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Expression analysis of AtTPS1 gene coding for trehalose-6-phosphate synthase in salt stressed olive (*Olea eurropaea* L.*)*

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Salinity is one of the major abiotic stresses that affect plant growth. The identification of novel genes, determination of their expression patterns, and the understanding of their functions in stress adaptation is essential to improve stress tolerance. The determination of genes such as AtTPS1 gene coding for trehalose-6-phosphate synthase (TPS) in plants under salt stress, contributes for the concerning about the cascade of responses in plants under water deficit and points out to target genes for plant breeding. The aim of the present work is to detect the change in the expression of AtTPS1 gene in two olive cultivars, salt moderate (Aggizi shame) and salt tolerance (Pecual) treated with validamycin A (30 µM) and grown under saline condition of 300 mM NaCl using semi-quantitative RT-PCR. An additional point of interest was to study the possible roles of exogenous trehalase inhibitor validamycin A (30 µM), on alleviating the harmful effects of salt stress on photosynthetic pigments (chlorophyll *a&b* and carotenoids), total protein, free proline, total soluble sugar, mineral contents and antioxidant enzyme activities (catalase, ascorbate peroxidase, peroxidase and polyphenol oxidase). Semi-quantitative RT-PCR indicated that the expression of this gene is up regulated in response to salt and validamycin treatments. The results indicated that validamycin A was effective osmoprotectant which offered protective roles for olive cultivars subjected to salt stress and presented a practical implication for olive cultivation in salt–affected soils.

Keywords: ***Olea eurropaea*** **L., Salt stress, Gene expression, Trehalose**

INTRODUCTION

Salinity is a major abiotic stress limiting growth and productivity of plants in many areas of the world due to increasing use of poor quality of water for irrigation and soil salinization (Ashraf and Foolad, 2007). Plant adaptation or tolerance to salinity stress involves complex physiological traits, metabolic pathways, and molecular or gene networks (Almeida et al. 2007). In response to osmotic stress, plants utilize a number of defense strategies to cope with unfavorable conditions. The early events include their sensing and subsequent signal transduction to initiate metabolic responses by activating various stress-responsive genes (Bray, 1997). It is important to analyze the functions of stress-inducible genes not only to understand the molecular mechanisms of stress tolerance and the responses of higher plants, but also to improve the stress tolerance of crops by gene manipulation (Seki et al. 2003).

A typical change in expression during a period of water deficit is the induction of genes involved in the synthesis of various osmolytes, e.g., proline, glycinebetaine, and trehalose, as well as of low molecular weight proteins (Ramanjulu and Bartels, 2002). Among these, trehaose a non-

reducing disaccharide plays a crucial role in metabolic homeostasis and abiotic stress tolerance in various organisms. It catalyzed by enzymes trehalose-6-phosphate synthase (TPS) and, subsequently, trehalose-6-phosphate phosphatase (TPP). This osmoprotectant stablizees proteins and membrane structures under stress (Blázquez et al. 1998). Over-expression of exogenous and endogenous gene encoding TPS is reported to be effective for improving abiotic stress tolerance in many plants (Almeida et al. 2007). The discovery of active genes such as AtTPS1 in plants under salt stress, contributes for the concerning about the cascade of responses in plants under water deficit and points out to target genes for plant breeding (Almeida et al. 2007). Trehalose levels are generally low in plants because the presence of the enzyme trehalase which hydrolyze trehalose to glucose. Hence, it should be possible to direct increased trehalose accumulation by down regulating plant trehalase activity or by expressing the trehalose biosynthetic genes under stress-specific regulation (Penna, 2003). Validamycin A ($C_{20}H_{35}NO_{13}$) is a specific competitive inhibitor of trehalase and raises trehalose in plant tissue.

Olea europaea L. is a traditional tree crop of the Mediterranean basin with a worldwide economical high impact. The expansion in cultivation of olive to areas irrigated with low quality water, mostly saline, limits growth and productivity. Although there are numerous studies on the response mechanism of olive trees to salinity, they are restricted to the ecophysiological level or to the study of a single pathway such as the mannitol metabolism in response to salt stress (Conde et al. 2007). Differently from other fruit tree species, little is known about the physiological and molecular basis of the olive fruit development and a few sequences of genes and gene products are available for olive in public databases. As a result, the molecular basis of salt tolerance in olive at a systems level has not been investigated yet (Bazakos et al. 2012).

