan, Pakistan*
[6]*Department of Horticulture Sciences, Bahauddin Zakariya University, Multan, Pakistan*
[7]*Fiblast, LLC., Tuskegee, USA*
[8]*Pulses Research Institute, Ayub Agricultural Research Institute Faisalabad, Pakistan*

**Corresponding author: awais.ghani@uaf.edu.pk*

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Abstract: In recent decades, adverse effects of unexpected contaminants on the quality of crops have threatened both the food security and human health. Vegetable production in heavy metal contaminated soils is a serious concern regarding the quality of food. Glutamic acid has been extensively studied as a stress-responsive antioxidant molecule and its function is involved in triggering plant growth during abiotic stress. Therefore, in the present study, the alleviating role of exogenously applied glutamic acid was examined in soil grown cucumbers (*Cucumis sativus* L.) under four levels of cadmium (0, 5, 10, and 20 mg Cd/kg) and two levels of glutamic acid (0, 10 mM). The results showed that the Cd stress reduced the plant growth and chlorophyll contents in the cucumbers. Significant decreases were more pronounced in the photosynthetic parameters under the Cd stress alone in both cultivars. However, pronounced deleterious effects were observed in the Ashly cultivar as compared to SSC-228 in respect to the plant growth and photosynthetic attributes. However, the exogenous treatment of glutamic acid significantly improved the plant growth and chlorophyll contents of plants under the Cd stress. The glutamic acid also decreased the Cd contents in the cucumber roots and leaves, and further decreased the reactive oxygen species (ROS) which were elevated by the high Cd concentrations. Interestingly, the antioxidant enzyme activities (SOD and POD) increased under the different elevated Cd levels in the leaves of the cucumber plants. However, the CAT and APX activities were reduced with an increasing Cd concentration in the soil in both cultivars. Meanwhile, the exogenously applied glutamic acid exhibited synergic effects and further activated the antioxidant enzyme activities in the cucumber leaves under the Cd stress. In this study, the SSC-228 cultivar was found to be more tolerant to Cd stress as compared to the Ashly cultivar. Furthermore, the findings of this study highlight that a glutamic acid application can play a significant role in enhancing the plant growth and stimulating the biochemical activities in cucumbers under Cd stress.

Keywords: heavy metal; vegetables; signalling molecule; plant growth; reactive oxygen species

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Cadmium is considered a harmful pollutant element that enters the atmosphere from different industrial practices, municipal activities and agricultural activities as well as from manure, sewage sludge and phosphate fertilisers (di-Toppi, Gabbrielli 1999; Liu et al. 2007; Meng et al. 2009). In heavy metals, Cd is a non–vital element, highly toxic for animals, humans and plants. It is in the 7th position among the top twenty toxins that affect human health by inclusion into the food chain (Chen et al. 2006; Saidi et al. 2014). Its toxicity induces several changes in physiological, ultrastructural and biochemical activities. The main symptoms are leaf chlorosis, growth reduction in roots and vines (Zhang et al. 2015).

Plants absorb Cd easily and accumulate it in various portions of plants, such as the edible leaves, fruits and seeds (Fang et al. 2014). Cadmium decreases the photosynthesis and transpiration rate of leaves (Liu et al. 2011) and inhibits the mineral uptake (Ouaritie et al. 1997; Ali et al. 2014). Cd toxicity causes lipid peroxidation and also changes the antioxidant activities (Ali et al. 2015a, b). It stimulates the addition of reactive oxygen species (ROS), causing the severe destruction of several cell components, such as lipids, proteins, DNA, and RNA (Foyer et al. 1994). Plants have an antioxidant system to protect itself against cell and tissue damage (enzymatic detoxification and non-enzymatic systems). Many techniques have been stated to diminish the Cd effects in plants, e.g., the exogenous application of substances such as amino acids, nutrient elements and plant growth regulators (Ahmed et al. 2016). Glutamate involves as receptors in stress condition and development of plant (Roy et al. 2008). Use of glutamic acid on wheat enhanced the seed number per spike, seed weight and spike quantity and dry substance production (Shih, Van 2001; Chang et al. 2010).

The cucumber (*Cucumis sativus* L.) belongs to the Cucurbitaceae family which has 825 species and 118 genera (Khan et al. 2015). The cucumber was used in the present study because it measures the environmental pollution in the soil polluted through external contaminants. Inadequate information is present about the lethal effects of Cd in this species (Pereira et al. 2006; Lin et al. 2012). Moreover, to our knowledge, there have not been any studies describing the ameliorating role of glutamic acid under Cd stress in cucumber cultivars. Therefore, in the current investigation, we examined the plant growth, Cd uptake and fluctuations in the biochemical activities, with an exogenous application of glutamic acid on the cucumber with a combination of different Cd levels.

