Journal of Stress Physiology & Biochemistry, Vol. 15, No. 3, 2019, pp. 50-61 ISSN 1997-0838

ORIGINAL ARTICLE

Regulatory Effect of Spermine on Growth and Antioxidant System in Tomato Seedlings Grown in the Presence of Cadmium

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Received July 28, 2019

The protective role of exogenous supplied polyamines such as spermine in detoxifying the cadmium induced toxicity was studied in the tomato plants *Solanum lycopersicum*, we applied a dose of (1 mM) spermine with cadmium (0, 20 and 100 µM) for 7 days. The results of the application of exogenous spermine with cadmium showed a significant attenuation of the toxic effects of metal for different growth parameters (fresh matter, dry matter, root length and aerial parts) and chlorophyll pigments, mainly after 3 days for leaves and roots. Moderation of spermine is more marked with 20 µM dose of cadmium. The stimulatory effect of cadmium on catalase activity (CAT), ascorbate peroxidase (APX) and superoxidedismutase (SOD) is partially lifted by presence of spermine in tomato. The accumulation of cadmium, proline and soluble sugars in cultivated plants with cadmium showed a reduction by presence of spermine. Moderation of cadmium toxicity by application of spermine in tomato could be explained by its antioxidants properties.

Key words: Tomato; Cadmium; Spermine; CAT; SOD; APX

Cadmium (Cd) is metal without any known biological function (Lane and Morel, 2000); it is a widespread non-essential metal, which enters the ecosystems through industrial processes or by the application of phosphate fertilizers (Mishra *et al.,* 2006). The dependence of agriculture on chemical fertilizers, sewage wastewater irrigation and rapid industrialization was raised and has added toxic metals to agricultural soils, and therefore, it resulting a harmful effects on the environment system (Kumar *et al.,* 2013). Many studies have revealed that Cd affect the absorption and translocation of nutrients in certain crops, such as tomato. Excessive accumulation of Cd can disrupt the absorption and distribution of nutrients; and may be a factor of mineral disorders and seedling growth perturbation. The polyamines are aliphatic compounds with low cellular PM and present in all plant cells (Igarashi and Kashiwagi. 2000); they act a central role in regulating physiological processes and plant development (Kusano *et al.,* 2007). Recent studies have shown that the exogenous application of polyamines is an efficient way to boost the tolerance of crops to stress in order to enhance their productivity (Aldesuquy *et al.,* 2014). Spermine is a natural polyamine, which is located almost in all eukaryotic cells due to its important role in cell growth and differentiation (Rawat *et al.,* 2017).

Some research has suggested that spermin can be presented in polycational form because it has a high affinity for polyanions such as nucleic acids and membrane phospholipids.

When bound, it can regulate the sythesis, activity of molecules and control of stages of cell division mitosis or meiosis (Galston and Kaur-Sawhney. 1995). Alternatively, it may covalently bind to specific proteins by trans-glutaminases enzymes and this association seems to play an important role in protecting chloroplast proteins from the action of proteases, thus preserving the effectiveness of photosynthesis (Luis *et al.,*, 2002).

Under the effect of metallic stress caused by the accumulation of heavy metals in the plant, spermine is involved in membrane protection, pH stabilization and free radical scavenging or inhibition of their production (Balestrasse *et al.,* 2005). Spermine is characterized by high antioxidant activity by protecting DNA against oxidizing agents; in addition, it decomposes hydrogen peroxide as an electron donor for peroxidase. (Mozdzan *et al.,* 2006). Tomato breeding has begun since the domestication of the species. It is one of the best known varieties in agronomy and, above all, it plays an important role in the human diet. Tomato crops can be affected by various environmental stresses such as abiotic and biotic stress. In this context, the main objective of this study is to examine the effect of cadmium on the morphological and physiological processes of the tomato plant through the intervention of an exogenous polyamine "spermine".

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of tomato (Solanum lycopersicum), were germinated on moistened filter paper at 25 ºC in the dark. The seedlings obtained were transferred to continuously aerated nutrient solutions containing KNO_3 (2 mM), $Ca(NO_3)_2$ (0.5 mM), KH_2PO_4 (2 mM), $MgSO_4$ (0.5 mM), Fe-K-EDTA (100 µM), and micronutrients: H_3BO_4 (30 µM), $MnSO_4$ (5 µM), $CuSO_4$ (1 µM), $ZnSO_4$ (1 µM), $(NH_4)_6Mo_7O_{24}$ (1 µM). Plants were grown in a growth chamber: 26° C / 70 % relative humidity during the light period and 20° C/ 90 % relative humidity during the dark period; photoperiod: 16 h daily with a light irradiance of 150 µmol m^{-2} s^{-2} at the plant canopy. Plants were grown for 21 days in control medium, and then cadmium treatments (20 and 100 µM $CdCl_{2)}$ were applied during 7 days.

