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Influence of Gamma Radiation, Glutathione and Ascorbic Acid on Some Antioxidant Enzymes in Egyptian Clover (*Trifolium alexandrinum* L.) Under Aluminum Stress

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Abstract: Experiments in the present investigation were conducted to study the effect of gamma radiation (15 Gy) and foliar application of glutathione and ascorbic acid on Egyptian clover (*Trifolium alexandrinum* L.) under heavy metal ($AlCl_3$) stress condition (0.5 mM). Low gamma irradiation is an important physical growth activator and productivity promotor. Ascorbic acid and glutathione are antioxidants that act as scavengers of toxic active oxygen species that evolve when oxidative stress occurs as a result of stress. In this investigation, seeds were irradiated (15 Gy) prior to sowing or plantlets were sprayed with glutathione (50 ppm) and ascorbic acid (250 ppm) or left untreated (control). Plantlets were either irrigated with tap water or with 0.5 mM $AlCl_3$ solution. Records after 50 days revealed decreased shoot and root length and fresh and dry weights of plants and increased peroxidase, phenoloxidase and catalase activity for plants under $AlCl_3$ stress. Moreover, glutathione and ascorbic acid treatment increased shoot and root length, plant fresh and dry weight, peroxidase and phenoloxidase activities compared to controls. Pre-sowing seed irradiation was not as useful as the glutathione and ascorbic acid treatment in helping plants to overcome aluminum stress conditions.

Key words: Clover · Stress · Aluminum · Gamma radiation · Antioxidant enzyme

INTRODUCTION

Egyptian clover (*Trifolium alexandrinum* L.) is a traditional forage crop that is grown annually in Egypt in winter and spring. After several cuttings for animal feeding and hay use, few fields are left for an extra month to harvest seeds, while the rest of the crop is plowed as a preparation way for summer crops cultivation. Egyptian clover is a part of a mandated crop rotation in the Nile Delta where cotton, rice and maize are grown [1 - 3].

Among the processes that have been reported to be affected by Al is oxidative stress and the most evident and primary symptom of Al toxicity is root growth inhibition [4, 5]. Several mechanisms of Al toxicity have been proposed [6]. However, the precise physiological and molecular bases are not completely understood [7]. In this regard, it is worth mentioning that plasmodesmata are the cytoplasmic channels responsible for symplastic intercellular transport in plants which interconnect cells to facilitate intercellular movement of water, nutrients and signaling molecules including hormones. Moreover, Al was found to be among the factors that affects this cell-to-cell transport process [8]. Sivaguru *et al.* [8] results suggest that Al-signal mediated localized alterations to calcium homeostasis may drive callose formation and plasmodesmata closure. Their data demonstrated that extracellular Al-induced callose deposition at plasmodesmata could effectively block symplastic transport and communication in higher plants. Šimonovicová *et al.* [9] reported that Al induces reactive oxygen species (ROS) and enhances lipid peroxidation in *Hordeum vulgare*. Levels of ROS in plant cells are normally controlled by protective antioxidant systems. Šimonovicová *et al.* [9] also found that superoxide dismutase (SOD) appeared to have a role in detoxification mechanisms at highly toxic Al doses. They noticed a significant correlation between elevated activities of peroxidase (POD) and ascorbate peroxidase (APX), H_2O_2

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consuming enzymes and root growth inhibition. They also stated that the signaling role of NADH-POD in oxidative stress seems to be more probable than that of oxalate oxidase (OXO), which might be involved in Al toxicity mechanism.

