American-Eurasian Journal of Scientific Research 3 (2): 139-146, 2008
ISSN 1818-6785

Alterations of Some Secondary Metabolites and Enzymes Activity by Using Exogenous Antioxidant Compound in Onion Plants Grown Under Seawater Salt Stress

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ABSTRACT: A possible survival strategy of plants under saline conditions is to use some compounds that could alleviate the deleterious effect of salt stress. This study was designed to examine the effect of exogenous application of α-tocopherol (Vit.E) and potassium dihydrogen phosphate (KH_2PO_4) to mitigate the deleterious effect of diluted seawater treatments (2500 and 5000 ppm) in onion (*Allium cepa* L.) plants. Generally, seawater salt stress induced changes in the content of phenolic and flavonoids compounds, as well as pungency levels. Total phenolic content were varied from 28.36 to 51.35 mg g^{-1} d.w., the highest content was found at 300 ppm salt combined with alpha-tocopherol. Whereas, total flavonoids content varied significantly from 24.81 to 48.75 mg g^{-1} d.w., the lowest content was found with control treatment (300 ppm) combined with Vit. E and KH_2PO_4 treatments. The overall means of pungency at 300, 2500 and 5000 ppm salts were 7.32, 9.3, and 11.8 μmol g^{-1} f.w., respectively. Glutathione S-transferase (GST) and phenylalanine ammonia-lyase (PAL) were also determined. Treatment with 5000 ppm seawater salt stress combined with Vit. E plus KH_2PO_4 had the highest GST specific activity among all other treatments (4.91 μmol/min/ mg protein). Significant increase in PAL activity (0.62 μmol trans-cinnamic acid min^{-1} mg^{-1} protein) was noticed when Vit. E and KH_2PO_4 were applied under 5000 ppm salt stress. The above mentioned results indicated that applying of alpha-tocopherol or elemental KH_2PO_4 could be used to ameliorate the deleterious effect of salt stress and could be enhance the level of some secondary metabolite in onion plants. This restoration may be a consequence of several roles played by such compounds, which can cause triggering of the internal cellular metabolism.

Key words: Flavonoids · Glutathione S-transferase · Onion · Phenylalanine ammonialyase · Seawater salt stress

INTRODUCTION

Plants are sessile organisms constantly challenged by a wide spectrum of biotic and abiotic stresses. Salt stress is one of the most serious factors limiting the productivity of different crops. Generally, plants produce secondary metabolites in nature as a defense mechanism against attack by pathogens and insects. Moreover, it could be possible to enhance a wide variety of useful metabolites in plant through applying different environmental stresses [1]. Some of these metabolites have light absorptive properties, harvest light for photosynthesis and protect the cells from damaging effects of high energy radiation, while others promote defensive action against herbivores and pathogens [2]. Onions (*Allium cepa* L.) was among the earliest cultivated crops and has been popular in folk medicine for centuries. It is well known for its health benefits, numerous therapeutic proprieties have been reported in onion i.e. anticarcinogenic, antibiotic, anti-bacterial, anti-fungal and antioxidant properties [3]. Onion flavor intensity has been studied in response to various growing conditions and little is known about its response to salt stress. Improving the content of some active compounds (volatile sulfur compounds, ascorbic acid, carbonyl compounds, vitamins and flavonoieds) are the most important pharmaceutical content and could be enhanced through different environmental stresses in some vegetables [1]. Moreover, salt stress may affect soluble solids content (SSC), bulb pungency as measured by total pyruvate (TPY), bulb sulfur (S) and sulfate (SO^{-}_{4}) accumulation, flavor precursors, and their biosynthetic intermediates in onion [4]. The soluble phenolics compounds are the most widely distributed secondary metabolites in plant kingdom and could be enhanced as a

