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Influence of salicylic acid (SA) and ascorbic acid (ASA) on *in vitro* propagation and salt tolerance of date palm (*Phoenix dactylifera* L.) cv. 'Nersy'

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Abstract

This study was carried out to investigate the antioxidant effects of salicylic acid (SA) and ascorbic acid (ASA) under two stress levels (75 and 150 mM NaCl) on growth and some biochemical constituents of date palm cv. Nersy cultured *in vitro*. Micro-propagated shoots of cv. Nersy at length 2.5-3 cm was excised from the proliferation medium and were separately cultured on MS medium. The data indicated that maximum growth and chlorophyll content of shoots was observed after 75 days of culturing in the medium supplemented with 50 mgl^{-1} salicylic acid and 100 mgl^{-1} ascorbic acid in both stress and non-stress conditions. It was also observed that the *in vitro*-grown plants resulted increased activities of antioxidant enzymes SOD and APX in medium containing of salicylic acid (75 mgl^{-1}) and ascorbic acid (100 mgl^{-1}) with increasing salt stress (up to 150 mM of NaCl) compared with other treatments. The study of shoot protein patterns using SDS-PAGE showed some remarkable changes in protein expression. The results suggests that the stress induced the synthesis of proteins bands with MW of 15, 29, 43 kD and 15, 22.6, 35 and 46 kD in medium supplement with 75 and 150 mM NaCl, respectively, compared with control treatment (20.9, 28.39, 37, 40 and 48.6) kD. Supplementing salicylic acid (75mgl^{-1}) and ascorbic acid (100 mgl^{-1}) under salt stress condition induced the synthesis of additional protein bands with MW of 19.28, 28.50, 32.61, 59.12 and 72.00 KD at 75 mM NaCl; and 17.96,22.6, 31.95 54.50 and 68.0 at 150 mM NaCl.

Keywords: Antioxidant compound, Date palm, Micropropagation, Salt stress, SDS-PAGE.
Abbreviations NAA_naphthalene acetic acid, NOA_naphthoxy acetic acid, SA_Salicylic acid, ASA_Ascorbic acid, SOD_Superoxide dismutase, APX_Ascorbate peroxidise.

Introduction

Date palm (*Phoenix dactylifera* L.) belongs to the monocotyledonous family Arecaceae and is an economically important tree species predominantly concentrated in arid regions of the Middle East and North Africa (Zaid, 2002). Plants are usually exposed to different environmental stresses which limit their growth and productivity (Shao et al., 2008). One of the most important factors affecting plant growth and the production of secondary metabolites is the salt stress (Nikolova and Ivancheva, 2005). It is estimated that about one-third of the world's cultivated land is affected by salt (Kaya et al., 2002). The physiological processes are affected by salt stress including ion toxicity, osmotic stress, nutrient deficiency and especially oxidative stress (Flowers, 2004). Formation and accumulation of reactive oxygen species (ROSs) can be induced by salt stress (Erda et al., 2011). Adaptation to salinity encompasses complex processes involving numerous changes such as weak growth, the induction or increased expression of special genes, transient increases in plant regulators levels, accumulation of osmolytes and protective proteins, elevated antioxidative activities and suppression of energy consuming pathways (Bartels and Sunkar, 2005). The free proline assays revealed that this amino acid over-accumulated in the roots and leaves of each stress treated plant. It is remarkably high when leaves are exposed to suboptimum temperatures and salinity stress. These results indicate that the production of proline is a common response to various abiotic stresses (Yaish 2015).
Addition of K^+ to the salt containing media of date palm has reduced the absorption of Na^+ and also balanced ions compartmentalization. This improvement in growth is accompanied by a decrease in Na^+ concentration and an increase in K^+ concentration in the plant tissues with lower Cl^- concentrations in leaves and roots of date palm (Alkhateeb et al., 2015). Some results have revealed the identification of miRNAs that share common biogenesis, structure, and expression features with the miRNAs that have been isolated from other plant species. These miRNAs and their targets could play a critical role in the date palm tree, which has the ability to grow under severe environmental conditions, including high temperature, drought, and salinity (Yaish et al., 2016). The development of methods to induce stress tolerance in plants is vital and still receives considerable attention. Choudhury and Panda, (2004) demonstrated that SA has ability to modulate plant response to wide range of oxidative stresses and regulate the activities of antioxidant enzymes and increase plant tolerance to a biotic stress. The ascorbic acid (ASA) plays a multiple roles in plant growth, functioning in cell division, cell wall expansion, and other development processes (Erda et al., 2011; Pignocchi and Foyer, 2003) .In addition, ascorbic acid is a key substance in the network of plant antioxidants (Noctor and Foyer, 1998). Yet, little information is available on the effect of salicylic acid and ascorbic acid on salt stress or enzyme activity in date palm plants. Therefore, the present research was conducted to evaluate the possibility of application of antioxidant compounds such as salicylic acid (SA) and ascorbic acid (ASA) to alleviate harmful effects of

salinity on date palm cv. Nersy plants growth on *in vitro* culture condition. We also study some important biochemical changes involved in tolerance to salinity.