MATERIAL AND METHODS

Plant materials. The experiment was conducted at the Institute of Horticultural Sciences, University of Agriculture Faisalabad, Pakistan. Two cucumber (*C. sativus* L.) cultivars 'V1 Ashly' and 'V2 SSC – 228' were purchased from a registered market. Before the experiment, different soil parameters were analysed, including the pH, electrical conductivity, and an element analysis was conducted. Four concentrations of cadmium chloride (0, 5, 10 and 20 mg/kg of soil) were selected in this study and applied in plastic pots filled with soil. For each treatment, three replications were maintained. Cadmium chloride was used and spiked in the soil one month before starting the experiment. The pot size was 19 cm in diameter and 28 cm in height. Each pot was filled with 6 kg of amended soil. A total of eight cucumber seeds were sown in each pot. As the cucumber is a vining plant, when the vine length reached the three to four leaf stage and the plants attained 3 to 6 inches in height, a foliar spray of "glutamic acid (10 mM)" was applied to the specified pots. At the flowering stage, harvesting was done. The morphological characteristics were immediately measured and samples were collected and preserved at –80 °C for the biochemical analysis.

Morphological parameters. Morphological observations were recorded after harvesting the plants and the plants were parted into the root, vine, and leaves. The length of the root, plant and leaves were measured with a scale. The fresh and dry weights of the cucumber plants were measured with an electronic weighing balance after the plants were harvested. After harvesting, the plants were kept in the sun to dry them. However, when the moisture level was lowered by up to 40% to 50%, the plant samples were placed into an oven at 65 °C. The dry plant samples were weighed until the biomass became stable instantly after removal from the oven (Momoh, Zhou 2001).

Determination of the Cd content. To determine the cadmium amount in the leaves and roots, the Hsu and Kao (2003) method was used. The tolerance index (TI) for the tested plants was deter-

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mined by using the formula: TI (%) = mean dry weight of the plant at 20 mg Cd/kg soil/mean dry weight of the plant at 0 mg Cd/kg soil × 100.

Photosynthetic pigment and Total soluble protein determination. The chlorophyll *a*, *b* (Chl *a*, *b*) and carotenoid contents were determined according to the Arnon (1949) method. To determine the total soluble protein (TSP) concentration, the Bradford (1976) protocol was followed.

Antioxidant enzyme activity determination. The ascorbate peroxidase (APX) activity was determined by the oxidation of the ascorbic acid and its absorbance was checked at 290 nm with a spectrophotometer (Asada, Takahashi 1987). The superoxide dismutase (SOD) activity was evaluated by following the process of Ries and Giannopolitis (1977) by constraining the photochemical reduction of nitro blue tetrazolium. The Peroxidase (POD) activity was analysed by using the process of Britton and Maehly (1955). The catalase (CAT) activity was investigated by using the process of Britton and Maehly (1955) and the reduction in the absorbance due to H_2O_2 was checked at 270 nm on the spectrophotometer.

Determination of the hydrogen peroxide and malondialdehyde (MDA) contents. The H_2O_2 content was determined by the technique of Velikova et al. (2000) and its absorbance was checked at 390 nm on the spectrophotometer. The lipid peroxidation of the membrane oxidative damage was evaluated by using the thiobarbituric acid (TBA) process to determine the MDA amount in the leaf tissue (Cakmak, Horst 1991).

Statistical analysis. All the treatments were arranged under completely randomised design under two factorials**.** The data were analysed with the mean and ± SD from three replicates. A two-way analysis of variance (ANOVA) was analysed with statistical software (version 8.1). Tukey's test was used to compare the significant means among the different treatments at a *P*-value of ≤ 0.05.

RESULTS

Effects of the glutamic acid on the cucumber growth under Cd stress. The cadmium (Cd) and glutamic acid effects on the plant fresh weight, root fresh weight, plant dry weight and root dry weight are shown in Table 1. The plants' exposure to Cd clearly decreased the plant fresh weight, fresh weight of the root and plant growth of the V1 cultivar (Ashly) when compared to the V2 cultivar (SSC-228). However, the decrease was more prominent in the plants with the addition of 20 mg Cd/kg in the V1 cultivar

Table 1. Effects of the different Cd and glutamic acid (Glu) treatments on the plant fresh weight, root fresh weight, plant dry weight and root dry weight of two cucumber cultivars