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powerful antioxidant in plant tissues under different stresses [5]. Phenolic compounds in onions are mainly formed by anthocyanins and flavonoids, these constituents may be involved in response of onion bulbs to abiotic stress [6]. Flavonoid is a (group of secondary metabolietes) polyphenolic compounds, widely present in onions and considered as an important factors of the overall antioxidant activity [7]. With regard to flavonoids, onion leaves are characterized by the highest total flavonoid content in comparison to other 62 common vegetables [8]. The pungent flavor is produced when onion cells are ruptured, releasing the enzyme alliinase (EC 4.4.1.4), which reacts with flavor precursors, S-alk (en)yl-cysteine sulfoxide, to produce many volatile sulfur compounds, along with pyruvic acid and ammonia [9]. Previous studies on onion pungency have reported that flavor strength could be greatly affected by cultivar, soil type and other environmental factors [10-11]. Most of the enzymes are stress-inducible and play an important role in the protection of plants from adverse effects of stresses [12]. Plant glutathione S-transferases (GSTs, EC 2.5.1.18) are a family of multifunctional enzymes involved in the intracellular detoxification of toxic compounds produced endogenously [13]. Phenylalanine ammonialyase (PAL) catalyses the elimination of ammonia from L-phenylalanine to give trans-cinnamate, this being the first committed step for the biosynthesis of plant specific phenylpropanoid derivatives such as phenolics [14]. Activity of PAL was found to vary greatly with stage of plant development and with various stresses [15]. On the other hand, saline water has been used to improve fruit quality of tomatoes grown in nutrients film culture [16]. However, Ågren [17] mentioned the effect of elements and exogenous application of antioxidant compounds on the primary metabolites of various crops, but these aspects have been less studied for secondary metabolites. Several reports have indicated that application of growth regulators and/or vitamins on crops alleviating the adverse effects of salt and ozone stresses [18-19]. Exogenous application of some antioxidants and other substances e.g. alpha tocopherol, salicylic acids (SA), acetyl salicylic acid, oleic acid, gamma antibutriec and elemental sulfur compounds enhanced date palm plantlet tolerance ability to environmental stress [20]. Alpha-tochopherol is low molecular weight lipophilic antioxidants which protect membrane from oxidative damage [21]. The effects of supplementary K^+ on some secondary metabolites were studied. Moreover, K^+ has mitigated negative effects of NaCl treatment on strawberry [16]. The role of potassium (K^+) is vital for osmoregulation and stimulating photosynthesis process [22]. The aim of the present work was to investigate the possibility of applying of alpha-tocopherol or elemental potassium di-hydrogen phosphate (KH_2PO_4) to ameliorate the deleterious effect of salt stress and could be enhance the level of some secondary metabolite in onion plant.

MATERIALS AND METHODS

Plant Materials and Growth Conditions: Seeds of onion *(Allium cepa* L.) named Behary red the most popular cultivar in Egypt was obtained from Onion Department, Agriculture Research Center, Giza, Egypt. Two pot experiments were conducted in the greenhouse of National Research Center, Dokki, Cairo, Egypt during 2005/2006 and 2006/2007-winter seasons. Seedling were grown under natural photoperiod and light intensity, temperature 25/21°C, relative humidity 75% and photosynthetic photon flux density 350 μmol m^{-2} s^{-1}. The practices of pot preparation with air dried clay loam soil, optimum fertilizer applications (P_2O_5, K_2O, and NH_4) and irrigation were standard for onion culture as described previously by [10]. Seedlings of the onion cultivar were transplanted after 40 days from sowing and salt stress treatments were started at 60 days from sowing. All treatments were designed in split-plots design with three replicates, where the three salinity treatments arranged randomized within the main plots.

Salt Treatments: Seawater (33.000 ppm) was collected from Mediterranean Sea, near Alexandria city (the major ions of seawater were: 487 mM Na^+, 10 mM K^+, 54 mM Mg^{2+}, 586 mM Cl^-, plus other less concentrated macro- and micro-nutrients). Tap water (300 ppm salts) used as control and diluting seawater (2500 and 5000 ppm) were applied. Additionally, spraying of vitamin E (Vit. E formulation containing 50% α-tochopherol, used at 200 ppm) and potassium di-hydrogen phosphate (KH_2PO_4 used at 200 ppm) were applied.