Cadmium content

Cadmium content in various plant tissues was analyzed by digestion of dried samples with an acid mixture ($HNO_3/HClO_4$, 4/1 v/v). Cadmium concentrations were determined by atomic absorption spectrophotometer (Perkin-Elmer, Analyst 300).

Chlorophyll determination

Chlorophyll was determined by the method of (Arnon. 1949). The leaf blades are previously immersed in acetone and stored overnight at 4°C. The plant material is crushed mortar in acetone at 80%. After centrifugation suspension for 5 min at 3000 g, the optical density of the supernatant is read at 460, 645, 652 and 663 nm, using a spectrophotometer. The concentration

of total chlorophyll *a* and chlorophyll *b*, expressed in mg/ g FW.

Determination of fresh and dry matter

Different collected parts (leaves and roots) are then weighed to determine the mass of fresh weight (FW) and then placed in the incubator at (60° C) for 3 days and reweighed to determine the mass of dry weight (DW).

Determination of proline content

Free proline content was determined according to (Bates *et al.,* 1973). Samples (100 mg) were homogenized with 5 ml 3% (w/v) sulphosalycylic acid. Homogenates were filtered through filter paper. After the addition of 2 ml acidic ninhydrin reagent (2.5 g ninhydrin/ 100 ml of solution containing glacial acetic acid, distilled water and 85% orthophosphoric acid at a ratio of 6:3:1) and 1 ml glacial acetic acid, the resulting mixture was incubated at 100° C for 1 h in a water bath. The reaction was then stopped using an ice bath. The mixture was extracted with toluene, and absorbance was measured at 520 nm.

Protein content

Soluble protein content was quantified using Coomassie Brilliant blue (Bradford.1976) with bovine serum albumin as a protein standard.

Determination of soluble sugars

The method used for the determination of soluble sugars is that described by (Staub 1963). The extraction is carried out from 25 mg of dry plant material in 80% ethanol. The samples are placed in a water bath at 70° C for 30 minutes. After cooling, the samples are centrifuged at 6000 g for 15 minutes. Subsequently, 25 µl of supernatant was taken and added to 5ml of anthrone and placed in a water bath at 100°C for 10 minutes. The absorbance was measured at 640 nm.

Determination of lipoperoxides (MDA)

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) (Buege & Aust. 1978). 500 mg Fresh material is mixed with 5 ml of tichloroacétique acid (0.1%, w/v), and after 15 minutes of centrifugation at 12000 g, the supernatant is added to an equal volume of thiobarbituric acid (TBA) 0.5% prepared in a solution of trichloroacetic acid (TCA) 20%. Then the mixture is incubated at 100°C for 30 minutes and a second centrifugation at 10000g for 5 minutes. The supernatant absorbance was measured at 530 nm and 600 nm. The concentration of malonyldialdehyde (MDA) is then calculated by extinction coefficient (155 mM. cm^{-1}).

Enzymatic activity assays

Catalase (CAT, EC 1.11.1.6)

Catalase activity was determined by measuring the disappearance of H2O2, as described by (Aebi. 1984). The activity of this enzyme is determined by measuring the amount of H2O2 decomposed by unit time. The assay is performed by measuring the optical density at 240 nm. The activity is expressed in mmol / min / mg of FW.

Superoxyde dismutase (SOD, EC 1.15.1.1)

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (Beauchamp &Fridovich .1971). This reaction is initiated by exposing for 25 minutes in a UV light source and the assay is performed at 560 nm. A unit of SOD equals the amount needed to halve NBT reduction.

Ascorbate peroxydase (APX, EC 1.11.1.11)

APX activity was measured according to (Nakano & Asada. 1981) as the decrease in absorbance at 290 nm due to ascorbate oxidation. Enzyme activity is determined by following the reduction in absorbance at 290 nm due to the oxidation of ascorbate in the presence of H2O2. The activity is expressed in mmol / min / mg of FW.