Darkó *et al*. [10] demonstrated that the roots of Al-tolerant wheat exhibited more intensive growth, while accumulating less Al and ROS than Al-sensitive wheat under Al stress conditions. They also found that among the antioxidant enzymes induced by Al stress, catalase and glutathione-S-transferase may play an important role in the detoxification of ROS in Al-tolerant wheat. Moreover, ascorbic acid (AA) and glutathione (GSH) have been implicated in the regulation of plant cell growth and division [11]. Recently, Devi *et al*. [12] reported that the higher content of AA in Al- tolerant cell lines of tobacco compared to Al- sensitive cell lines are responsible for its higher tolerance to Al. They also reported that high content of GSH has shown to be responsible for the tolerance mechanism of tobacco cells. Such results suggest that AA and GSH play an important role in Al-inhibited growth. On the other hand, gamma irradiation is known to affect physiological and biochemical processes in plants along with morphological, structural and functional changes depending on the strength and duration of gamma irradiation [13]. In this investigation, alteration of enzyme activity in response to seed irradiation reported earlier by Stoeva *et al.* [14] is of special interest because this study aims at evaluating the response of Egyptian clover plants to seed irradiation and AA + GSH treatments under both, control and $AlCl_3$ stress conditions.

MATERIALS AND METHODS

Plant Material and Treatments: This pot experiment was conducted in the experimental field in the National Center for Radiation Research and Technology (NCRRT), Nasr City, Cairo, Egypt. Egyptian clover seeds (cv. Giza 6) were obtained from the Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Giza. Eighteen grams of seeds were divided into 3 equal groups and were treated as follows:

First group: Seeds were untreated and used as a control. Second group: seeds were irradiated with a dose of 15 Gy at a dose rate of 0.7 Gy/ min using a self-contained dry- storage biological gamma irradiator [Canadian Gamma Cell- 40 (^{137}Cs)] installed at NCRRT.

Third group: seeds were germinated in pots, then plantlets were treated by spraying them with (50 ppm GSH + 250 ppm AA) after 20 and 30 days from sowing.

Seeds (0.5 g/ pot) were sown on the 5th of Oct. 2014 in each of the thirty six pots (12 x 40 cm diameter pots for each group of seeds) that were used in this experiment after being filled with 10 Kg medium. Soil analysis conducted in the Faculty of Agriculture, Cairo university showed that the medium had a loamy sand texture (87 % sand: 7.3 % silt: 5.7 % clay) and an initial pH of 7.19. Its EC was 1.23 dS/ m and the concentrations of soluble anions were 3.0, 5.0 and 4.6 meq/ l of HCO_3, Cl and SO_4, respectively. The concentrations of soluble cations were 6.0, 2.5, 0.10and 4.0 meq/ l for Ca, Mg, K and Na, respectively. The medium's field capacity was 11.5, its porosity (%) was 38.6 and its bulk density was 1.72 g/ cm3.

Of each group of 12 pots, six were irrigated with tap water and the other six were irrigated with a 0.5 mM $AlCl_3$ solution. Similar quantities of water/ $AlCl_3$ solution (1.5 l/ pot) were used in the irrigation of different pots on a weekly basis. Fertilization rate applied during medium preparation was according to recommendations of the Ministry of Agriculture and Land Reclamation [(200 kg super phosphate calcium + 75 kg potassium sulfate + 15 Nitrogen units)/ Fed]. The following data was recorded 50 days after sowing and results were statistically analyzed.

Morphological Characteristics: Shoot length (cm), root length (cm), number of branches as well as plant fresh weight and dry weight (g) were measured 50 days after sowing.

Antioxidant Enzymes Activity: Samples were extracted as described by Gong *et al.* [15]. Leaf samples were homogenized with 0.05 M phosphate buffer (pH 7.0) containing 10% polyvinil pyrrolidone and 0.1 M Ethylene Diamine Tetra Acetic acid (EDTA).The homogenate was centrifuged at 14000 x g for 16 min at 4°C. The supernatant was used for the peroxidase assay. Peroxidase activity was measured by the method of Vetter *et al.* [16] as modified by Gorin and Heidema [17]. The assay mixture contained 0.1 ml enzyme extract, 1.35 ml 100 mM MES buffer (2-morpholino ethane sulfonic acid, monohydrate) (pH 5.5), 0.05% H_2O_2 and 0.1% p- phenylenediamine. Changes in absorbance were recorded at 485 nm for 3 min with a spectrophotometer. The activity of peroxidase was presented as $\Delta OD_{485nm}\ min^{-1}\ mg^{-1}$ protein.