Experimental Design: The plants were divided into three groups: (i) Plants which irrigated by tap water (300 ppm) as a control and diluted seawater salts (2500 and 5000 ppm) (ii) Plants received seawater salt level (300, 2500 and 5000 ppm) plus spraying α-tocopherol (iii) Plants received seawater salt level (300, 2500 and 5000 ppm) plus spraying α-tocopherol combined with KH_2PO_4. It means that, 9 treatments which were the interaction within

3 salinity levels, 3 spraying α-tocopherol and 3 spraying α-tocopherol combined with KH_2PO_4
Onion bulbs were used for the following measurements:

Determination of Phenolic Content: Total phenolic were determined using the Folin-Ciocalteau reagent [23]. Bulb tissues (1 g) were homogenized in 80% aqueous ethanol at room temperature and centrifuged at 10000 rpm for 15 min and the supernatant was saved. The residue was re-extracted twice with 80% ethanol and supernatants were evaporated to dryness at room temperature. Residue was dissolved in 5 ml of double distilled (dd) H_2O. One-hundred microlitres of this extract was diluted to 3 ml with dd H_2O and 0.5 ml of Folin-Ciocalteau reagent was added. After 3 min, 2 ml of 20% of sodium carbonate was added and the contents were mixed thoroughly. The absorbance was measured at 650 nm by spectrophotometer after 60 min using pyrogallol as a standard. The results were expressed as mg pyrogallol equivalent/g dry weight.

Determination of Total Flavonoid: Total flavonoid contents were measured by the aluminum chloride colorimetric assay as described previously [24]. One ml aliquot of previous extracts or standard solutions of quercetin (QE) at different concentrations were added to a 10 ml volumetric flask containing 4 ml dd H_2O, then 0.3 ml 5% $NaNO_2$ was added. After 5 min, 0.3 ml of 10% $AlCl_3$ was added. Finally, after 6 min, 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with dd H_2O. The solution was mixed thoroughly and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid contents were expressed as mg quercetin equivalent (QE)/ g. dry weight.

Pungency Determination: Pungency was determined as pyruvic acid [9, 25]. The content of pyruvic acid was estimated using dinitrophenylhydrazine (DNPH) reagent. Bulb tissue was homogenized for 1 min in a blender at a ratio of 2 ml of water per gram of tissue; the homogenate was filtered through two layers of cheesecloth. An aliquot of this filtrate was transferred to a 1.5 ml centrifuge tube and clarified by centrifugation at 12000 rpm for 5 min. Then 100-fold dilution of onion juice was made by adding 10 µl of the clarified onion filtrate to 1.0 ml of water in test tube, then 0.5 ml of 0.125 g l^{-1} DNPH in 2 M HCl was added. The samples were placed in water bath at 37°C, and removed after 10 min; finally 2.5 ml of 0.6 M NaOH was added. The absorbance at 420 nm was determined. Standards were prepared by adding 25–200 µl of 1mM sodium pyruvate. The concentration of pyruvate in the samples was expressed as µmol g^{-1} f.w.

Determination of Enzymes Activity

Preparation of cytosolic fraction: Tissues (about 1g) were excised and homogenized using mortar and pestle in 4 ml of ice-cold grinding buffer containing 250 mM sucrose, 25 mM Tris and the pH was adjusted to 7.2. The homogenate was centrifuged at 12000 rpm for 15 min at 4°C. The resulting supernatant was used for analyses of GST and PAL enzyme activities.

Glutathione S-transferase Assay: Activity of GST (EC 2.5.1.18) was assayed in a reaction mixture containing 50 mM phosphate buffer, pH 7.5, 1 mM of 1-chloro- 2, 4 dinitrobenzene (CDNB) and crude extract equivalent to 100 µg of protein. The reaction was initiated by adding 2 mM reduced glutathione (GSH) and formation of S-(2,4-dinitro phenyl) glutathione (DNP-GS) was monitored as an increase in absorbance at 334 nm as described previously [26]. The enzymes activity is expressed in units (U). One unit represents the conversion of 1µmol substrate to product per min.

Phenylalanine Ammonia-lyase Assay: Activity of PAL (EC 4.3.1.5) was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid as described by Dickerson [27]. A sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer at 30°C. The optical density (O.D) values were recorded at 290 nm for approximately 5 minutes. The amount of trans-cinnamic acid formed calculated using its extinction coefficient of 9630 M^{-1} cm^{-1}. Enzyme activity was expressed as µmol trans-cinnamic acid min^{-1} g^{-1} protein.