Statistical analysis

Results was performed at least three independent experiments. All values are expressed as mean± standard deviation (SD), and the statistical significance of differential finding between experimental groups was determined by one-way ANOVA test using GraphPad Prism 6 software and SPSS. $P<0.05$ was considered statistically significant and is indicated with asterisks over the value (*: $p<0.05$; **: $p<0.01$; *** $p<0.001$).

RESULTS

The results show a decrease in fresh weight reduction in leaves (Fig. 2A) and roots (Fig. 2B) in

presence of cadmium compared to the control. Growth is stopped almost in presence of 100 µM of $CdCl_2$. whereas when the exogenous application of (1 mM) of spermine, compared to control plants, there is significant increase in fresh weight of leaves and roots. This improvement in growth by spermine proves that this polyamine reduced the cadmium inhibitory effect on plant growth (Fig. 1).

The reduction in growth of tomato seedlings treated with the cadmium is most important with (100µM) than (20 µM) of $CdCl_2$, there is a reduction of length of the different organs of seedlings (Tab. 1). The visual symptoms of phytotoxicity are expressed especially in young leaves and are manifested by chlorosis and reduced leaf surface also the appearance of some necrotic spots on the leaves (Fig. 1).

In fact, in presence of spermine in the culture medium, there is improvement in growth with stem elongation, expansion leaf surfaces and absence of chlorosis, so this polyamine has reduced the harmful effects of this metal.

Effect on soluble proteins content

Control samples show a high levels soluble protein content in leaves than the roots. In stressed plants, there is a significant reduction of total soluble protein contents, and this reduction was noticed in leaves (Fig. 2C) and roots (Fig. 2D) by control after 7 days of treatment. Indeed, this reduction is explained by the stimulation of proteolytic pathways. For against, the combination with spermine (1 mM) leads to increased levels of soluble protein compared to stressed plants, so that there was an inhibition of proteolytic pathways.

Effect on total soluble sugar content

The addition of 100 µM of $CdCl_2$ to culture medium leads to strong elevation of carbohydrate content by control on leaves (Fig. 2E) and roots (Fig. 2F). The combination treatment with spermine reduces the stimulatory effect generated by cadmium.

Estimation of photosynthetic pigment content

For against, the presence of spermine (1 mM) promotes the restoration of chlorophyll *a* and chlorophyll *b* content in the presence of $CdCl_2$. This polyamine stimulated the production of chlorophyll *a* and *b* to ensure good photosynthetic efficiency by reducing the inhibitory effect of this metal (Fig. 3A).

Effect on proline content

Exposure to 100 µM of $CdCl_2$ leads to a significant accumulation of proline in leaves (Fig 3D) and roots (Fig. 3E) whose content is multiplied by factor of 5 for both leaves and roots by control, but accumulation is more intense in roots than leaves. The application of spermine at plants exposed to $CdCl_2$ allows a reduction in accumulation of proline.

Determination of lipid peroxidation content (MDA)

The exposure to 100 µM $CdCl_2$ significantly increases the production of malondialdehyde in leaves (Fig. 3B) and roots (Figure 3C) by control, but this increase was higher in the levels of leaves than roots. The application of spermine (1 mM) combined treatment with $CdCl_2$ leads to attenuation of malondialdehyde (MDA) production.

Effect on soluble proteins content

Control samples show a high levels soluble protein content in leaves than the roots. In stressed plants, there is a significant reduction of total soluble protein contents, and this reduction was noticed in leaves (Fig. 2C) and roots (Fig. 2D) by control after 7 days of treatment. Indeed, this reduction is explained by the stimulation of proteolytic pathways. For against, the combination with spermine (1 mM) leads to increased levels of soluble protein compared to stressed plants, so that there was an inhibition of proteolytic pathways.

Cadmium content

Results related to cadmium accumulation in leaves and roots of tomato seedlings treated with increasing doses of $CdCl_2$ in the presence of 1 mM spermine for a week are shown in Figure 3. Significant increase intra tissular cadmium content, proportional to external concentration of the metal is obtained. In fact, cadmium ions were accumulated at higher levels in roots (Fig. 3F) than in leaves (Fig. 3E).

Catalase activity

Catalase is an important antioxidant enzyme in peroxisomes, in control plants; catalase activity is low in leaves (Fig. 4A) and roots (Fig. 4B). In leaves, the application of cadmium, even at a high dose does not vary catalase activity which remains relatively stable.