Results

Effect of salicylic acid (SA) and ascorbic acid (ASA) on some growth criteria

Data in the Table 2 indicate that SA and ASA treatment improved the growth characters at all levels of salt stress and also non-stress plants, compared with control. The greatest shoot length, number of leaves and root length are related to the plants which were cultured under the salt stress with concentration (75 mM) of NaCl in combination with 50 mgl^{-1}salicylic acids and 100 mgl^{-1} ascorbic acid (Fig.2C). The maximum reduction in growth criteria was observed in plants which were cultured under the salt stress with concentration of 150 mM in combination with 0 mgl^{-1} salicylic acid and 0 mgl^{-1} of ascorbic acid.

Impact of salicylic acid and ascorbic acid on some biochemical traits

Photosynthetic pigments

Table 3 shows that the greatest amount of *chl a*, *chl b* and total chlorophyll are related to the plants which were cultured under the salt stress with concentration 75 mM in combination with 50 mgl^{-1} salicylic acids and 100 mgl^{-1} ascorbic acid. The least amount of *chl a*, *chl b* and total chlorophyll were related to the plants cultured under the salt stress (150 mM) in combination with 0 mgl^{-1} salicylic acid and 0 mgl^{-1} ascorbic acid.

Superoxide dismutase (SOD) and ascorbate peroxidise activity (APX).

Table 4 shows that the high levels of antioxidant enzymes activity of SOD and APX were occurred in the shoots cultured under the salt stress (150mM) in combination with 75 mgl^{-1} salicylic acids and 100 mgl^{-1} ascorbic acid, compared to other treatments. The results of experiment also indicate the least activities of SOD and APX were achieved in control treatment, followed by salt stress (75 mM) in combination with 0 mgl^{-1} salicylic acid and 0 mgl^{-1} ascorbic acid.

SDS-PAGE protein patterns

Changes of protein patterns were analyzed in leaves of date palm plants cv. Nersy *at in vitro* culture condition to understand any possible alterations in gene expression in plants cultured under two levels of salt stress (75 and 150 mM) of sodium chloride (NaCl) in the absence or presence of SA and ASA comparing with non-salt stressed "control treatment", using SDS-PAGE. The salinity treatments resulted in the induction of new bands with molecular weight of 15, 29 and 43 KD in shoots under 75 mM of NaCl; and 15, 22.6, 35 and 46.0 KD in plants treated with 150 mM NaCl (Fig. 2, Lane 2 and 3). We observed that salt stress in SA and ASA treated plants induced variation in the appearance of new protein bands and disappearance of some others (differentially expressed proteins) with different molecular weights. The analysis of protein patterns indicates that in non-stressed plants and those cultured in media supplemented with SA, ASA no protein bands and pattern was changed, compare to control treatment, while in salt-stressed plants application of SA, ASA individually or in combination allowed the synthesis of additional new protein bands. The salt-stressed plants treated with 75 mgl^{-1} SA + 100 mgl^{-1} ASA showed the most enhancement of protein synthesis, compared to those treated with individual application of salicylic acid and ascorbic acid. In plants treated with 75 mgl^{-1} SA + 100 mgl^{-1} ASA bands were revealed as 19.28, 28.50, 32.61, 59.12 and 72.00 KD at 75 mM NaCl; 17.96, 22.6, 31.95 54.50 and 68.0 at 150 mM NaCl (Fig. 2, Lane 18 and 19), while treatment of shoots with SA or ASA individually, caused a little change and induced only two or three new polypeptides at 75 and 150 mM NaCl (Fig. 2, Lane 6, 7, 8, 9, 12, 13, 14 and 15) .

Discussion

Reduction in growth parameters with increasing the salinity levels can be attributed to different physiological changes like photosynthesis reduction, protein dehydration and toxic effect of ions accumulation in plant tissue. This study clearly demonstrated that salt stress on date palm plants can significantly be alleviated by the application of salicylic acid (SA) and ascorbic acid (ASA) treatments. The SA and ASA play important roles in the defense response to stresses (salts, water, etc.) in many plant species (Senaratna et al., 2000). It is reported that SA-induced plant growth could be attributed to the enhanced activity of antioxidants that protect the plants from oxidative damages (El-Tayeb, 2005). Application of salicylic acid has helped increasing of plant growth in saline conditions (Stevens et al., 2006). Ascorbic acid perhaps also minimized the oxidative damage by increasing the amount of antioxidant enzymes that, in turn, leads to better growth in the date palm plant cv. Nersy at *in vitro* conditions.