Determination of Protein: Protein levels of cytosolic were determined spectrophotometrically at 595 nm, using comassie blue G 250 as a protein binding dye [28]. Bovine serum albumin (BSA) was used as a protein standard.

Statistical Analysis: Data were statistically analyzed using Costate Statistical Package [29].

RESULTS AND DISCUSSION

Total phenolic content of the extracts varied significantly from 28.36 to 51.35 mg g^{-1} d.w. as shown in Table 1, the lowest content was found at 300 ppm salt

Table 1: Effect of α-tocopherol and KH_2PO_4 on total phenolic, flavonoids and pungency levels of onion plant grown under seawater stress

Sprayed compounds	Diluted seawater (ppm)	Total phenolic mg g^{-1} d.w.	Flavonoids content mg g^{-1} d.w.	Pungency content μmol g^{-1} f.w.
Unsprayed	*T.W. (300)	39.68±0.06^{e}	30.08±0.108^{d}	7.32±0.036^{e}
	2500	37.38±0.34^{f}	34.08±0.140^{c}	8.31±0.059^{c}
	5000	34.51±0.15^{g}	29.96±0.057^{de}	11.8±0.037^{a}
α-tocopherol	T.W. (300)	51.35±0.10^{a}	48.75±0.086^{a}	6.92±0.088^{f}
	2500	45.37±0.05^{c}	33.87±0.063^{c}	6.01±0.043^{h}
	5000	28.72±0.05^{h}	26.67±0.052^{f}	7.76±0.069^{d}
α-tocopherol + KH_2PO_4	T.W.(300)	28.36±0.06^{h}	24.81±0.073^{g}	6.83±0.058F^{g}
	2500	50.84±0.04^{b}	39.87±0.023^{b}	9.30±0.070^{b}
	5000	42.37±0.03^{d}	29.75±0.053^{e}	6.87±0.077^{g}

$^{a,b,c,....}$Means within same column followed by different letters are significantly different at $P < 0.05$. Values are means of three replicates (±SE)
*T.W. - Tape water

combined with Vit. E and KH_2PO_4, whereas the highest content was found at 300 ppm salt combined with α-tocopherol (Vit. E). In this concern, the amount of total phenolics varied widely and ranged from 845 to 2075 mg.kg^{-1} and from 75 to 115 mg.kg^{-1} of lyophilized tissues of different onion and garlic, respectively [30]. Moreover, the increasing of phenolic compounds in the tissue ameliorates the ionic effect of salt stress [31]. It could be explained that exogenous application of phosphate anion (HPO^{-}_{4}) may be responsible for amelioration of photosynthesis and its related parameters; also this anion is important for the growth and development of the plant. Moreover, potassium cation (K^{+}) increased the osmotic activity causing a reduction in water potential and an influx of water from the surrounding cells. Phenol accumulation could be a cellular adaptive mechanism for scavenging oxygen free radicals during stress, and this free radical scavenger could be radical oxidized in the system of this tissue preventing sub-cellular damage. Flavonoids are a second class of health-enhancing compound produced by the *Alliums* genus. Total flavonoids content (Table 1) varied significantly from 24.81 to 48.75 mg g^{-1} d.w., the lowest content was found at 300 ppm salts combined with α-tocopherol (Vit. E) plus KH_2PO_4. Whereas, the highest content was found at the 300 ppm salt combined with Vit. E treatment. Flavonoids have been linked to defense against various stresses [32]. Generally, salt stress treatments were capable of acting as activators of flavonoid accumulation. Flavonoids were shown to be highly effective scavenger of most types of oxidizing molecules, [(including singled oxygen and varies free radicals such as superoxide radical (O^{-}_{2}), hydroxyl radical (.OH), hydrogen peroxide (H_2O_2) and alkoxyl radical (RO)] many of them play an important physiological and ecology role, being involved in resistance to different types of stress [33]. Meanwhile, Selmar and Mohamed [34] stated that the content of secondary plant metabolites indeed is higher in plants that suffer from drought and salt stresses than those cultivated under optimal conditions. Water shortage and salt stress leads to stomata closure and an enhanced CO_2-deficiency that generates a high oversupply of reduction equivalents. In order to prevent damage by oxygen radicals, $NADHH^{+}$ is reoxidized by photorespiration and violaxanthine cycle. However, the markedly higher concentration of reduction equivalents also leads to a stronger rate of synthesis of highly reduced compounds, like isopernoids, phenols or flavonoids. The pungency of onion is one of the most important parameters should take into account in the quality evaluation of the onion cultivars. The pungency (measured as pyruvate) level in onion bulbs was affected by salt stress as declared in Table 1. The overall means of pungency at 300, 2500 and 5000 ppm salts were 7.32, 8.31 and 11.80 μmol g^{-1} f.w. respectively (unsprayed treatments), it means that pungency was gradually increased as salts stress increased. Similar results were found when onion was grown under salt stress [35]. On the other hand, the pungency level changed moderately in response to α-tocopherol and KH_2PO_4 treatments under salt stress. Tocopherol compounds play an important role as antioxidant in plant cells and act as stabilizer of membrane lipids. However, flavor strength could be affected by cultivar, soil type and other environmental factors [36]. Enhance pungency due to salt stress could be explained through, total flavor precursors (methyl cysteine sulfoxide) were increased significantly in response to salt stress, continuously pungency increased as salt treatments increased. The possibility of applying α-tocopherol to ameliorate salt stress at specific growth stages in order to enhance the accumulation of some active metabolites still needs to be determined because salinity affects onion production as well.