In the present study, we investigated the effect of exogenous validamycin A (30 µM) on physiological parameters and antioxidant enzyme activities in two olive cultivars under high concentration of NaCl (300 mM). An additional point of interest was to detect the change in the expression of AtTPS1 gene in two olive cultivars (salt moderate (Aggizi shame) and salt tolerance (Pecual) using semi-quantitative RT-PCR. semi-quantitative RT-PCR indicated that the expression of this gene is up regulated in response to salt and validamycin A treatments.

MATERIALS AND METHODS

Plant material and growth conditions.

This investigation was conducted through the two successive seasons of 2015 and 2016, on two olive cultivars, salt tolerance (Pecaul**)** and salt moderate (Aggizi shame), grown at the orchard of Horticultures Institute Research, Giza governorate. Stress experimental work was carried out on uniform and healthy one year old transplants propagated by cuttings, grown in plastic pots of 25 cm. in diameter and 30 cm. in depth contained 6 kg/pot of loamy sand soil. Each pot contained one plant, the plants were irrigated twice every week intervals by tap water before the application of saline solution. The pots were grouped into four sets. The first set of pots irrigated with tap water and designated as control (T_1), the second set was irrigated with saline solution containing 300 mM NaCl (T_2), the third one were treated with validamycin A (30 µM) (T_3) as a foliar spray (twice weekly) for 90 days. The fourth set was irrigated with saline solution containing 300 mM NaCl plus validamycin A (30 µM) (T_4) as a foliar spray. Irrigation with saline solution was carried out twice weekly for three month. Then, fresh samples of leaves were taken and stored in (-40°C), to be used in total RNA isolation, physiological and biochemical parameters.

Chemical analysis.

Photosynthetic pigments (chlorophyll *a, b* and carotenoids) were measured in olive leaf using the formula of Lichtenthaler (1987). Free proline content was determined following the method of Bates et al. (1973). Total protein was determined according to the method described by Bradford (1976).Total soluble sugars (TSS) were extracted according to Homme et al. (1992). Phosphorous was determined according to Snell and Snell (1967). Potassium and sodium were determined photometrically using a flame photometer (Jackson, 1973). Calcium was determined photometrically by using the atomic absorption method described by Allen (1989). Total nitrogen content was determined using the micro-kjeldahl method Jacobe (1958). Catalase (EC 1.11.1.6) assay method was that adopted by Aebi (1984). Ascorbate peroxidase (APX; EC 1.11.1.11) and peroxidase (POD, EC1.11.1.7) and polyphenol oxidase activities was determined by the method of Yingsanga et al. (2008).

RNA extraction and AtTPS1 gene expression analysis.

The expression pattern of AtTPS1 was analyzed by semi quantitative RT-PCR using gene specific primer. For stress-induced expression assays, RNA was isolated from untreated two cultivars and treated with 300Mm NaCl in combination with validamycin A. Samples were ground to a fine powder under liquid nitrogen in a mortar and pestle and total RNA was isolated using mini kit (Promega A3500, Madison, WI, USA) with an optional RNase-free DNase treatment. First strand cDNA was synthesized with 1 µg of total RNA and oligo (dT) 20 primers using Super Script III RN_{ase} H Reverse Transcriptase (Promega). The RT-PCR was carried out using the following AtTPS1 gene-specific primer: 5'-TTGAGGTCCCCGAAGTCAAAC-3' (forward), and 5'- TGCGGCCAACAATTTCATG-3' (reverse). Amplification of gene specific products from cDNA used the PCR cycle: initial denaturation 94°C for 3 minutes, denature (94°C) for 45 seconds, anneal (54°C) for 45 seconds and extend (72°C) for 2 minutes each for 30 cycles and final extension 72°C for 6 minutes. The experiments were replicated at least three times. After RT-PCR, the PCR products from each sample were analyzed on 1% agarose gels.

Statistical analysis.