While the combination of spermine with $CdCl_2$ causes a stimulation of catalase activity. In roots, a considerable stimulation of catalase activity was observed upon application of 20, 100 µM $CdCl_2$ after 7 days of treatment. In contrast, addition of spermine to medium culture induces an increase in catalase activity.

Ascorbate peroxydase activity

Under our experimental metallic stress, application of 20 and 100 µM $CdCl_2$ stimulate APX activity in leaves (Fig. 4C) and roots (Fig. 4D) compared to control plants after 7 days of treatment. However, treatment with spermine combined to 100 µM of cadmium induced a significantly increase of APX activity in leaves as well as in roots; however, APX activity is still very much higher than that measured in the control plants.

SOD activity

Changes in specific activities of superoxide dismutase in tomato leaves and roots are supported by Figure 4. Note that cadmium induced in leaves (Fig. 4E) and roots (Fig. 4F) a significantly increase in activity of this enzyme compared to control plants after 7 days. The combination of spermine with $CdCl_2$ induces a greater stimulation of SOD specific activity in plants treated with 20 and 100 µM $CdCl_2$, after 7 days of treatment.

Table 1. Effect of Spermine (1mM) on aerial organs and roots length in tomato seedlings treated with cadmium (20 µM and 100 µM) after different exposure times (1, 3, 5 and 7 days).

		Control	Cd (20 µM)	Cd (20 µM) +Sp	Cd (100 µM)	Cd (100 µM) +Sp
Day 1	Root (cm)	18.5	14.3	22	12.	18
	Aerial organs (cm)	18	16.5	18.4	10.1	15
Day 2	Root (cm)	19	12.6	23	11.1	14.5
	Aerial organs (cm)	20	17	19.5	10.5	13
Day 5	Root (cm)	20.5	14.2	24	11.6	14
	Aerial organs (cm)	20.5	18	20	11	13.2
Day 7	Root (cm)	25.75	18	25.9	10.65	15.5
	Aerial organs (cm)	25.2	19	21	10	13.1

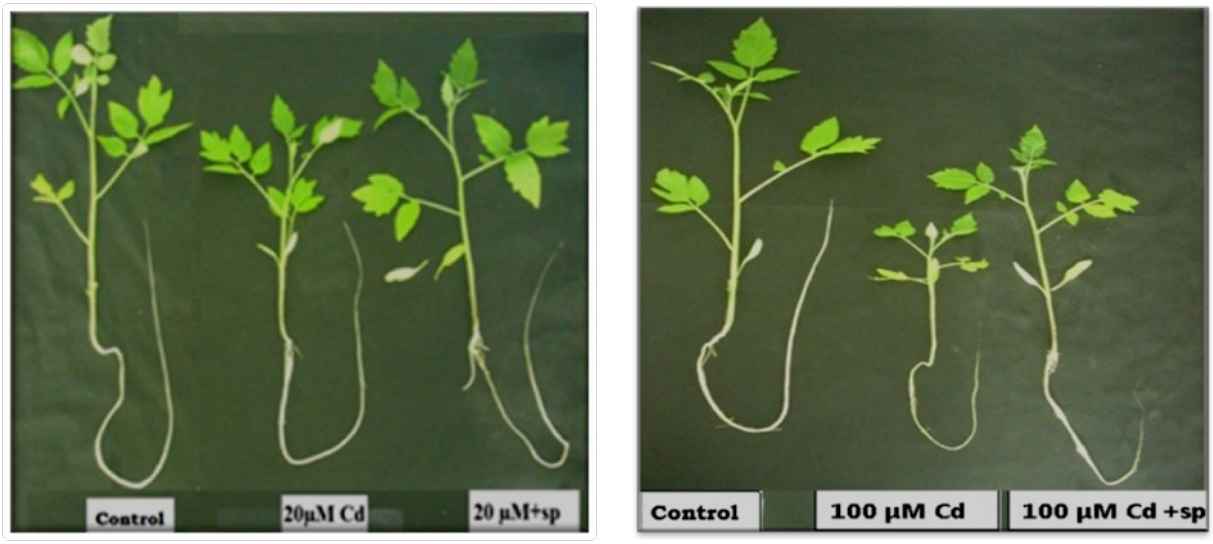

Figure 1. Effect of spermine on morphology of tomato seedlings grown on hydroponic medium and treated with $CdCl_2$ (20 and 100 µM) of $CdCl_2$ after one week of treatment.