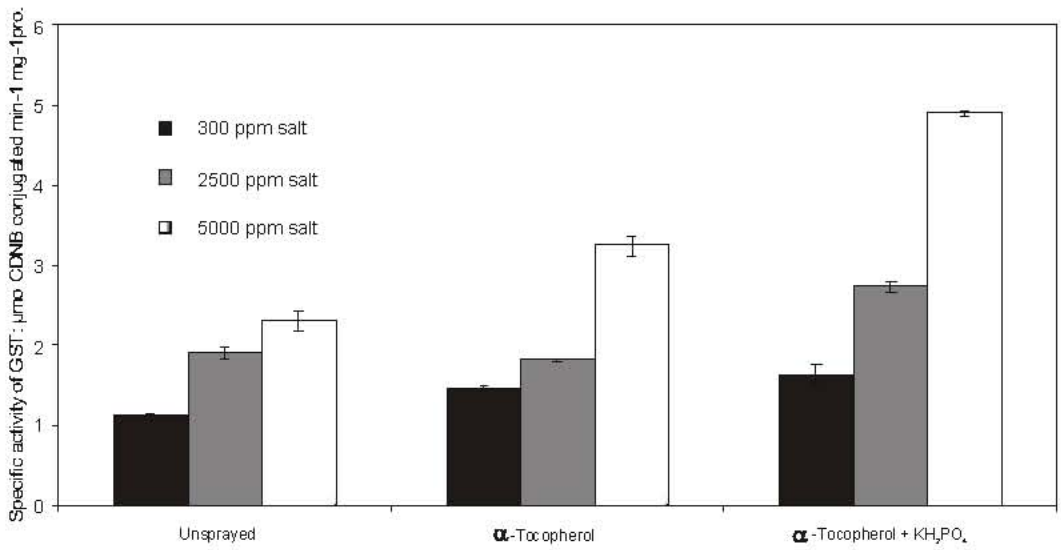

Fig. 1: Change in GST specific activity of onion bulb subjected to α-tocopherol and KH_2PO_4 under salt stress (bars indicate standard error)