Phenoloxidase activity was determined according to a modification made to the method described by Ishaaya [18], in a reaction mixture consisting of 0.5 ml phosphate buffer (0.1 M, pH 7), 200µl sample extract and 200µl catechol solution (2%). Prior to the initiation of the reaction, samples and other ingredients of the reaction mixture were separately incubated at the optimum temperature of reaction (25° C). Enzyme reaction was initiated by adding catechol solution, then after exactly 1 min, the optical density was determined. Zero adjustment was against sample blank. The phenoloxidase activity was determined as $\Delta OD_{405\ nm} \times 10^3\ min^{-1}\ mg^{-1}$ protein.

Catalase activity was measured according to Aebi [19]. The assay mixture contained 0.1 ml of enzyme extract, 0.1 mM phosphate buffer (pH 7.5), 0.1 M EDTA and 0.3% H_2O_2 and the absorbance was measured at 240 nm. Catalase activity was expressed as $\Delta OD_{240\ nm}\ min^{-1}\ mg^{-1}$ protein. For the calculation of catalase activity, the extinction coefficient of 0.036 $mM^{-1}cm^{-1}$ was used.

Statistical Analysis: Pots were arranged in a randomized complete block design with three replications (two pots each). Experimental data obtained was treated with Analysis of Variance (ANOVA) at confidence level of 95 %, which is the procedure used for testing the differences among means of two or more treatments and the differences between means were detected using least significant difference (LSD) at $P \leq 0.05$. All data was analyzed using statistical software (MSTATC 2.10, Russell D. Freed).

RESULTS AND DISCUSSION

Morphological Characters: Data presented in table (1) show that irrigation using a 0.5 mM $AlCl_3$ solution significantly reduced shoot length, root length and number of branches compared to irrigation using $AlCl_3$ – free water. Moreover, statistically, reductions in shoot and root length were significant. On the other hand, the (50 ppm GSH + 250 ppm AA) treatment significantly increased shoot and root length compared to untreated controls. Contrarily, pre-sowing seed irradiation had a significant negative impact on root length and resulted in an insignificant increase in shoot length compared to the control. Meanwhile, both treatments effect on the number of branches was trivial. Regarding the interaction of both factors under investigation, plantlets sprayed with GSH and AA and irrigated with $AlCl_3$ – free water recorded the highest values while pre-sowing seed irradiation followed by $AlCl_3$ solution irrigation resulted in the least value for all three morphological criteria studied compared to the other five treatments. Moreover, GSH + AA treatment was found to be effective in maintaining a significantly high shoot length and no. of branches under $AlCl_3$ stress. Pre-sowing seed irradiation had a similar effect on no of branches only.

Although symptoms of Al toxicity are manifested in the shoots, these are usually regarded as a result of root damage [20]. Haug and Vitorello [21] reported that Long-term exposure to Al and inhibition of root growth generally leads to P, K, Ca and Mg deficiencies. Vitorello *et al.* [22] added that the most common responses in shoots to Al toxicity are cellular modifications in leaves, reduced stomatal opening, decreased photosynthetic activity, chlorosis and foliar necrosis. In this regard, Ciamporova [23] and Vardar *et al.* [24] primarily attributed root growth inhibition to inhibited cell elongation and expansion, which are followed by inhibited cell division. Generally, limited growth recorded in response to $AlCl_3$ stress can be simply justified by the expected limited nutrients uptake as a result of root damages caused by Al toxicity [25]. In this regard, Wang and Kao [26] reported that AA and GSH protective role in counteracting rice roots growth inhibition in response to $AlCl_3$ was unlikely caused by blockage of Al uptake.

Table 1: Effect of pre-sowing seed irradiation and plantlet (GSH + AA) spraying on shoot length, root length and number of branches of Egyptian clover plants under $AlCl_3$ stress.