The obtained data were statistically analyzed using the one-way analysis of variance as described by Snedecor and Cochran (1969). Means were compared by LSD at 5% using SPSS program version16

RESULTS AND DISCUSSION

Photosynthetic pigments:

Chlorophyll (Chl. *a & b*), total chlorophyll, carotenoids and total pigments content of olive leaves were significantly decreased by salinity stress (300 mM NaCl) as compared with control (Table 1). Application of 30 µM validamycin A to stressed plants increased total chlorophyll content as compared to salt stressed plants.

Upon validamycin A and salt stress treated plants, carotenoids contents of salt moderate (Aggizi shame) recorded non significant variation while slightly increased in salt tolerance (Pecual) as compared with control. Our results revealed that application of validamycin A was effective in reducing the inhibitory action of salt stress on photosynthetic pigments of two olive cultivars. The decrease in chlorophyll content can be attributed to the sensitivity of this pigment to increasing environmental stresses, especially to drought and salinity (Ashraf and Foolad, 2007). Our results revealed that application of validamycin A greatly increased trehalose content which may reduce the inhibitory action of salt stress on photosynthetic pigments of both olive cultivar plants (Jun *et al.*, 2008). Zeid (2009) suggested that trehalose may preserve the stability of the chloroplast envelope and maintained the osmotic potential of the chloroplast.

Proline:

Proline is one of the so-called 'compatible compounds that are commonly found at high concentrations when plants are exposed to salt stress and which confer them with salt stress tolerance (Pagter *et al.,* 2009). The rate of salt stress- induced proline accumulation was considerably higher in Aggizi shami than Pecual (Fig 1). The application of 30 µM validamycin A significantly decreased proline contents of both cultivars as compared with their respective control and corresponding salt stressed plants. Interestingly, the priming of olive plants with validamycin A were decreased the concentration of this osmoticum when compared with control. Similar results were obtained by Nounjana *et al.* (2012) which reported that supplements of rice with trehalose negatively affected proline amounts in both control and salt–stressed conditions resulting in a significant reduction in proline. Furthermore, exogenous trehalose also reduced proline accumulation in two maize cultivars under drought stress while increasing biomass production, improving plant water relations and some key photosynthetic attributes (Ali and Ashraf, 2011). It appears that treatment of olive plants with validamycin A in response to irrigation with saline water has a significant role in high alleviation of salinity stress, it may be presumed in this case that osmoprotective effects of the accumulated trehalose reduced the need for plants to accumulate proline.

Total soluble sugars (TSS):

The effect of validamycin A on the total soluble sugars of both olive cultivars under saline condition are shown in Figure (1). As with other cellular constituents sugar levels are affected by stress. In validamycin A treated plants contents of sugar were significantly increased as compared with non-treated plants.

Table 1: Change in photosynthetic pigments (chlorophyll *a&b* and carotenoids, mg g^{-1}FW), in leaves of two olive cultivars treated with 30 µM validamycin A under salt stress of 300 mM NaCl.

Cultivars	Treatments	Chlorophyll *a*	Chlorophyll *b*	Total Chlorophyll	Carotenoids	Total pigments
Pecual	T_1	1.31	1.05	2.38	0.30	2.68
	T_2	1.11	0.84	1.95	0.15	2.10
	T_3	1.20	0.94	2.14	0.22	2.36
	T_4	1.17	0.92	2.10	0.19	2.29
Aggizi shame	T_1	1.01	0.82	1.84	0.06	1.90
	T_2	0.45	0.37	0.23	0.06	0.29
	T_3	1.05	0.88	1.93	0.06	1.99
	T_4	1.03	0.86	1.92	0.05	1.97
	LSD 0.05	0.133	0.134	0.126	0.013	0.186

Where, T_1 (Control); T_2 (300 mM NaCl); T_3 (30 µM of validamycin A) and T_4 (300 mM NaCl + 30 µM of validamycin A). Means having the same letters in a column were not significantly different at $p<0.05$.