Treatment	Cultivars	Fresh plant weight (g)	Fresh root weight (g)	Plant dry weight (g)	Root dry weight (g)
Control	V1	31.27 ± 0.066^{e}	6.44 ± 0.063^{d}	15.747 ± 0.014^{e}	3.22 ± 0.031^{d}
	V2	33.71 ± 0.035^{b}	7.31 ± 0.003^{a}	16.021 ± 0.012^{b}	3.65 ± 0.001^{a}
10 mM Glu	V1	32.81 ± 0.008^{c}	6.94 ± 0.020^{b}	16.405 ± 0.004 5^{c}	3.47 ± 0.01^{b}
	V2	34.35 ± 0.037^{a}	7.45 ± 0.011^{a}	17.44 ± 0.271^{a}	3.72 ± 0.005^{a}
5 mg Cd	V1	27.96 ± 0.036^{h}	5.43 ± 0.012^{g}	14.235 ± 0.255^{h}	2.71 ± 0.006^{g}
	V2	31.14 ± 0.035^{e}	6.52 ± 0.008^{d}	15.34 ± 0.019^{f}	3.26 ± 0.004^{d}
10 mg Cd	V1	25.01 ± 0.021^{j}	4.47 ± 0.011^{j}	12.50 ± 0.008^{j}	2.23 ± 0.005^{j}
	V2	28.11 ± 0.038^{h}	5.37 ± 0.007^{g}	14.3 ± 0.242^{h}	2.68 ± 0.004^{g}
20 mg Cd	V1	21.78 ± 0.037^{n}	3.56 ± 0.026^{l}	10.876 ± 0.020^{n}	1.78 ± 0.013^{l}
	V2	23.74 ± 0.068^{l}	4.73 ± 0.056^{i}	11.863 ± 0.020^{l}	2.36 ± 0.028^{i}
5 mg Cd + 10 Glu	V1	29.48 ± 0.132^{g}	5.92 ± 0.005^{f}	14.725 ± 0.050^{g}	2.96 ± 0.002^{f}
	V2	32.02 ± 0.015^{d}	6.74 ± 0.020^{c}	16.021 ± 0.012 4^{d}	3.37 ± 0.001^{c}
10 mg Cd + 10 Glu	V1	27.30 ± 0.026^{i}	5.13 ± 0.006^{h}	13.665 ± 0.014^{i}	2.56 ± 0.002^{h}
	V2	30.38 ± 0.017^{f}	6.23 ± 0.005^{e}	15.206 ± 0.017^{f}	3.11 ± 0.002^{e}
20 mg Cd + 10 Glu	V1	22.58 ± 0.084^{m}	4.04 ± 0.021^{k}	11.288 ± 0.029^{m}	2.02 ± 0.010^{k}
	V2	24.43 ± 0.281^{k}	5.06 ± 0.076^{h}	12.426 ± 0.230^{k}	2.53 ± 0.038^{h}

$^{a-n}$Different lower-case letters are representing the significant difference between treatments by Tukey's test ($P \leq 0.05$)
Each data values are represented as means and ± SD of four replications

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when compared to the V2 cultivar. The exogenous application of glutamic acid significantly enhanced the plant fresh biomass under the Cd stress conditions. The Cd stress under the various concentrations only decreased the dry weight of the different plant parts of both cultivars, but a larger reduction occurred in the V1 cultivar at 20 mg Cd/kg (Table 1). The foliar application significantly enhanced the dry weights in different parts of the plants and the maximum weight was found at 5 mg Cd/kg in the V2 cultivar when compared to the V1 cultivar (Table 1). The effects of the different Cd and glutamic acid treatments on the growth attributes, such as the root length and height of the plant, are presented in Table 2. The plants exposed to the Cd stress had a decreased plant growth, plant height and root length when compared to their respective controls. However, a significant reduction was more prominent in the V1 plants treated with the 20 mg Cd/kg soil. The exogenous application of glutamic acid significantly enhanced the plant growth in the V1 and V2 cucumbers. The glutamic acid maximally enhanced the leaf length and leaf width, but more pronounced ameliorating effects were observed in the V2 cucumber at 5 mg Cd/kg (Table 2).

SPAD index. The effects of the various Cd and glutamic acid concentrations on the chlorophyll aspects are highlighted in Figure 1. The cadmium addition alone significantly reduced the Chl *a*, *b* and carotenoids and total chlorophyll concentration in the V1 cultivar when compared to both cultivars at 20 mg Cd/kg (Figure 1). The foliar glutamic acid application alone significantly improved the chlorophyll content in the V1 and V2 cucumbers when compared to their relevant controls. The higher values were detected at 5 mg Cd/kg with the exogenous glutamic acid application in the V2 cultivar when compared to V1 (Figure 1).

Cd concentration in the plant parts. A significant linear proportional amount of Cd was found in the cucumber leaves and roots with an increasing amount of Cd in the soil (Figure 2). The maximum Cd content was found in the leaves and roots of the V1 cultivar, but less in the V2 cultivar in all the Cd treatments. The exogenous glutamic acid application significantly decreased the Cd content in the leaves and root (Figure 2A, 2B). The tolerance index of both cultivars under different Cd stress concentrations with the exogenous glutamic acid application is presented in Figure 2C. The SSC-228 cultivar showed a higher tolerance index at 20 mg Cd/kg with the exogenous glutamic acid application when compared to the V1 cultivar.