The results are in agreement with those reported by Amin et al. (2008), who found a progressive increase in plant height, by increasing ascorbic acid level. Smrinoff and Wheeler, (2000) reported that the ASA counteracts with the harmful effects of salinity on plant height and root length at all salinity levels. These results are in coincidence with that cited by Azooz et al. (2004). Athar et al. (2008) suggested that ascorbic acid could accelerate cell division and improve the growth. Some signs of environmental stresses in plants are reduction of chlorophyll. However, this reduction remarkably depends upon the plant genotype (Colom and Vazzana, 2001). The chlorophylls (a and b) play important role in photosynthesis. The chlorophyll content reduction was reported in salt stressed date palm (AL- Mayahi, 2015). Decreases in photosynthetic pigments were due to instability of protein complexes and destruction of chlorophyll by increased activity of chlorophyll degrading enzymes and chlorophyllase under high stress condition. Based on the theory of Schutz and Fangmir (2001), the reduction of chlorophyll due to stress is related to the increase of production of reactive oxygen species (ROS) in the cell. These free radicals cause peroxidation, disintegration and reduction of chlorophyll content in plants under stressful conditions. The SA application triggers scavenging of ROS that may increase Chl content in date palm. The SA-induced salt tolerance in date palm plants might be associated with an increase in the activity of ascorbate peroxidase and superoxide dismutase.

Table1. Treatments that applied in this study.

No.	Treatments	No.	Treatments
1	0mM NaCl (control)	11	0mM NaCl+100 mg.l^{-1} ASA
2	75mM NaCl	12	75 mM NaCl+50 mg.l^{-1} ASA
3	150mM NaCl	13	75 mM NaCl+100 mg.l^{-1} ASA
4	0 mMNaCl+50 mg.l^{-1} SA	14	150 mM NaCl+50 mg.l^{-1} ASA
5	0mM NaCl+75 mg.l^{-1}SA	15	150 mM NaCl+100 mg.l^{-1} ASA
6	75 mMNaCl+50 mg.l^{-1}SA	16	75 mMNaCl+50 mg.l^{-1}SA+100 mg.l^{-1}ASA
7	75mM NaCl+75 mg.l^{-1}SA	17	150 mM NaCl +50mg.l^{-1}SA+100 mg.l^{-1}ASA
8	150 mMNaCl+50 mg.l^{-1}SA	18	75 mM NaCl +75mg.l^{-1} SA +100mg.l^{-1}ASA
9	150 mM NaCl+75 mg.l^{-1}SA	19	150 mM NaCl+75mg.l^{-1}SA+100 mg.l^{-1}ASA
10	0mM NaCl+50 mg.l^{-1} ASA		

(All treatments have 0.5 mgl^{-1} NAA and 0.5 mgl^{-1} NOA).

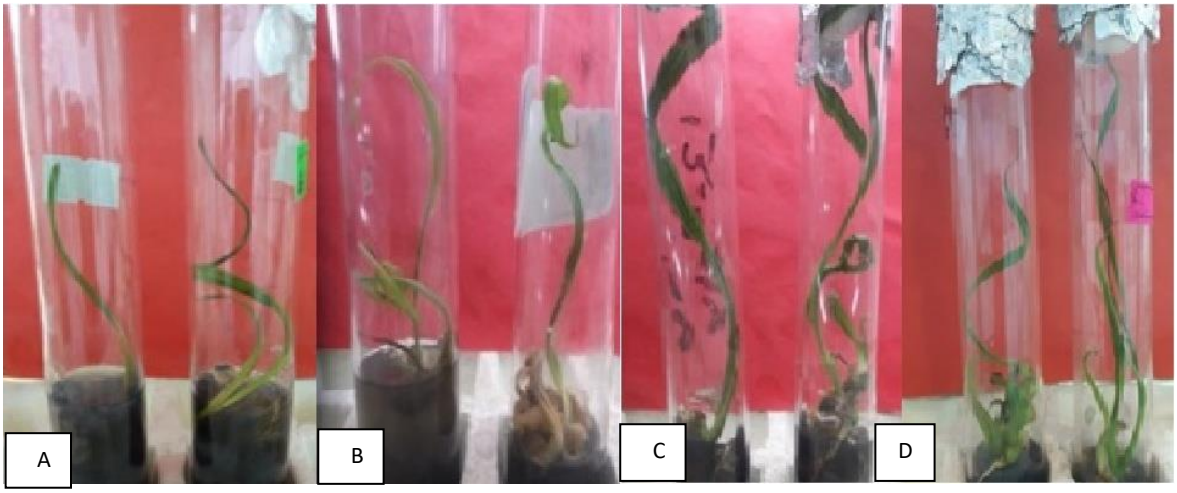

Fig 1. Effect of salicylic acid (SA) and ascorbic acid (ASA) on shoot and root length, number of leaves and roots of date palm cv. Nersy plantlets under different concentrations of NaCl. (A) Treated with 75 mM NaCl; (B) Treat with 75 mM NaCl + 100 mg.l^{-1} ASA; (C) Treated with 75 mM NaCl + 50 mg.l^{-1} SA +100mg.l^{-1} ASA (D) Treated with 75mM NaCl + 75 mg.l^{-1} SA +100mg.l^{-1}ASA.