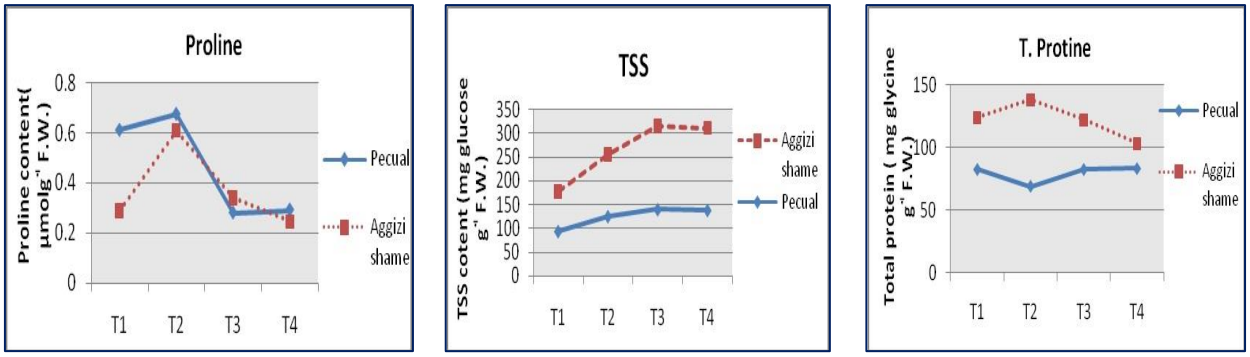

Fig.1: Change in proline, total soluble sugars (TSS), and total protein (T. protein) contents in two olive cultivars treated with 30 µM validamycin A under saline conditions (300 mM NaCl).

Under saline conditions, treated with validamycin A caused 1.5 and 2 fold increases than untreated in total sugar content at Pecual and Aggizi shame, respectively. Soluble sugars may play a key role in osmotic adjustment at the cellular level of plants under salt stress (Gupta and Kaur, 2005). The osmoregulation was performed in glycophytic plants (such as olive) through accumulation of organic compounds such as soluble sugars within the cytoplasm to reduce the harmful effects of salt stress (Levit, 1980).

Total protein:

The results in Figure (1) shows that, protein content was significantly decreased by salinity stress (300 mM NaCl) as compared with control in salt tolerant (Pecual), in contrast, protein content significantly increased in stressed Aggizi shame (salt moderate). Total protein contents decreased in Aggizi shame when compared with validamycin treated plants. This decrease may have resulted from an adverse effect of NaCl on protein synthesis or proteolysis (Joshi and Misra, 2000 and Best et al. 2011).

Mineral content:

When the plants were stressed with NaCl, the concentrations of most elements differed significantly from those in the control. Results in Figure (2) showed the response of K, Na and Na/K ratio of both olive cultivars subjected to (300 mM NaCl), the significant accumulation of sodium was increased under salt stress conditions in both cultivars. Exogenous application of validamycin A inhibited the accumulation of Na in both cultivars of NaCl-stressed plants.

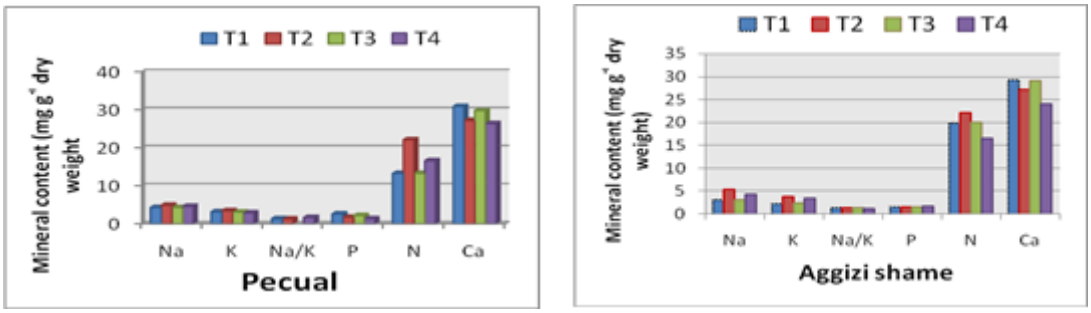

Fig. 2: Change in minerals content (mg g^{-1}DW) in leaves of two olive cultivars treated with 30 µM validamycin A, grown under saline conditions (300 mM NaCl).