Table 2. Effects of the different Cd and glutamic (Glu) acid treatments of on the plant height, root length, leaf length and leaf width of two cucumber cultivars

Treatment	Cultivars	Plant height (cm)	Root length (cm)	Leaf length (cm)	Leaf width (cm)
Control	V1	37.26 ± 1.674^{bcd}	6.3 ± 0.057^{d}	13.45 ± 0.017^{e}	7.886 7 ± 0.008 8^{e}
	V2	41.6 ± 1.222^{ab}	7.13 ± 0.072^{b}	14.195 ± 0.016^{b}	8.350 0 ± 0.01^{b}
10 mM Glu	V1	40.43 ± 0.463^{bc}	6.7 ± 0.047^{c}	13.94 ± 0.006^{c}	8.197 5 ± 0.003 3^{c}
	V2	47.83 ± 1.359^{a}	7.8 ± 0.047^{a}	14.465 ± 0.013^{a}	8.493 3 ± 0.006 6^{a}
5 mg Cd	V1	32.93 ± 1.462^{defgh}	5.3 ± 0.057^{f}	12.26 ± 0.012^{j}	7.207 ± 0.006 6^{h}
	V2	39.2 ± 1.135 7^{bcd}	6.3 ± 0.047^{d}	13.312 ± 0.013^{e}	7.823 ± 0.008 8^{e}
10 mg Cd	V1	30.46 ± 0.638^{fghi}	4.30 ± 0.057^{h}	11.27 ± 0.006^{k}	6.625 ± 0.003 3^{k}
	V2	33.96 ± 1.894^{cdefgh}	5.3 ± 0.047^{f}	12.37 ± 0.015^{h}	7.270 ± 0.01^{h}
20 mg Cd	V1	23.83 ± 1.257^{i}	3.66 ± 0.088^{i}	10.110 ± 0.008^{m}	5.940 ± 0.005 7^{m}
	V2	28.76 ± 1.072^{ghi}	4.8 ± 0.047^{g}	11.48 ± 0.023^{j}	6.750 ± 0.015^{j}
5 mg Cd + 10 Glu	V1	37.13 ± 1.033 3^{bcdef}	5.8 ± 0.057^{e}	12.783 ± 0.063^{g}	7.513 ± 0.038^{g}
	V2	41.03 ± 0.938^{b}	6.85 ± 0.047^{bc}	13.647 ± 0.005^{d}	8.043 ± 0.023^{d}
10 mg Cd + 10 Glu	V1	33.03 ± 0.952^{defgh}	4.76 ± 0.088^{g}	12.088 ± 0.011^{i}	7.106 7 ± 0.006 6^{i}
	V2	35.43 ± 2.098^{bcdefgh}	5.7 ± 0.047^{e}	13.075 ± 0.005^{f}	7.686 ± 0.003 3^{f}
20 mg Cd + 10 Glu	V1	28.1 ± 1.352 7^{hi}	4.11 ± 0.057^{h}	10.983 ± 0.014 2^{l}	6.45 ± 0.008 8^{m}
	V2	32 ± 0.731 1^{efgh}	5.11 ± 0.047^{fg}	11.985 ± 0.039^{l}	7.04 ± 0.023^{i}

$^{a–m}$Different lower-case letters are representing the significant difference between treatments by Tukey's test ($P \leq 0.05$)
Each data values are represented as means and ± SD of four replications

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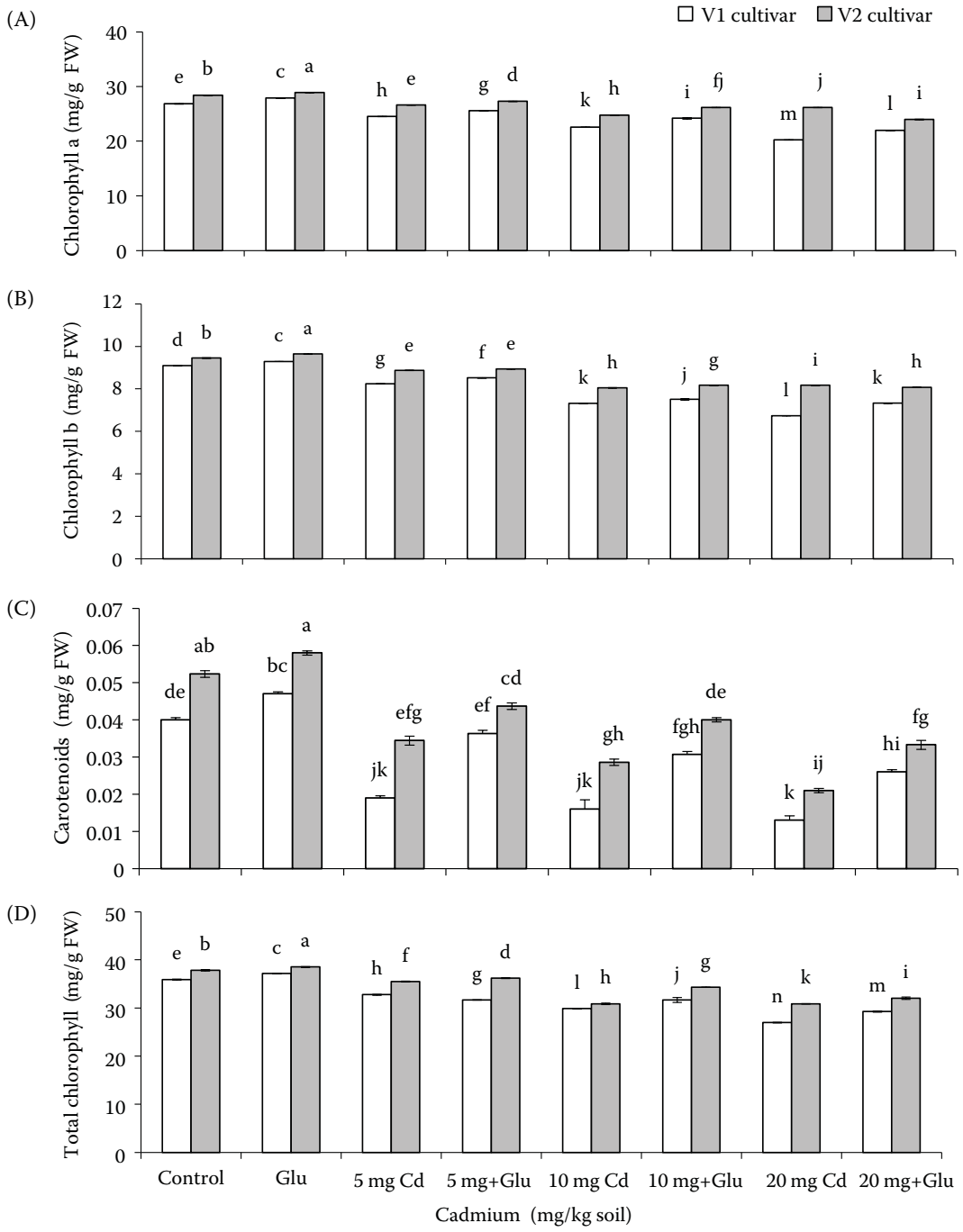