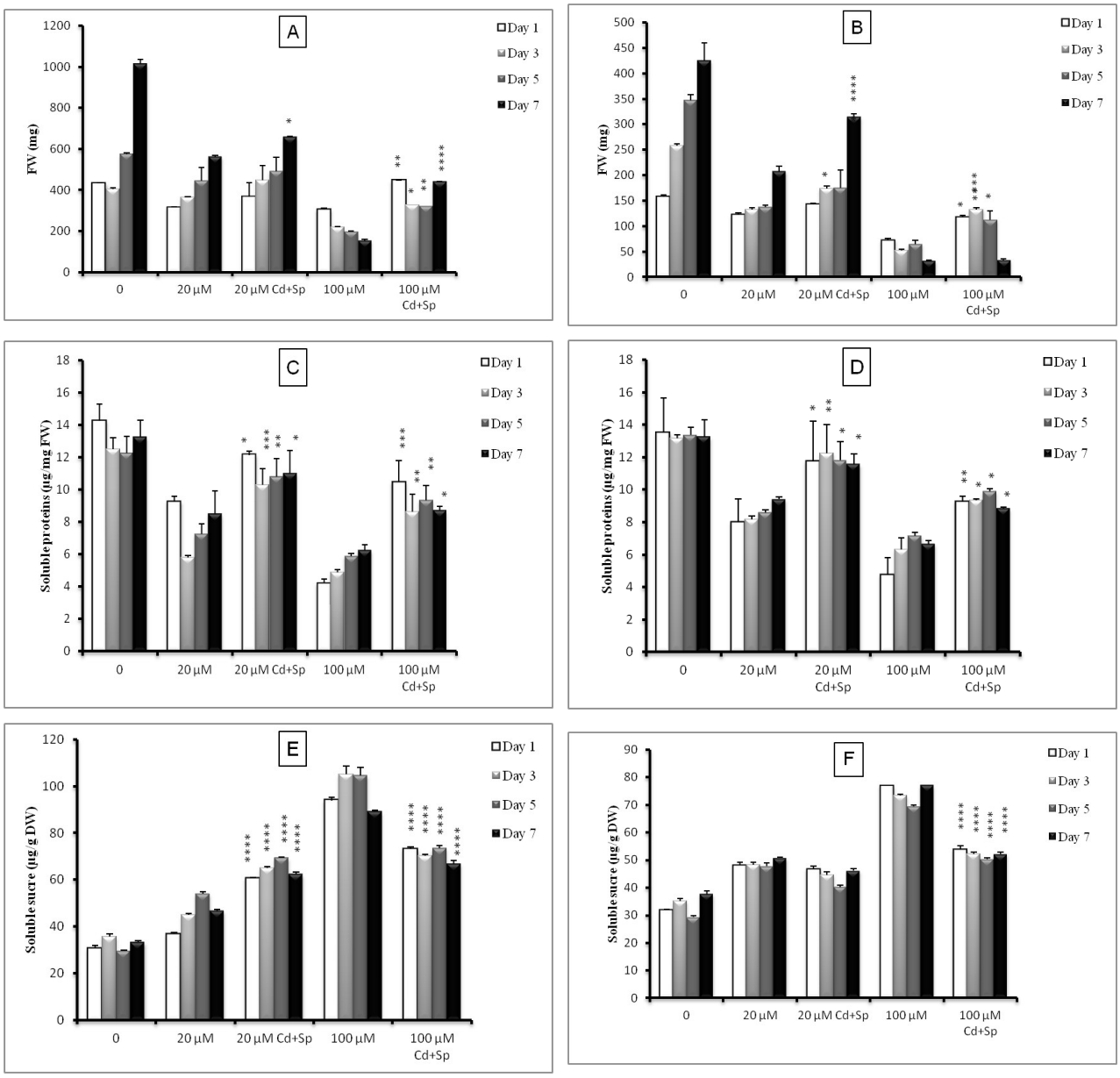

Figure 2. Effect of Spermine (1mM) on fresh weight of leaves (A) and roots (B), soluble proteins production in leaves (C) and roots (D) total carbohydrates contents in leaves (E) and roots (F), on tomato seedlings treated with cadmium (20 µM and 100 µM) after different exposure times (1, 3, 5 and 7 days). Values are expressed as the mean ± SD; $p < 0.05$. Data indicate were analyzed using one-way ANOVA followed by Turkey's test.