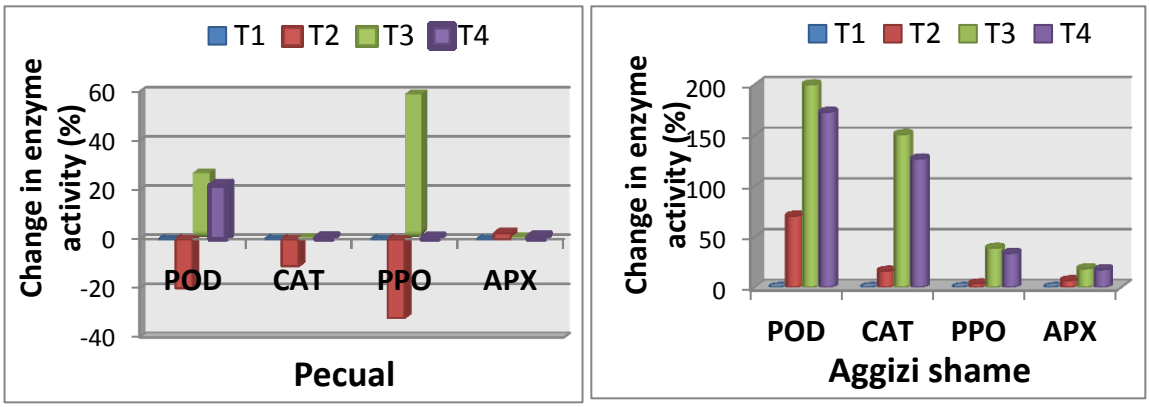

Fig. 3: Change in antioxidant enzyme activities, peroxidase (POD), catalase (CAT), polyphenol oxidase (POD), and ascorbate peroxidase (APX) in leaves of two olive cultivars treated with 30 µM validamycin A, grown under saline conditions (300 mM NaCl) as compared to control.

The increase of Na contents and Na/K ratio under salinity conditions was affected by exogenous validamycin A supplement. This result was corroborated with Garg et al. (2002) who showed that treatment with exogenous trehalose significantly reduced the salt induced accumulation of Na in rice leaves. Salinity stress caused significant decreases in phosphorus contents of two olive cultivars. Regarding the effect of validamycin A (30 µM) of salt tolerant (Pecual) on Ca contents the results recorded non significant variation between treatments as compared to salt stressed without validamycin A at 30 µM (Zeid , 2009).

Antioxidant enzyme activities:

The activity of peroxidase enzyme significantly decreased in salt tolerant (Pecual) and slight increase in Aggizi shame (salt moderate) as compared to corresponding control under salinity conditions (300 mM NaCl). On the other hand, application of validamycin (30 µM) as foliar spray increased POD activity in both cultivars recording 21.9% for pecual and 127.9 % for Aggizi shami (Fig. 3).

As shown in Figure (3), application of 300 mM NaCl slight increased catalase activity in Aggizi shami and decreased in Pecual as compared to the control. On the other hand, application of validamycin (30 µM) increased CAT activity in both cultivars recording 0.57% for Pecual and the highest activity observed 126.8 % for Aggizi shami. The activity of polyphenol oxidase decreased in salt stressed Pecual comparing with the control. Results of both cultivars showed that, treatment with validamycin (30 µM) in response of salt stress induced significant increase in PPO activity as compares with corresponding control (Fig. 3). Figure (3) show that activity of APX significantly increased in both olive cultivars treated by NaCl concentrations as the compared with non-treated control. Validamycin A increased activity of APX in salt moderate cultivar but declined in tolerance cultivar under salt stress compared with plants treated with NaCl alone.

Our results demonstrated that, exogenous validamycin A supplied to NaCl-stressed olive cultivars caused increase in all antioxidant enzymes investigated compared with plants treated with NaCl alone. The beneficial effect of exogenous validamycin A on antioxidant enzymes was the most pronounced in salt moderate olive cultivar by greatly stimulating POD, CAT, PPO and APX activity. However, application of validamycin A caused an overall enhancement in the activities of all the enzymes under salt stress, suggesting the presence of an effective scavenging mechanism to remove ROS from the plant system and acting as a potential mechanism of plant salt tolerance (Luo et al. 2010). Both trehalose-producing transgenic plant and exogenous application could reduce generation of ROS, but it would affect the subsequent plant defense response (Fernandez et al. 2010). Less oxidative damage was reported in trehalose treated plants in terms of reduced malondialdehyde (MDA) contents and enhanced levels of enzymatic, SOD, POD and CAT (Duman et al. 2011).