Figure 1. Effects of the glutamic acid (0, 10 mM) and cadmium (0, 5, 10, 20 mg/kg) treatments on the (A) chlorophyll *a*, (B) chlorophyll *b*, (C) carotenoids, (D) total chlorophyll in *Cucumis sativus* L.

Antioxidant enzymatic activities. The antioxidant enzyme activities (POD, SOD, APX and CAT) were affected by the various Cd and glutamic acid treatments of two cucumber cultivars (Figure 3). The results revealed that the SOD and POD enzyme activities were enhanced in the leaves of both cucumber cultivars under the different elevated levels of Cd alone (Figure 3A, 3B). A higher amount of Cd in the soil also significantly increased the SOD and POD contents in the leaves of both cucumber cultivars. The SOD and POD contents were higher in the V1 cultivar when compared to the V2 cultivar under the stress conditions. However, the glutamic acid application significantly decreased the Cd effect in the V2 cultivar when compared to the V1 cultivar. Furthermore, the CAT activity

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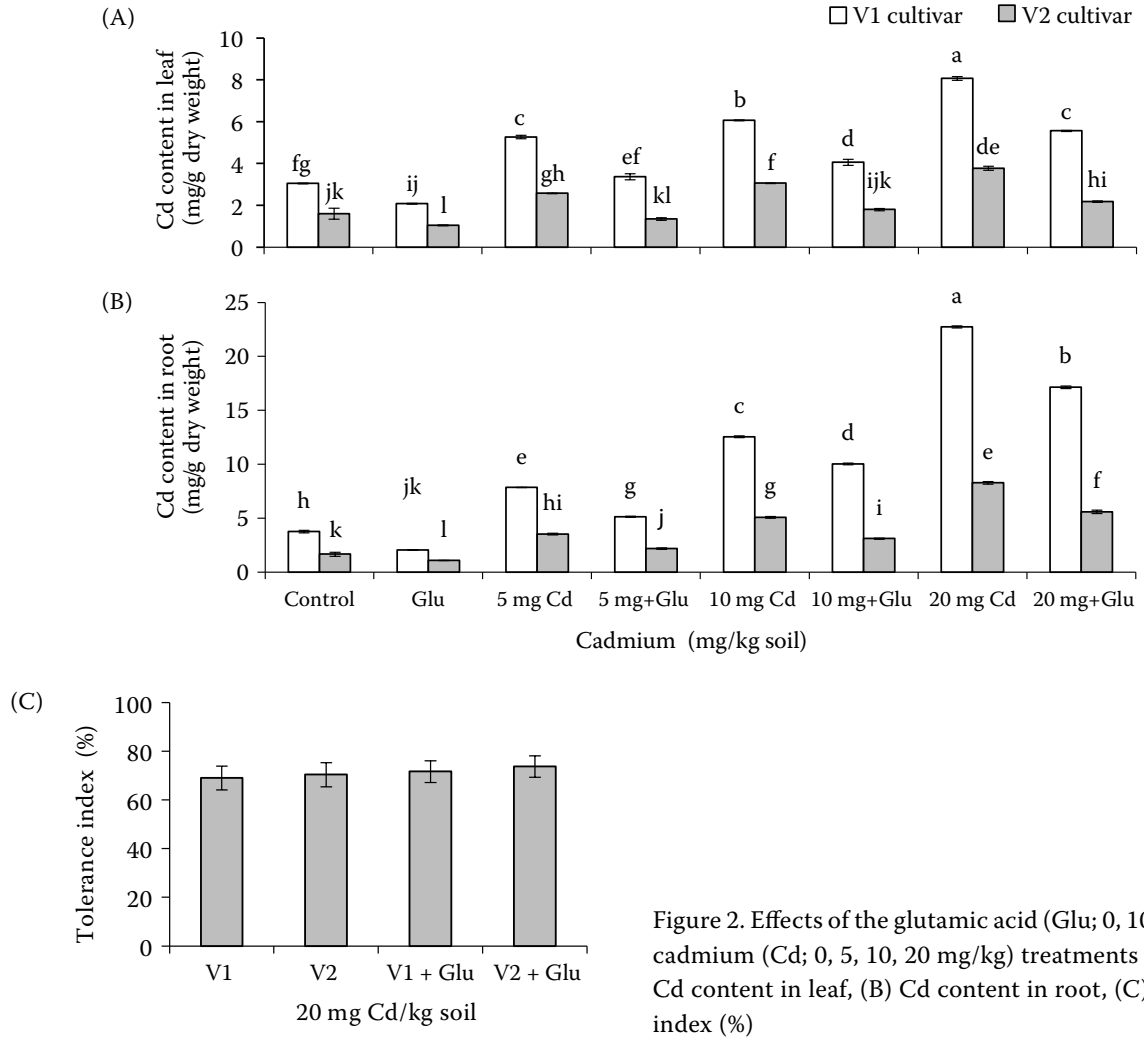