Therefore, impact of salicylic acid on chlorophyll may be related with its influence on the anti-oxidative enzyme activities. Salicylic acid regulates physiological and biochemical processes in plants and can be used as a potential growth regulator to improve plant growth under saline conditions. This positive effect of SA could be attributed to an increased CO_2 assimilation and photosynthetic rate and increased mineral uptake by the stressed plant under SA treatment. On the other hand, ascorbic acid also beneficially influences damage reduction which is caused by salt. This may be due to salinity resulting in increased activity of reactive oxygen species (ROS) which may cause severe cellular damage. In this study, it was found that ascorbic acid plays an important role in photosynthetic pigments to defend system against oxidative stress. It is a powerful reducing agent found usually in small concentrations in plants, and is proposed to play an important role in scavenging reactive oxygen species (O_2, H_2O_2, OH- etc.) generated during stress conditions in plants (Smirnoff, 2005). Furthermore, ascorbic acid (ASA) also benefitted growth which may be due to the antioxidant activity of ascorbic acid protecting plants from damage due to a biotic stress (Beltagi, 2008). The present study confirmed pervious observations and reports that application of SA under stressed conditions improved plants performance in terms of enhanced chlorophyll contents (Agarwal et al., 2005). This result is in agreement with those reported by Khan et al. (2003), who found that SA increased the photosynthetic rate in corn and soybean. Also, Farahat et al. (2013) found that the combined treatment of ascorbic acid with salinity level gave significantly increased chlorophyll a, b, and total chlorophyll content of shoots, compared with control plants. The role of application of ASA in wheat has also been reported earlier (Athar et al., 2008), where an increase in photosynthetic activity was noted as a result of ASA application resulted in overcoming damaging effect of salt stress. This result indicated that oxidative stress is one of the main consequences of salinity stress on date palm and SA with ASA has an ameliorative effect on this process. The SA application may cause a temporary and low level of oxidative stress in plants, which acts as a hardening process improving the anti-oxidative capacity of plants and helping to induce the synthesis of protective compounds and; therefore, the acclimation to stress (Janda et al., 2007). These results indicated that increased APX activity could be an adaptive mechanism to an increased oxidative stress which caused by salinity. Furthermore, SA intensified APX activity to facilitate oxidative damage protection. Salicylic acid has an affinity to bind with the enzymes APX (Slaymaker et al., 2002) which are involved in ROS metabolism and redox homeostasis. Alteration in this homeostasis leads to induction of a defense response in plants (Mittler, 2002). Increasing APX activity as a consequence of exogenous SA application was also reported by (Agarwal et al., 2005).

Regarding SOD, the results indicated that the activity of SOD was affected variously under salinity, SA, ASA levels. We also noticed interaction among effects of salinity, SA, ASA levels. The results of this study showed that salt stress had significant effects on the enzymes activity in shoots. Different abiotic stresses may provoke oxidative stress, leading to cellular adaptive responses such as acceleration

Table 2. Effect of salicylic acid (SA) and ascorbic acid (ASA) on in *in vitro* growth of date palm cv. Nersy under different concentrations of NaCl.