	Shoot length (cm)			Root length (cm)			No. of branches		
Treatment	Water Irrigation	$AlCl_3$ (0.5 mM)	*Mean (B)*	Water Irrigation	$AlCl_3$ (0.5 mM)	*Mean (B)*	Water Irrigation	$AlCl_3$ (0.5 mM)	*Mean(B)*
Control	9.2 b	8.3 b	*8.8 b*	14.2 b	10.2 c	*12.2 b*	9.1 a	7.0 ab	*8.0 a*
50 ppm GSH + 250 ppm AA	19.3 a	17.1 a	*18.2 a*	25.1 a	13.1 b	*19.1 a*	10.8 a	7.3 ab	*9.1 a*
15 Gy irradiated seeds	14.5 a	4.4 b	*9.5 b*	12.7 bc	4.8 d	*8.7 c*	8.1 ab	3.8 b	*6.0 a*
Mean (A)	*14.3 a*	*9.3 b*		*17.3 a*	*9.4 b*		*9.3 a*	*6.0 b*	

For each factor (A, B or A x B) means bearing a common letter are insignificantly different at $P < 0.05$.

Table 2: Effect of pre-sowing seed irradiation and plantlet (GSH + AA) spraying on Egyptian clover plant fresh weight and dry weight under $AlCl_3$ stress.

	Fresh weight (g)			Dry weight (g)		
Treatment	Water Irrigation	$AlCl_3$ (0.5 mM)	*Mean (B)*	Water Irrigation	$AlCl_3$ (0.5 mM)	*Mean (B)*
Control	0.753 d	0.537 de	*0.645 b*	0.230 bc	0.113 d	*0.171 b*
50 ppm GSH + 250 ppm AA	3.517 a	2.153 b	*2.835 a*	0.320 a	0.283 ab	*0.302 a*
15 Gy irradiated seeds	1.253 c	0.203 e	*0.728 b*	0.200 c	0.160 cd	*0.180 b*
Mean (A)	*1.841 a*	*0.964 b*		*0.250 a*	*0.186 b*	

For each factor (A, B or A x B) means bearing a common letter are insignificantly different at $P < 0.05$.

GSH is the most abundant low molecular weight tri-peptide in all mitochondria-bearing eukaryotes including plants. In plants, it's involved in many cellular processes, including defense against ROS [27]. El-Awadi *et al.* [28] attributed significant increases in plant height in two wheat cultivars to GSH and AA, which is in accordance with our findings. The authors also reported that the number of leaves were relatively less responsive to treatments, which is in harmony with the insignificant effect recorded for GSH + AA on the number of branches.

Stimulated shoot length in response to seed γ- irradiation found in this trial is in harmony to some extent with significant shoot length increases reported by Soliman and Abd El-Hamid [29] for *Phaseolsus vulgaris* cv.Giza 6 in response to 25- 50 Gy seed irradiation. Unlike clover in this trial, kidney beans recorded a significant increase in branch numbers as a result of low gamma dose seed irradiation. Similar results were reported for chamomile plants [30].

Plant Fresh Weight and Dry Weight: As shown in table (2), irrigation with a solution containing 0.5 mM $AlCl_3$ significantly reduced both FW and DW compared to irrigation using $AlCl_3$- free water. On the other hand, both FW and DW records followed a similar trend in response to pre-sowing seed irradiation and (GSH + AA) plantlet spraying, where the radiation treatment resulted in insignificant increases compared to controls, while the antioxidants mixture caused significant increases in both FW and DW. FW values recorded for the interaction between both factors under investigation followed a trend which was not much different than that noticed for the main factors. Meanwhile, both, (GSH + AA) and irradiation treatments led to insignificant differences in DW between plants irrigated with $AlCl_3$- free water and 0.5 mM $AlCl_3$ solution, which implies insignificantly affected assimilates accumulation in plants as a result of investigated treatments.