Figure 2. Effects of the glutamic acid (Glu; 0, 10 mM) and cadmium (Cd; 0, 5, 10, 20 mg/kg) treatments on the (A) Cd content in leaf, (B) Cd content in root, (C) tolerance index (%)

presented a declining trend with an increasing Cd concentration in the soil of the cucumber plants (Figure 3C). Meanwhile, the exogenous application of glutamic acid under the different Cd concentrations exhibited a synergic effect and increased the CAT activity in the cucumber leaves.

Effects of glutamic acid on the reactive oxygen species and TSP under Cd stress. The changes in the malondialdehyde (MDA) content, hydrogen peroxide (H_2O_2) contents, and total soluble protein contents are shown in Figure 4. The different Cd treatments significantly increased the hydroxyl ion (OH^-) and superoxide radicle (O_2^-) contents in the cucumber leaves. Moreover, the glutamic acid application significantly ameliorated the Cd stress in both cultivars (Figure 4A) and reduced the contents in both cultivars. Furthermore, the data showed that the MDA contents significantly increased in the V1 cultivar when compared to the V2 cultivar under different Cd treatments. However, the exogenously applied glutamic acid significantly lowered the MDA contents under the Cd stress in both cultivars, but a larger synergetic effect was observed in the V2 cultivar (Figure 4B). Furthermore, the data delineated that the TSP contents significantly increased in both cultivars under the higher Cd stress concentration (20 mg Cd/kg soil).

DISCUSSION

In this study, glutamic acid was applied to induce Cd tolerance in cucumber cultivars. The results explained that the cucumber plant growth was significantly affected due to the Cd impairing its morphological characteristics. Similar results have been found in *Brassica napus* (Ali et al. 2014), *Sedum alfredii* Hance (Yang et al. 2003), mung beans (Wahid, Ghani 2008) and cucumbers (Sun et al. 2015). In this experiment, plant growth regulators (PGRs)