Treatments	Growth attributes	NaCl Cons (mM)		
		0.0	75	150
0.0	Shoot Length	6.90±0.55ghi	6.77±0.81hi	5.50±0.43g
	No. of leaves	1.67±0.19ef	1.67±0.21ef	1.16±0.26h
	Root Length	4.0±0.54c	3.8±0.84d	2.6±0.64g
50 SA	Shoot Length	7.80±0.65defg	7.94±0.4cdef	6.91±0.75ghi
	No. of leaves	1.67±0.019ef	1.83±0.15de	1.33±0.21gh
	Root Length	4.6±0.38abc	3.8±0.45d	2.8±0.47g
75 SA	Shoot Length	7.19±067fghi	7.37±0.48efghi	6.40±0.43ij
	No. of leaves	1.33±0.13gh	1.50±0.0.2fg	1.16±0.0.08h
	Root Length	3.8±0.45d	3.77±0.42def	2.5±0.64g
50 ASA	Shoot Length	7.51±0.0.76efgh	7.63±0.50defgh	6.65±0.081hi
	No. of leaves	1.67±0.19ef	1.83±0.15de	1.33±0.13gh
	Root Length	4.3±0.42bcde	4.55±0.0.37abcd	3.1±0.47fg
100 ASA	Shoot Length	8.25±0.0.65bcde	8.88±0.0.95abc	7.45±0.34efghi
	No. of leaves	1.83±0.15de	2.00±0.0.2cd	1.50±0.0.13fg
	Root Length	4.5±0.43abcd	4.6±0.38abc	3.6±48ef
50 SA+100 ASA	Shoot Length	9.10±0.55ab	9.50±0.22a	7.95±0.18cdef
	No. of leaves	2.33±0.12ab	2.50±0.11a	1.83±0.04de
	Root Length	4.8±0.39ab	5.1±0.23a	4.0±0.47c
75 SA+100 ASA	Shoot Length	8.53±0.75abcd	9.07±0.55ab	7.63±0.5defgh
	No. of leaves	2.00±0.2cd	2.16±0.2bc	1.67±0.19def
	Root Length	4.75±0.25abc	4.8±0.39ab	3.75±0.45def

± Standard error

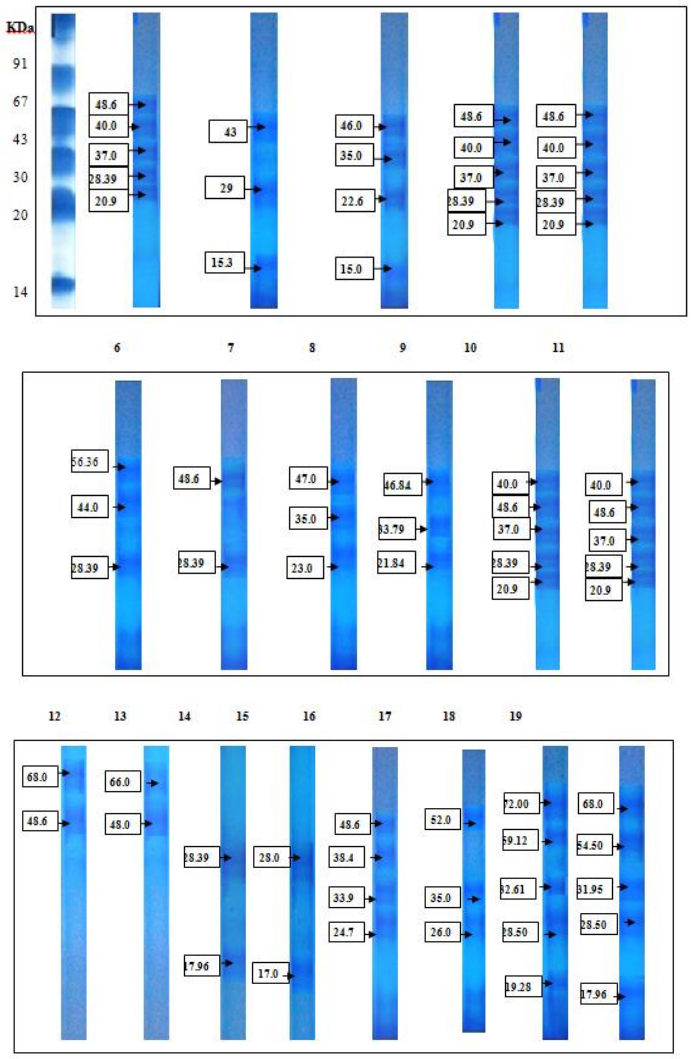

Fig 2. SDS-PAGE of protein extracted from Shoots of date palm cv. Nersy. treated with NaCl, Salicylic acid "SA" and Ascorbic acid "ASA" as shown in Table 1.

Table 3. SDS-PAGE of protein extracted from Shoots of date palm cv. Nersy . treated with NaCl, Salicylic acid "SA" and Ascorbic acid "ASA", as shown in Table 1.