Reduced growth characteristics discussed earlier in this study are well reflected in FW and DW values recorded. The ultimate consequence of inhibited root and shoot growth in response to Al toxicity is reduced plant biomass. This is attributed to the fact that root damage caused by Al toxicity consequently results in a damaged root system with an impaired efficiency in nutrient uptake [25]. El-Awadi *et al.* [28] reported that GSH and AA treatments (50 and 100 ppm) caused significant increases in shoot FW of the wheat cultivars Sakha 93 and Giza 168. Moreover, increases in fresh weight were associated with increases in dry matter, with 100 ppm GSH foliar application resulting in maximum shoot DW. Our findings are also confirmed by results of Bakry *et al.* [31] and Talaat and Aziz [32] who demonstrated that AA and GSH, respectively, increased plant growth. Such increase might be attributed to enhanced cell division. In this regard, Noctor *et al*. [33] stated that plants cannot survive without GSH that has functions in plant development which cannot be performed by other thiols or antioxidants.

Under standard growth conditions, ROS levels in a plant cell are under tight control of scavenging systems that include GSH. Oxidative stress occurs when ROS, the generally very reactive molecules possessing an unpaired electron are not adequately removed. Excess ROS formed within cells can provoke oxidation and modification of cellular amino acids, proteins, membrane lipids and DNA. These changes lead to oxidative injuries and result in the reduction of plant growth and development [34]. Role of GSH in ROS detoxification starts at early plant developmental stages.

Generally, insignificant increases in FW and DW in response to pre- sowing seed irradiation found in this study are in line with significant increases reported by Soliman and Abd El-Hamid [29] in kidney bean shoots. Meanwhile, the authors reported significant reductions in growth attributes in response to high (100- 150 Gy) γ- irradiation doses. An enhancing effect for γ- irradiation on chamomile shoot and root fresh and dry weights

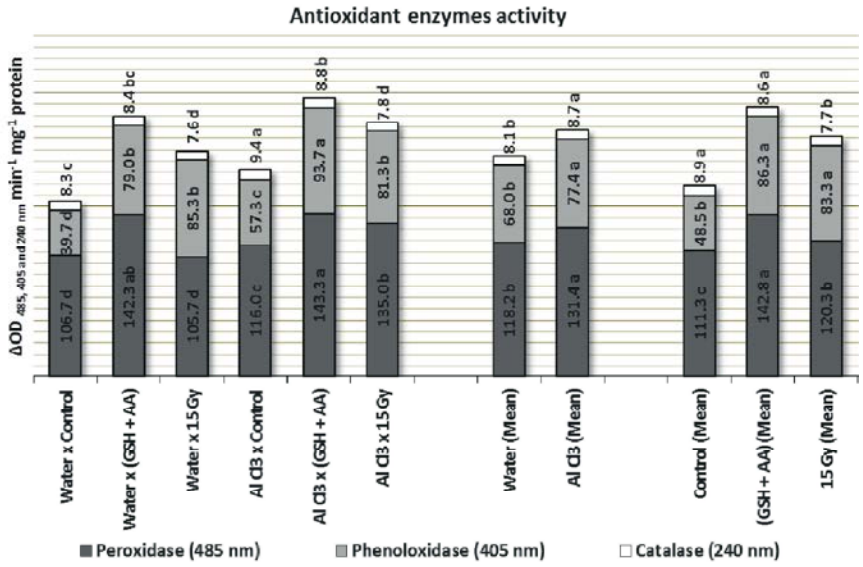

Fig. 1: Effect of pre-sowing seed irradiation and plantlet (GSH + AA) spraying on some antioxidant enzymes (peroxidase, phenoloxidase and catalase) activity in Egyptian clover plants grown under $AlCl_3$ stress.

compared with plants produced from non-irradiated seeds was also reported by Nassar *et al*. [30]. Similarly, maize (*Zea mays* L.) pure strain G4 produced plants that recorded enhanced biomass under both, normal irrigation and drought conditions when its grains were treated with γ- radiation [35].