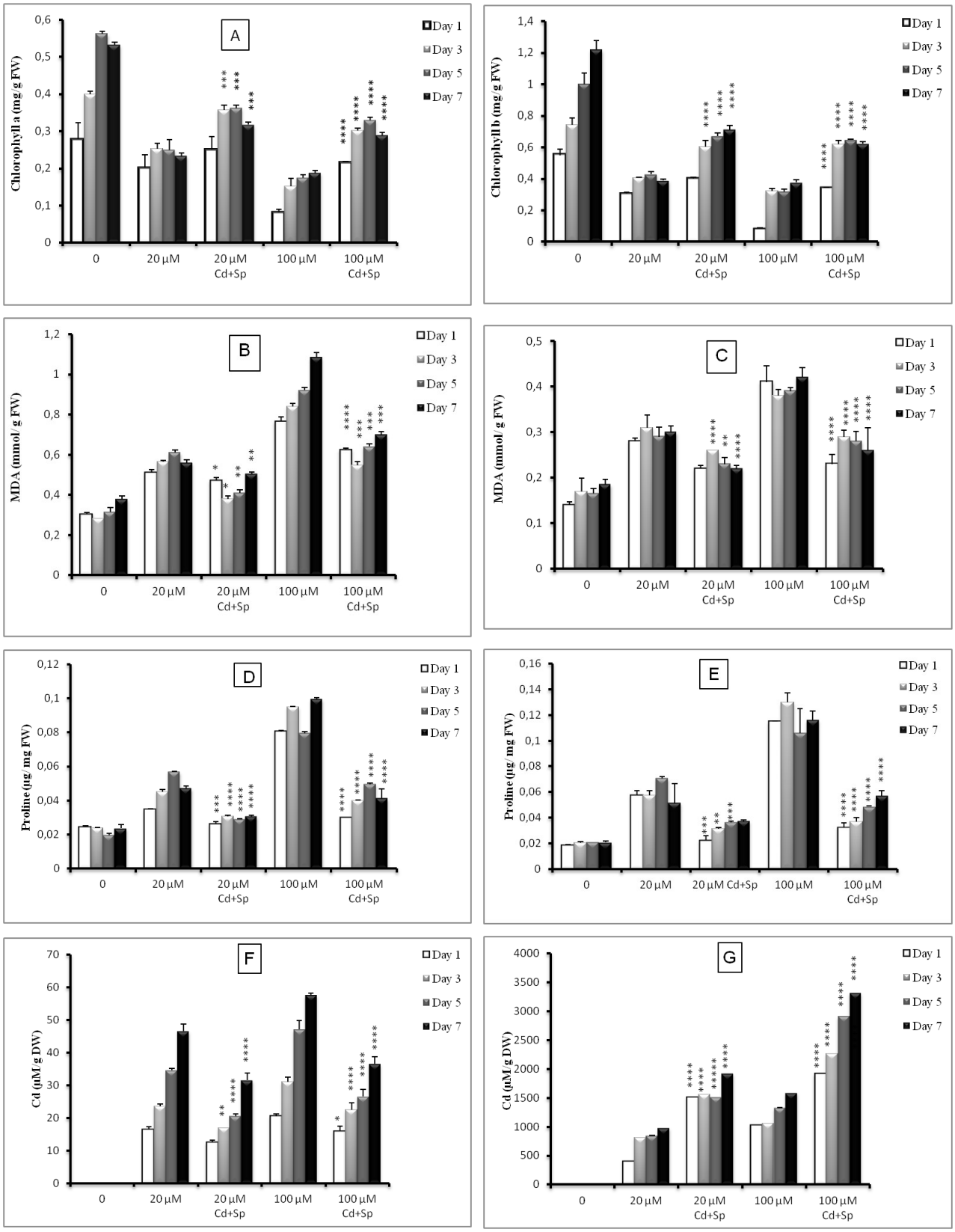

Figure 3. Effect of Spermine (1 mM) on chlorophyll a content and b (A), malondialdehyde production in leaves (B) and roots (C), Proline content in leaves (D) and roots (E), Cd content in leaves (F) and roots (G) on tomato seedlings treated with (20 µM and 100 µM) $CdCl_2$ after different exposure times (1, 3, 5 and 7 days). Values are expressed as the mean ± SD; $p < 0.05$. Data indicate were analyzed using one-way ANOVA followed by Turkey's test.