Treatments Mg. l^{-1}	Chlorophylls (mg/100 g FW)	NaCl Cons. (mM) 0.0	75	150
0.0	Chl a	0.783±0.067ij	0.779±0.023ij	0.584±0.04l
	Chl b	0.247±0.002de	0.239±0.007de	0.197±0.003e
	Chl t	1.030±0.04ghi	1.018±0.0016ghi	0.781±0.20j
50 SA	Chl a	0.816±0.019i	0.943±0.071h	0.739±0.023jk
	Chl b	0.268±0.004cde	0.273±0.005cde	0.249±0.002de
	Chl t	1.084±0.13g	1.216±0.16f	0.988±0.0.12hi
75 SA	Chl a	0.809±0.03i	0.935±0.04h	0.718±0.023k
	Chl b	0.251±0.002cde	0.269±0.004cde	0.237±0.009de
	Chl t	1.060±0.061gh	1.204±0.046f	0.955±0.04i
50 ASA	Chl a	0.983±0.071gh	1.008±0.13g	0.815±0.019i
	Chl b	0.280±0.058cd	0.300±0.0001cd	0.257±0.005cde
	Chl t	1.263±0.30f	1.308±0.08f	1.072±0.0gh
100 ASA	Chl a	1.130±0.061e	1.169±0.013de	0.941±0.04h
	Chl b	0.293±0.004cd	0.329±0.008bc	0.266±0.002cde
	Chl t	1.423±0.0.23de	1.498±0.023cd	1.207±0.046f
50 SA+100 ASA	Chl a	1.390±0.08b	1.520±0.20a	1.200±0.046d
	Chl b	0.328±0.008bc	0.419±0.009a	0.289±0.005cd
	Chl t	1.718±0.41b	1.939±0.49a	1.489±0.20cd
75 SA+100 ASA	Chl a	1.266±0.064c	1.380±0.08b	1.067±0.13f
	Chl b	0.307±0.08bcd	0.384±0.016ab	0.279±0.004cd
	Chl t	1.573±0.20c	1.764±0.21b	1.346±0.0.08ef

± Standard error

Table 4. Effect of salicylic acid "SA" and ascorbic acid on Superoxide dismutase (SOD) and Ascorbate peroxidise (APX) activity in date palm cv. Nersy plant leaves under concentrations of NaCl *in vitro.*

Treatment	Enzymes	NaCl Cons. (mM) 0.0	75	150
0.0	SOD	12.50±0.41q	14.80±0.66p	18.60±0.76m
	APX	0.912±0.08m	1.231±0.26l	1.650±0.19j
50 SA	SOD	17.30±0.62n	20.86±0.46jk	21.20±1.6ij
	APX	1.850±0.15i	2.150±0.20g	2.350±0.06f
75 SA	SOD	20.5±1.4jkl	22.69±0.43e	25.80±2.15e
	APX	2.150±0.42g	2.500±0.28e	2.850±0.18e
50 ASA	SOD	15.80±0.30o	19.64±1.01l	20.10±0.17kl
	APX	1.350±0.26k	1.800±0.15i	2.200±0.12g
100 ASA	SOD	19.88±1.07l	21.92±1.16ij	23.54±2.07g
	APX	2.000±0.42h	2.350±0.4f	2.600±0.19d
50 SA+100 ASA	SOD	24.50±1.75f	28.00±1.20d	32.00±1.95c
	APX	2.350±0.06f	2.600±0.3d	3.000±0.48b
75 SA+100 ASA	SOD	31.68±1.65c	35.70±1.75b	40.01±2.40a
	APX	2.850±0.47c	3.000±0.36b	3.350±0.24a

of ROSs scavenging systems and alteration in antioxidant enzyme activities in plants (Hamdia and Shaddad, 2010). Previously, role of SA was indicated in the induction of antioxidant defenses, maintaining the redox state of the gluthatione pool and plant protection against oxidative stress (Borsani et al. 2001). Therefore, the effects of SA and ASA on date palm plant, grown on salt stress *in vitro*, were investigated in the present study. Different abiotic stresses may provoke oxidative stress, which lead to cellular adaptive responses such as acceleration of ROSs scavenging systems and alteration in antioxidant enzyme activities in plants (Hamdia and Shaddad, 2010). Modulation of antioxidant systems and the levels of substrates can correlate to tolerance to salinity stress in higher plants (Jahnke and White, 2003). Changes in the levels of antioxidant molecules and the activity of antioxidant enzymes, which are signals of plant tolerance/adaptation to stress conditions, are correlated into oxidative stress tolerance of plants (Lee et al., 2001). Variations in the antioxidant levels can serve as a signal for the modulation of ROSs scavenging mechanisms and ROSs signal transduction (Mittler, 2002). In the present study, enhanced activities of SOD due to SA and ASA addition might have been one of the factors contributing to improved growth in date palm cv. Nersy growing *in vitro* under saline conditions.