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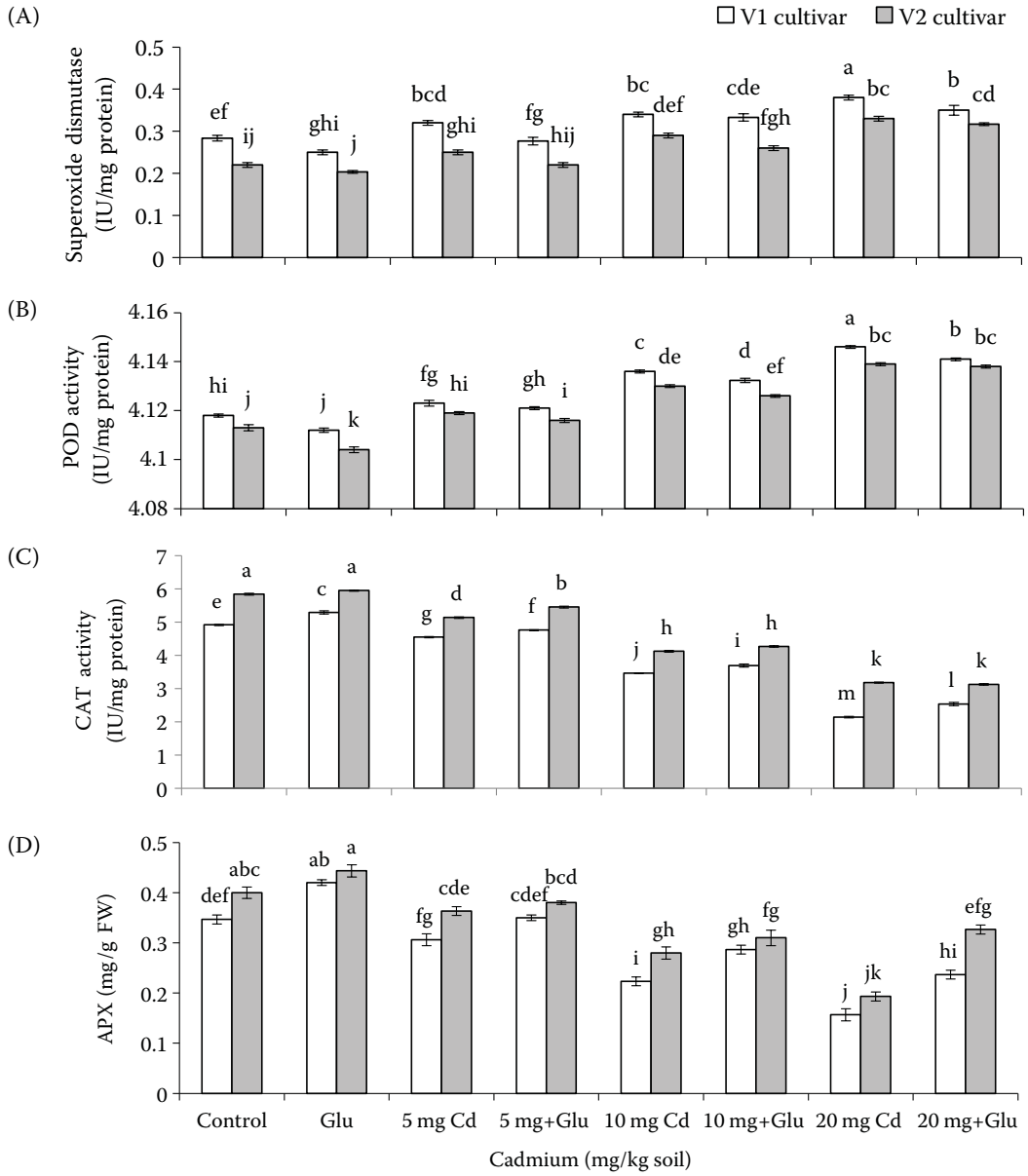

Figure 3. Effects of treatments of glutamic acid (Glu; 0, 10 mM) and cadmium (Cd; 0, 5, 10, 20 mg/kg) on (A) superoxide dismutase, (B) peroxidase (POD), (C) catalase (CAT), (D) ascorbic peroxidase (APX)

were used to respond positively towards the morphological characteristics and growth of cucumber plants by enhancing the leaf length, dry and fresh weight, plant height and root length. A significant reduction in the growth was found in the V1 cultivar (Ashly) under the Cd stress. This might be due to that the plants were not capable of taking up nutrients to continue their metabolic activities, with the antagonistic impacts of the Cd on the roots (Ali et al. 2014).

In a previous study, the exogenous application of glutamic acid increased the seed germination under salt stress in cucumbers (Chang et al. 2010). This effect might be linked to the fact that glutamate promoted a role in the regulation of the metabolic processes, and had a possible role as a signalling molecule in plants (Forde, Lea 2007). The reduction in the SPAD index was found when compared to the control group under Cd stress. This might be due to the damage to the chloroplast structure, photosynthetic apparatus and protein complexes. Our results also presumed that the Cd stress might be due to stopping the electron transport chain in the photosynthesis (Mohanty et al. 1989). The glutamic acid