Antioxidant Enzymes Activity: As illustrated in figure (1), both factors under investigation and their interaction together significantly affected peroxidase, phenoloxidase and catalase activities in Egyptian clover plants. Significant increases in activity were recorded for all three antioxidant enzymes in response to $AlCl_3$ stress. Meanwhile, (GSH + AA) foliar application led to significant increases in the activitiy of peroxidase and phenoloxidase only, compared to controls. It was also found that pre- sowing seed irradiation resulted in a significant decrease in catalase activity and significant increases in peroxidase and phenoloxidase activities compared to controls. A significant difference in activity between the (GSH + AA) treatment and the seed irradiation treatment in favor of (GSH + AA) was recorded for peroxidase and catalase only, while statistical insignificance was recorded for phenoloxidase.

As shown in Figure (1), water- irrigated and $AlCl_3$ treated plants recorded insignificant differences in-between in peroxidase and catalase activities while phenoloxidase activity recorded a significant increase in favor of plants under $AlCl_3$ stress. On the other hand, only peroxidase activity showed a significant increase in samples collected from plants under $AlCl_3$ stress compared to samples from plants irrigated with $AlCl_3$- free water.

In this regard and for different plant species, several studies have reported that oxidative stress resulting from heavy metals stress was coupled with increased antioxidant enzymes activity [36 - 39]. Enhanced oxidative defense systems have been reported to be correlated with tolerance to different stress conditions, thus allowing plants to develop normally under adverse environmental conditions [38, 39]. In this regard, Apel and Hirt [40] stated that catalase and peroxidase are part of the repertoire of enzymatic antioxidant defense system that provides protection against the ROS. Mikani *et al.* [41] explained that peroxidase and the antioxidant enzymes superoxide dismutase and catalase convert the potentially dangerous O_2 and H_2O_2 to water through their combined action. Moreover, Singh and Singh [42] stressed on the important role catalase plays in the removal of hydrogen peroxide (H_2O_2) from the cells to avoid its accumulation to toxic levels. Unlike the increased activity of catalase in response to $AlCl_3$ stress observed in this study, Sandalio *et al.* [43] reported a decrease in catalase activity in pea seedlings under cadmium stress.

Antioxidant enzymes are very good biochemical markers for stress and increasing their activities could have a remediation potential [39]. Morsy *et al.* [44] reported increases in peroxidase in two *Zygophyllum* species (*Z. album* and *Z. coccineum*) and concluded that soil heavy metal pollution enhanced oxidative stress in

both species. They added that both species overcame heavy metal stress through the production of scavenging systems, though, *Z. album* recorded higher antioxidant enzyme activities and greater lipid peroxidation scavenging compared to *Z. coccineum*.

Increased activity of peroxidase in response to (GSH + AA) treatment is in harmony with results reported by Ejaz *et al.* [45] who found that the activity of peroxidase in sugarcane plants was increased under salt stress as well as after AA application. This higher level of antioxidant enzyme is probably attributed to its role in helping plant's resistance against oxidative damage. Similarly, Athar *et al.* [46] reported increased antioxidant enzymes activity in wheat plants after AA application. Earlier work suggested that increases in activity of antioxidant enzymes helps plants maintain their growth under stress conditions and may be regarded as an indicator of tolerance [47].

Gamma irradiation was reported to induce oxidative stress with overproduction of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxides, which react rapidly with almost all structural and functional organic molecules [48]. To avoid oxidative damage, plants have evolved various protective mechanisms to counteract the effects of ROS in cellular compartments [49]. One of the protective mechanisms was the enzymatic system, which operates with the sequential and simultaneous actions of a number of enzymes including Peroxidase [50]. Radiation is a well-known factor that affects antioxidant status and increases free oxygen radical generation. In this regard, it worth mentioning that Sreedhar *et al.* [51] revealed a variable degree of stimulation in the activities of peroxidase in leaves of 21 day old seedlings of groundnut (*Arachis hypogaea* L.) irradiated with selected doses of gamma rays. Enzyme induction was significantly correlated with the dose of irradiation.

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