The beneficial effect of SA was also reflected on membrane stability, chlorophylls contents and growth parameters. Similar to the results of this study, some reports have shown that salt stress induces an increase in SOD activity. This has frequently been correlated with plant salt tolerance (Sudhakar et al., 2001). This view was further supported by the arguments that major detoxification of ROS produced during photosynthesis is mediated by superoxide dismutase and by reductive processes involving the major redox buffers of plant cells such as ascorbate (Foyer and Noctor, 2003). Ascorbat has been shown to have a critical role in several physiological processes in plants, including growth, differentiation and metabolism (Foyer, 1993). Likewise by application of ascorbic acid, antioxidant enzyme activities increased significantly in salt-treated date palm plants *in*

vitro. Up-regulation in the activity of ascorbat peroxidase and superoxide dismutase indicates that these enzymes are somehow involved in the neutralization process of reactive oxygen species as well. Ascorbic acid protects metabolic processes against H_2O_2 and other toxic derivatives of oxygen, which affect many enzyme activities, minimizes the damage caused by oxidative processes through synergistic function with other antioxidants, and stabilizes membranes (Shao et al., 2008). These results are in agreement with those of Dolatabadian and Jouneghani, (2009) who reported that major enzymes as (superoxide dismutase) involved in scavenging reactive oxygen species increase significantly by the application of ascorbic acid to salt stressed bean plants. Higher SOD activity in leaves of plants subjected to foliar application of ASA, SA and H_2O_2 at suboptimal temperature stress suggests a more efficient scavenging system, which may protect membranes from injurious effect of ROS (Foyer and Noctor, 2003).

Changes in protein synthesis under salt stress may be due to changes in the efficiency of mRNA translation or the regulation of RNA transcription, transport and stability. The expression of salt-stress proteins is related to the adaptation process of plants to salinity as well as to the genetic constitution of selected salt tolerant genotype. These results confirmed the results reported by El-Bassiouny et al. (2008), who concluded that one of the important mechanism involved in the cell protection against salinity stress is the induction of *de novo* synthesis of a set of new protein. The salinity altered the protein patterns of two *Anabaena* strains by inducing the synthesis of specific proteins called the salt-stress proteins that are strain dependent **(**Apte and Bhagwat, 1998).

The new bands of high molecular weight proteins in salt stressed plants treated with SA and ASA might be due to synthesis of these proteins (Gopala Roa et al., 1987). These new proteins may have a specific function to protect date palm plants from further dehydration damage and considered as a defense mechanism to salt stress. Salt induced polypeptides have been observed in many studies and are assumed to play a role in salt stress tolerance (Jiang and Huang, 2002). The observation of new protein in the present study was confirmed by the earlier work that the proteins are specific to adaptation to salt stress (Gomathi et al., 2013). Expression of 15 kDa polypeptide was observed under a salt stress. In present study, enhanced expression of 72 kDa protein upon SA and ASA indicated that SA and ASA might have induced the protein synthesis under salt stress condition. Such altered and enhanced expression of protein may be responsible for the survival and growth of the plants under high level of NaCl.

Disappearance of certain polypeptides in salt stressed plants in the absence of SA or ASA may be related to increased hydrolysing enzyme RNAase activity (Kong-Ngern et al., 2005). These results were supported by previous studies in other plant species (Azooz et al., 2004; Azooz and Al-Fredan, 2009). The polypeptides that were disappeared during salinity stress might be compensated by others. We also noticed that the applied ASA has a stimulatory effect on the quantitative and qualitative changes in protein biosynthesis. These new protein bands may be due to *de novo* synthesis of new proteins in plants under 75 and 150 mM NaCl or in combination with ASA, as reported by other investigators (Azooz et al., 2004; El-Bassuony et al., 2008; Azooz and Al-Fredan, 2009). They reported that, vitamin treatments induced alterations in the enzymes related to protein metabolism. These enzymes might act as activators of protein synthesis that appeared in plants treated with salinity or/and vitamins. This may play an inductive role in triggering a special defense system, helping these plants to improve their salt tolerance and consequently their growth. El-Bassuony et al. (2008) has shown that vitamin treatments induces a significant alterations in the enzymes related to protein metabolism, which indicates that vitamins might act as activators of protein synthesis.

In addition, the cells exposed to salt stress tend to the production of ABA. This hormone has a relationship to tolerance to the salt stress through the signal transduction leading to the induction of gene 'Gene activation' and the formation of proteins that have the function of protecting osmotic (Munns, 2005).

Materials and methods

Plant material

This study conducted in Tissue Culture Laboratory, Date Palm Research Center, Basra University during the period from 2013 and 2014. Micro propagated shoots of date palm cv. Nersy (This cultivar has been chosen because it is one of the rare cultivars and with good quality) at length 2.5-3 cm was excised from the proliferation medium and were separately cultured on MS medium (Murashige and Skoog, 1962). The culture media consisted of MS salts, supplemented with Myo-Inositol (100 $mg.l^{-1}$), Glutamine (200 $mg.l^{-1}$), Thiamine-hydrochloride (1 $mg.l^{-1}$), nicotinic acid (1$mg.l^{-1}$), Pyridoxine-hydrochloride (1$mg.l^{-1}$), sucrose (30$g.l^{-1}$), activated charcoal (0.5 $g.l^{-1}$) and agar (7 $g.l^{-1}$) and two antioxidant compounds to tolerance salt stress salicylic acid (SA) and ascorbic acid (ASA). The pH was adjusted to 5.7-5.8 and then the media were autoclaved at 121°C for 20 min. Cultures were incubated in the in the growth chambers at 25 ± 2°C under 16 h photoperiods. Treatments were consisted of 19 media, Salicylic acid (SA), Ascorbic acid (ASA), as well as their interaction with three concentrations of Sodium Chloride as shown in Table 1.