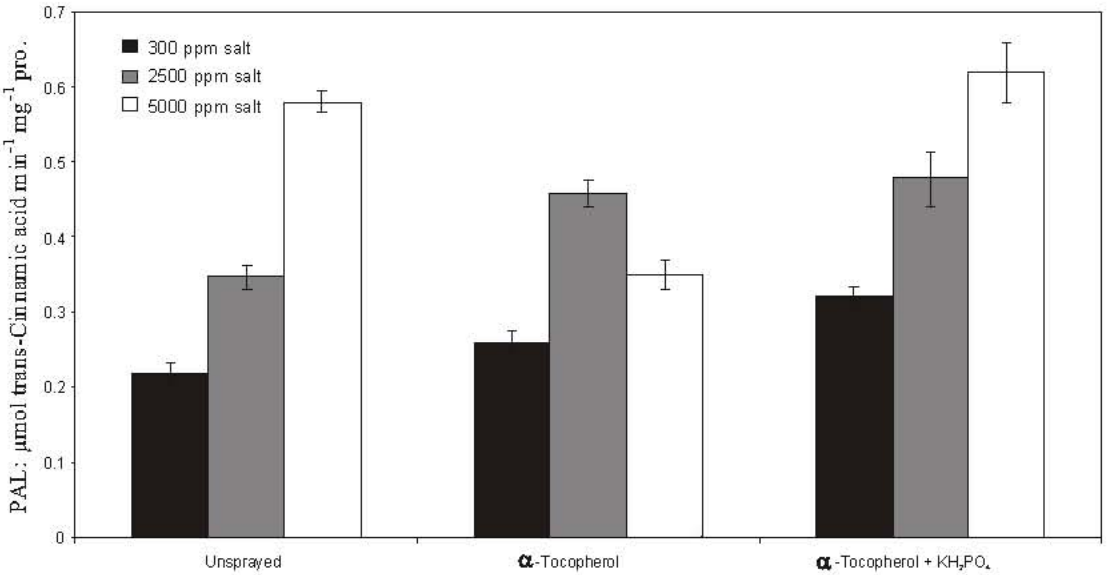

Fig. 2: Change in PAL specific activity of onion bulb subjected to α-tocopherol and KH_2PO_4 under salt stress (bars indicate standard error)

Activity of some antioxidant enzymes such as GST was increased by salt treatments (Fig. 1). However, application of α-tocopherol (Vit. E) to plant enhanced the activity of GST under salt stress; treatment with 5000 ppm salts combined with Vit. E plus KH_2PO_4 had the highest GST specific activity among all other salt treatments (4.91 µmol/min/ mg protein). Excess concentrations of salt stress are known to cause cellular oxidative damage. The induction of GST under salt stress provide additional defense against oxidative stress and keeps the metabolic activities in onion tissue functional. It could be noticed that exogenous application of antioxidant compound (α-tocopherol) caused an inhibition of free radical generation, quench preformed free radicals and continuously enhance the activity of antioxidant enzymes such as GST. The essential for reactive oxygen species (ROS) detoxification during normal metabolism and particularly during stress, are antioxidant enzymes defenses system. In agreement with the present results, Gapińska *et al.* [37] stated that the

severe salt stress caused an increase in GST activities. On the other hand, Hossain *et al.* [38] stated that onion bulb had a higher level of specific GST activity than the activity levels of other vegetables. However, the efficiency of exogenous application of antioxidant (α-tocopherol) to alleviate the deleterious effect of oxidative stress (paraquat stress) was found [39]. Under seawater stress, the antioxidant enzymes activity (SOD, POD, CAT) were relatively higher in Behary red onion cultivar than that detected in Giza 20 and Giza 6 cultivars [40].

The activity of lignifications related-enzymes, i.e. phenylalanine ammonia-lyase (PAL) is generally recognized as a marker of environmental stress in different plant tissues. The results presented in Fig. 2 indicated that under salt stress (5000 ppm), the application of Vit. E plus KH_2PO_4 increased the activity of PAL significantly (0.62 μmol trans-cinnamic acid min^{-1} mg^{-1} protein) than any other treatments. A positive relationship between PAL activity and total phenolics compounds in lettuce were found [41]. The hypothesis indicated that PAL activity was increased under salt stress and this enzyme is involved in the biosynthesis of phenolic and flavonoids compounds. Partially, the present results showed that exogenous application of alpha-tocopherol and K^+ could be improved the tolerance ability of plants against salt stress of onion plant. In addition to the scientific information derived from these studies, the exogenous application of the (α-tocopherol and α-tocopherol + KH_2PO_4) was partially effective in overcoming the adverse effects of salinity; mediated by restoring the metabolic alterations imposed by salt stress.

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