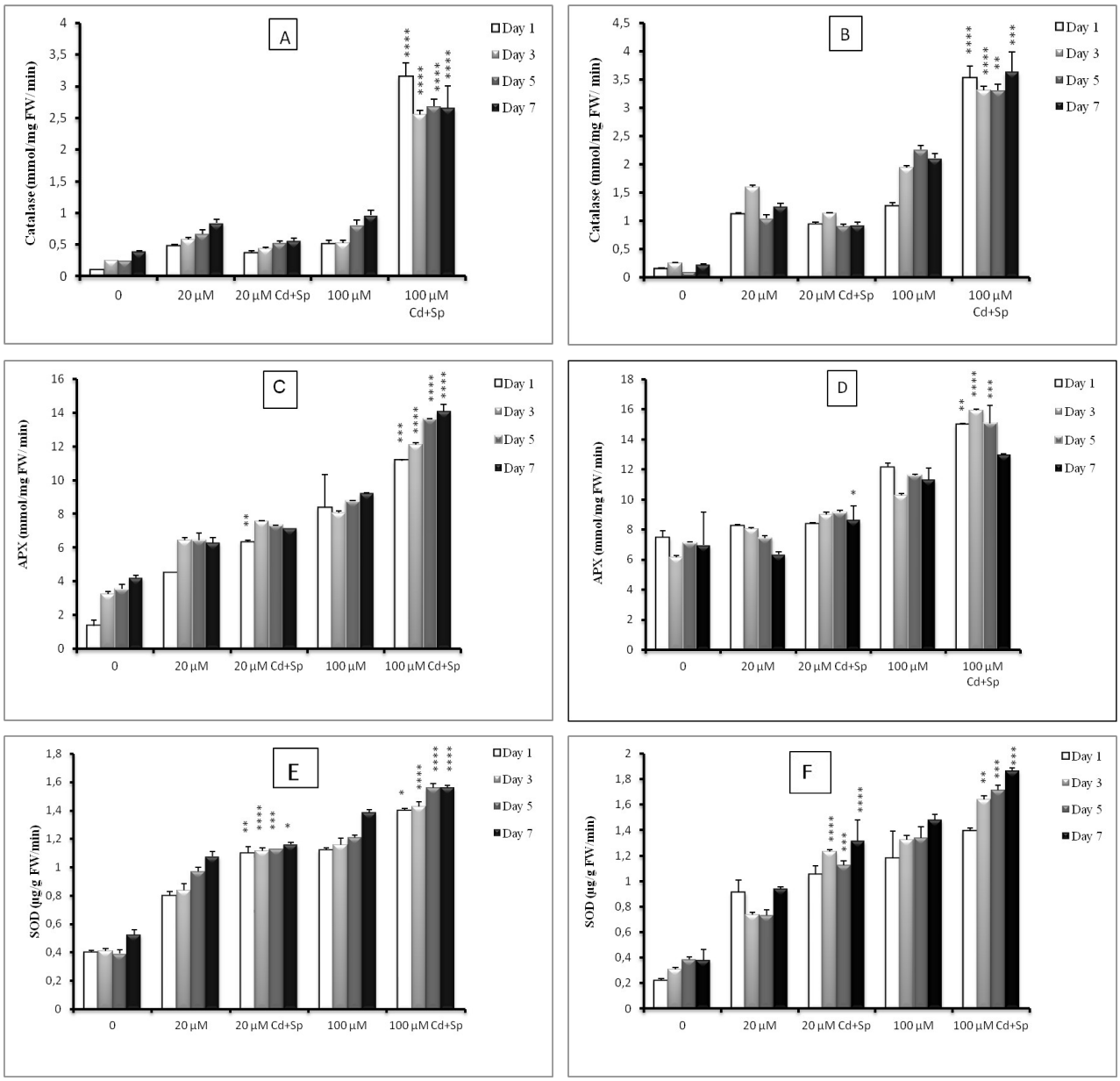

Figure 4. Effect of Spermine (1mM) on catalase activity in leaves (A) and roots (B), APX activity in leaves (C) and roots (D) and SOD activity in leaves (E) and roots (F) on tomato seedlings treated with cadmium (20 µM and 100 µM) after different exposure times (1,3,5 and 7 days). Values are expressed as the mean ± SD; $p < 0.05$. Data indicate were analyzed using one-way ANOVA followed by Turkey's test.