Effect of salicylic acid (SA) and ascorbic acid (ASA) on some growth criteria

Growth criteria were recorded after 75 days from culture of shoots included all of length of shoots (cm), number of leaves formed and length of roots (cm).

Biochemical and physiological parameters determination

Assessment of chlorophyll content

The amount of chlorophylls in the leaves was estimated by the method described by (Porra, 2002).

Estimation of antioxidant enzyme activities

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was assayed by measuring the inhibition of superoxide-driven nitrite formation from hydroxylamine hydrochloride according to Das et al. (2000). The reaction mixture was prepared by mixing 1.110 ml of 50 mM phosphate buffer (pH 7.4), 0.075 ml of 20 mM L-methionine, 0.040 ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM hydroxylamine hydrochloride and 0.1 ml of 50 µM EDTA. A 100 µl of enzyme extract (50 µg protein) and 80 µl of riboflavin (50 µM) were added to this mixture. The cocktail was mixed and then illuminated for 10 minutes in an aluminium foil-coated wooden box containing two 20 W-Philips fluorescent lamps fitted parallel to each other. The control tube contained equal

amount of buffer instead of sample. The sample and its respective control were run together. After 10 minutes of exposure, 1 ml of Greiss reagent (prepared freshly by mixing equal volumes of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-1-napthyl ethylene diamine) was added to each tube and the absorbance was measured at 543 nm.

The APX (EC 1.11.1.11) activity was measured according to the methods of (Nakano and Asada 1981). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM hydrogen peroxide, and 0.1 mL of enzyme extract in a total volume of 1 ml. The concentration of oxidized ascorbate was calculated by the decrease in absorbance at 290 nm. The absorption coefficient was 2.8 mM^{-1} cm^{-1}. One unit of APX was defined as 1 mmol ml^{-1} ascorbate oxidized min^{-1} (Hossain et al., 2006).

Extraction of protein and gel electrophoresis

Protein were extracted by homogenizing the 0.333 gm freeze-dried shoot sample in pre-chilled mortar and pestle using 1 ml of extraction buffer consisting of 0.2 M, tris hydroxymethyl aminomethane (Tris); 0.001M ethylene diamine tetra acetic acid (Na2+EDTA); 12%, glycerol; 0.01M, dithiothreitol (DTT); and 0.05mM phenyl methyl sulfonyl fluoride (PMSF). The samples were centrifuged at 15000 × g for 15 min, and the supernatant was used for determination of total protein content. The protein sample was added with an equal volume of cracking puffer (0.125M, Tris.Cl; pH 6.8; 4%, SDS; 20%, glycerol; 10%, β-mercaptoethanol and 0.01%, bromophenol blue) and was denatured by boiling in water bath at 90°C for 3 min. Protein samples (~500µg) were electrophoresed in a discontinuous SDS polyacrylamide gel following Laemmli (1970), using a 12% resolving gel (0.375 M, Tris. Cl; pH 8.8) and 4% stacking gel (0.125M, Tris-Cl; pH 6.8) in Tris-glycine buffer (0.025M, Tris; pH 8.3; 0.192M, glycine; 0.1%, SDS) for 16 hr, constantly at 20mA. Staining of the gel was done using 0.2% (w/v) Commassie Brilliant Blue R-250 in 12.5% (w/v) trichloroacetic acid (TCA). The position of the protein band in the gel was expressed to compare with standard protein markers with known molecular weight.

Statistical analysis

All the data were statistically analyzed by two-way analysis of variance (ANOVA). The least significant difference (LSD) method was used to test the difference between treatments and $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS packet software.

Conclusions

This study proved the beneficial effects of salicylic acid (SA) and ascorbic acid (ASA) on growth characters, some biochemical constituents under salinity stress conditions of date palm cv. Nersy plants at *in vitro* conditions. These effects may be attributed to the protective role of SA and ASA in plant cells from the oxidative stress induced by salinity and by increasing the antioxidant activity of enzymes such as SOD and APX, appearing new protein bands. We also concluded that application of SA and ASA combined together to media are the most effective treatment to enhance growth date palm plants under salinity stress conditions. The combined effect of SA and ASA leads to synthesis of additional proteins bands, providing a reason for bearing more salt stress.

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