AtTPS1 gene expression analysis induced by validamycin A and salt stress:

Olive AtTPS1 gene expression has been determined in both salt moderate and salt tolerance cultivars treated with validamycin A under salt stress at 300 mM NaCl. A 230-bp TPS fragment was amplified in both cultivars by RT-PCR, Figure (4). In salt tolerance Pecual another amplified fragment appears at 210bp. The results revealed that, PCR amplification of the AtTPS1 gene was successfully obtained for plant treated with validamycin A in combination with NaCl, meanwhile amplification was not successful for the untreated control. Trehalose expression has two-step pathway involving two enzymes: trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). From the literature, it is clear that trehalose and T6P accumulate in plants treated with the potent trehalase inhibitor "validamycin A". When the cytoplasmic trehalose level increases, its feedback inhibition of trehalose phosphate phosphatase (TPP) activity enhanced the level of T_6P (Ahmed et al. 2013). These results suggested that olive TPS participates in the response to salt stress with validamycin A in both cultivars. These findings suggest that exogenous trehalose acts as an elicitor of genes involved in abiotic stress responses (Fernandez et al. 2010). Genes coding enzymes of the trehalose biosynthetic pathway have been studied in several organisms, with special relevance to *E. coli* and *Sacharomyces cerevisae*. Similar genes have also been isolated in plants. Isolation and molecular characterization of *A. thaliana* TPS gene (AtTPS1) has been described Blázquez et al. (1998), as well as a similar gene (SlTPS1) for *S. lepidophylla* (Zentella *et al*., 1999). More recently, a family of 11 *A. thaliana* TPS genes has been described, although only one encodes for trehalose-6-phosphate synthase Leyman et al. (2001). A trehalose-6-phosphate phosphatase (AtTPP) gene was also reported in *A. thaliana* (Vogel *et al.*, 1998) and tobacco, *Nicotiana tabacum* (Wang et al. 2005), and more recently in rice (Pramanik and Imai, 2005).

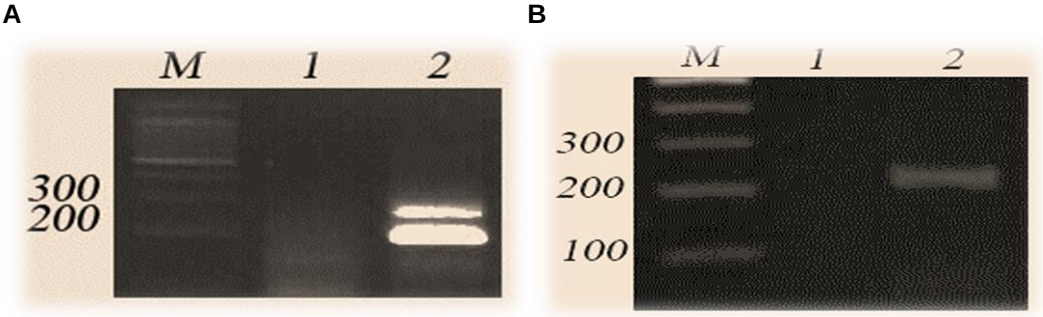

Fig. 4. Expression analysis of AtTPS1 gene for two olive cultivars. (a) salt tolerance (Pecual) and (b) salt moderate (Aggizi shame), Lane M: 100 bp. Ladder, lane1: control, lane 2: Treated cultivar with 300 mM NaCl+ 30 µM validamycin A.

CONCLUSION

Olive AtTPS1 gene expression has been determined in both salt moderate and salt tolerance cultivars treated with 30 µM of validamycin A under salt stress at 300 mM NaCl.

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CONFLICT OF INTEREST

The present study was performed in absence of any conflict of interest.

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