DISCUSSION

Decreased growth is one of the characteristic responses of plants to cadmium stress (Gratao *et al.,* 2012). Our data illustrate a significant drop in tomato plant growth revealed by the analysis of the fresh and dry weight of roots and leaves after 7 days of cadmium exposure. To date, numerous studies have proven the effectiveness of the application of salicylic acid in improving cadmium toxicity in different plant species (Asgher *et al.,* 2015, Meng *et al.,* 2009). The data obtained suggest that the protective effect of spermine treatment on growing tomato seedlings is probably due to a decrease in cadmium uptake by plants, which, for example, was detected in soybean pretreated with salicylic acid. The results suggest that the reported protective effect of spermine treatment on growing tomato plants is probably due to a decrease in cadmium uptake, which has been detected in particular in soybean traited with salicylic acid (Noriega *et al.,* 2012) and luzerne plants (Cui *et al.,* 2012). The comparative analysis of the amount of cadmium in untreated and

treated tomato plants with spermine showed (Figure 2) that the maximum accumulation of toxic ions was measured in the roots, while in the leaves, the cadmium content was about an order of magnitude lower, which is consistent with the results showing that underground organs are the preferred area for cadmium accumulation (Shakirova *et al.,* 2016).

Our study showed that tomato seedlings exposed to cadmium have a high content of MDA, which causes a disruption in the functionality and integrity of the cell membrane, as well as photosynthetic pigments are damaged in tomatoes. But when we add spermine to the culture medium, the content of the MDA accumulation has been reduced. Plant genotypes that are more stress tolerant have a high ROS trapping capacity and this activity is due to the high antioxidant content.

The activation of several enzymatic (SOD, APX and CAT) or non-enzymatic antioxidants is involved in the detoxification of ROS in stressed cells (Blokhina *et al.,* 2003). The increase in proline content is an indication of the elimination of free radicals (Mohanty & Matysik 2001), this amino acid is involved in maintaining cell turgorcence and the production of a macromolecular structure (Roychoudhury *et al.,* 2008).

The spermine act as a key protective molecule and present as a direct effectors of stress tolerance (Peremarti *et al.,* 2009). For the past several years, many researchers have been investigating molecular, biochemical and physiological aspects of the major PAs in plants, using rice as a model. An exogenous use of PAs, intended to elevate endogenous PAs content , has been applied before or during stress (Velikova *et al.,* 2000), which can prevent the degradation of chlorophyll (Demetriou *et al.,* 2007), and minimize the activity of protease and Rnase (Chattopadhayay *et al.,* 2002).

In the present work, the impact of exogenous spermine (1 mM) on cadmium stress-induced cell damage has been illustrated by restoring root and leaf weight in tomato plants, recovering chlorophyll, MDA and proteins degradation. It has been shown previously that polyamine could act indirectly by increasing the gene expression and level of activity of antioxidant enzymes (Tang &Newton . 2005, Verma &Mishra. 2005, Wi *et al.,* 2006) resulting in tolerance to various abiotic stresses. The elevation of APX, SOD and CAT activity in tomato seedlings in this work asserts the protective role of enzymes during cadmium stress.

Substantial increase in proline content in cucumber roots with spermidine exogenous and under saline conditions has been reported earlier (Duan *et al.,* 2008). However the protective impact of spermine is more strong visible in tomato plants where the inversion of inhibitory effect of cadmium stress was conferred by preventing growth inhibition or various forms of cellular damages, activation the level of osmolytes and activity of antioxidant enzymes.

CONCLUSIONS

By dint to their antioxidant property, exogenous spermine exert a stimulating effect on several Physiological processes in the tomato plant. As shown in this study, the combination of this polyamine with cadmium makes it possible to reduce the disturbances caused by this metal stress, such as the recovery of weight, and the content of photosynthetic pigments as well as the stimulation of antioxidant enzymes. Nevertheless, because of the expensive price of polyamine, this has further research needed on the treatment time of exogenous spermine application to take advantage of their large-scale.

ACKNOWLEDGEMENTS

We acknowledge the ‘‘Ministère Tunisien de l’enseignement supérieur et de la recherche scientifique” for the financial support of this study.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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