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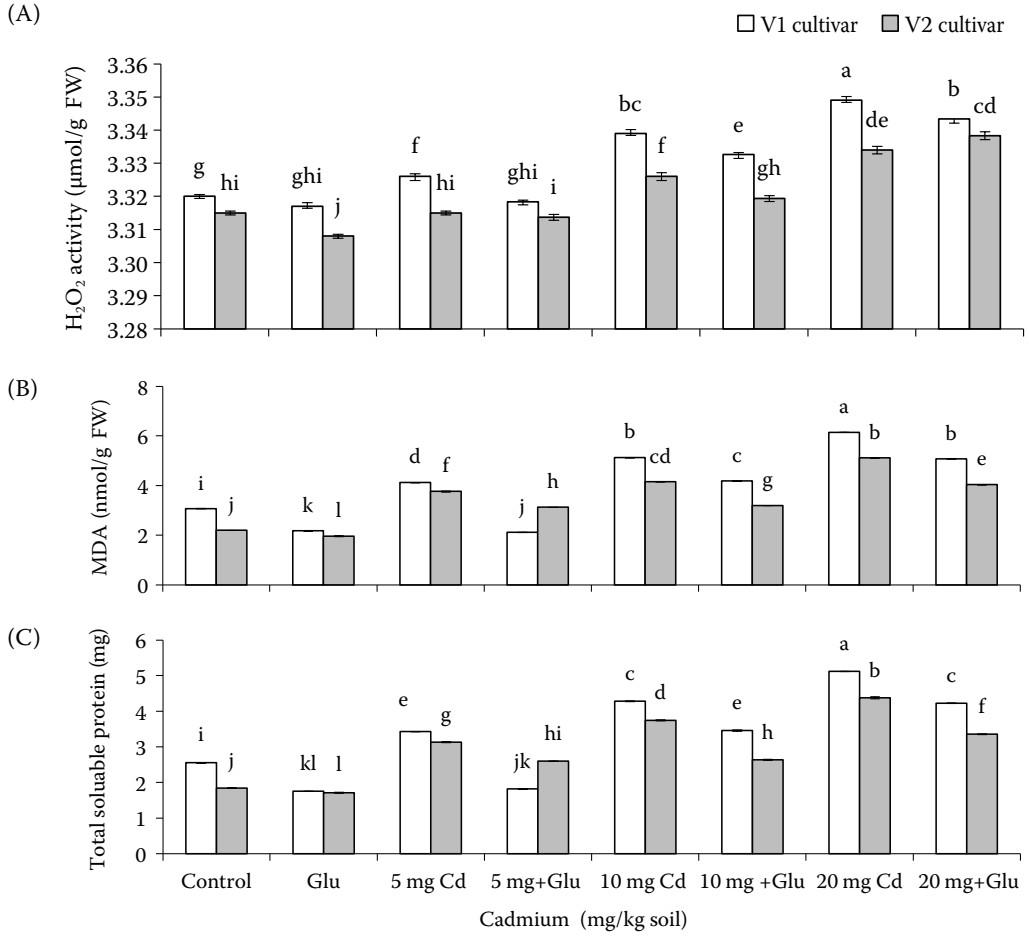

Figure 4. Effects of the glutamic acid (Glu; 0, 10 mM) and cadmium (Cd; 0, 5, 10, 20 mg/kg) treatments on the (A) hydrogen peroxide, (B) malondialdehyde, (C) total soluble protein

increased the plant resistance system, thus avoiding the uptake or transportation of the Cd in the plant tissues. It can be proposed that foliar glutamic acid could be applied to resolve the phytoextraction.

To escape oxidative damage and scavenge ROS, plants have non-enzymatic and enzymatic antioxidant activities. In the detoxification process, the first enzyme is SOD which scavenges the superoxide radicals at a very fast rate to H_2O_2 (Gratão et al. 2005). A higher SOD activity was found in the V1 cultivar when compared to the V2 cultivar under different Cd treatments, but this was higher in the V1 cultivar due to larger accumulation of Cd in the plants. The SOD activity was higher in the V1 cultivar which caused higher cellular damage in the V1 cultivar (Gossett et al. 1994). However, the efficient scavenging increased due to the CAT and APX activities which resulted in a reduced Cd induced ROS. However, the exogenous application of glutamic acid enhanced the activity of these enzymes that protect the plants from ROS. The enhanced activity of APX in the V2 cultivar suggested that the ascorbate-glutathione cycle evenly worked, and therefore, the Cd stress tolerance was better than the V1 cucumber cultivar. Gill and Tuteja (2010) proposed that these enzymes are significantly important in reducing the ROS with highly stressed environments. However, the application of glutamic acid also plays a promotional role to increase the antioxidant enzyme activity under stress conditions.

In the current study, the Cd concentrations alone increased the MDA content in the cucumber cultivars. Recently, another study also showed that Cd stress increased the MDA and ROS contents in lettuce seedlings (Xu et al. 2015). The total soluble protein content and protein oxidation addition increased due to the increasing Cd concentration in the cucumbers. In another finding, Cargnelutti et al. (2006) also presented that the total soluble protein and oxidation of proteins in cucumbers in-

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creased due to the metal-treatment. The increase in the protein content by metal exposure may be due to the synthesis of de novo stress proteins (Verma, Dubey 2003). Mostly, Cd toxicity caused oxidative stress, but higher plants have a highly developed resistance system to detoxify the Cd stress. In plant tissues, the H_2O_2 content could actively scavenge with these two enzymes. This result is also found in wheat, where fulvic acid ameliorated the APX and CAT activities with the addition of metal stress (Ali et al. 2015b). These results suggested that additional ROS is excluded because the glutamic acid may serve as an antioxidant, or act as stimulus molecule to produce antioxidants.

CONCLUSION

The findings of the present study demonstrated that an exogenous glutamic acid application impairs the Cd toxicity in both cucumber cultivars by decreasing the interior Cd concentration and inhibiting the ROS accumulation in the plants. The results suggested that the foliar application of glutamic acid on the cucumber cultivars increased the plant growth and chlorophyll contents by stimulating the antioxidant activities and decreasing the lipid peroxidation. Subsequently, the V2 cultivar was presumed to be best cadmium-tolerant cultivar. To study the glutamic acid ameliorative effects with Cd stressful conditions, a controlled environmental condition based method is required.

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