



## Growth responses and aquaporin expression in grape genotypes under salinity

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### Abstract

The effects of salinity on growth, leaf area and water relations of two grape genotypes (Gharashani and Shirazi) were studied under 2-week salinity (25, 50 and 100 mM NaCl). Growth and fresh weights of all plant parts were significantly ( $p < 0.05$ ) decreased by increasing salinity in both genotypes. Shirazi showed a higher decrease in shoot and root lengths and fresh weights than Gharashani. It also had a higher decrease in water potential and relative water content and a lower increase in leaf area as compared with Gharashani. There was a significant positive correlation ( $p < 0.05$ ) between water potential and leaf area in two genotypes. Under salinity stress the expression of aquaporin PIP2.2 decreased significantly ( $p < 0.05$ ) in roots and leaves of Gharashani, but it was higher in roots than in leaves. In roots and leaves of Shirazi the expression of this gene was partly increased. Considering growth factors and water relations, it seems that Shirazi is a sensitive genotype, but Gharashani can partly tolerate salinity.

**Keywords:** grape; salinity; leaf area; water potential; aquaporin; expression profile

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### Introduction

Salinity is one of the most important factors limiting production of horticultural crops. In fact, it is a widespread environmental stress for crop plants in arid and coastal regions. The salinity of the soil and irrigated water is a problem that restricts yield on almost 40 million hectares of irrigated land, which is approximately one-third of the irrigated land on earth (Norlyn and Epstein, 1984). Naturally occurring salt stress is generally due to NaCl (Levitt, 1972). Salinity limits vegetative and reproductive growth of plants by inducing severe physiological dysfunctions and causing widespread direct and indirect harmful effects, even at low salt

concentrations (Shannon et al., 1994). Salinity can damage the plant through osmotic effect, specific toxic effects of ions, and disturbance of essential nutrient uptake (Marschner, 1995).

Several studies have shown that changes in grapevine water status, at critical phenological stages, have a direct effect on grape composition and quality attributes by influencing vegetative growth, yield, and fruit metabolism (Pellegrino et al., 2005; Ezzaouani et al., 2007).

The difference in water potential ( $\Psi_w$ ) between the substrate and the plants, caused by salinity or water availability, limits the water flux from soil to leaves. Therefore, soil salinity and drought can have similar effects on the physiology of plants (Munns, 2002). Under both sources of stress, the leaf osmotic potential ( $\Psi_\pi$ ) should always be lower than the soil water potential ( $\Psi_w$ ) in order to maintain the water flux and leaf cell turgor pressure (Turner and Jones, 1980).

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Many authors have proposed the use of the pressure chamber method (Scholander et al., 1965) as an excellent tool to measure vine water status under irrigated and non-irrigated conditions (Girona et al., 2006; Sibille et al., 2007). Vine water status can be assessed using different pressure chamber approaches, such as leaf water potential and stem water potential (Girona et al., 2006; Sibille et al., 2007). These methods are widely used and constitute reference measurements of vine water status, from low to very high levels of water restriction on vine (Tisseyre et al., 2005; Acevedo-Opazo et al., 2008).

An accurate and simple measurement of leaf area (LA) of a plant is essential to understand the interaction between plant growth and environment, since it is an indicator of plant growth and productivity. Salinity affected the expansion of the plant's total leaf surface through the expansion of individual leaves rather than through the rate of production of new leaves (Martínez-Vilalta et al., 2009).

Grapevine is one of the world's most economically important fruit crops. It is the first fruit crop to have its genome fully sequenced (Velasco et al., 2007), and now has the potential to be a model organism for future studies of fruit trees (Troggio et al., 2008). Although grapevines are able to survive in a range of soil moisture conditions, their growth and yield is determined by their total water use (Sarker et al., 2005), so it is important to identify and characterize the molecular components of water transport. The discovery and characterization in plants of membrane intrinsic proteins (MIPs), some of which function as water permeable pores (aquaporins), has shown that aquaporins can have primary roles in regulating plant water transport (Maurel et al., 2008). In grapevine, there are few studies characterizing the MIPs in general and identifying the roles of the MIPs in water transport (Picaud et al., 2003; Vandeleur et al., 2009).

The MIP superfamily can be divided into four subfamilies; the plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-like intrinsic proteins (NIPs) and the small basic intrinsic proteins (SIPs) (Forrest and Bhavé, 2008). The PIPs are further divided into two

subclasses. In general PIP1s have little or no water channel activity *in vitro*, whereas PIP2s show high water permeability when expressed in *Xenopus laevis* oocytes (Chaumont et al., 2000).

Our aim here was to investigate growth factors (root and shoot lengths, fresh and dry weights, leaf numbers and leaf size, relative water content, leaf growth rate, water potential) and expression of the most highly expressed PIP aquaporin gene (VvPIP2.2) in the roots and leaves of two grape genotypes under salinity. Also, correlations between these parameters were discussed statistically.

## Materials and Methods

### Plant materials and growth conditions

Hardwood cuttings of two genotypes of grapevine (Gharashani and Shirazi) were obtained from Kahriz vineyard (Agricultural Research Center, Urmia, Iran). The cuttings were disinfected and then the basal parts of the cuttings were soaked in IBA 0.1% (w/v) for 5-10 s. All cuttings were put in a mist house (relative humidity 80%) with a heat-bed temperature of 20-30 °C. After two weeks, the rooted cuttings were transferred in pots (2L) containing Hoagland solution. Plants with 4-5 fully expanded leaves were treated with NaCl (0, 25, 50, 100 mM NaCl) in full strength Hoagland solution for 2 weeks. NaCl was added to the nutrient solution at the desired concentration incrementally until the final desired concentrations were reached. Leaf water potential was measured by pressure chamber (Scholander et al., 1965) at different treatments in time points. Also, leaf area variations in different salinity treatments were measured by Compu Eye, LSA software on days 0, 7, and 14 after salinity. Leaf growth rate and relative water content [RWC = (fresh weight-dry weight) / (turgid weight-dry weight) × 100] were also studied under salinity. Plants were harvested after 2 weeks and plant parts were weighed separately and dried at 70 °C for 48 hours. For RT-PCR the root and leaf tissues were collected in 50 mM NaCl and different time points and were frozen in liquid nitrogen immediately and stored at -80 °C until RNA isolation.

## RNA isolation, cDNA synthesis and RT-PCR conditions

Total RNA was extracted from root and leaf tissues by Louime et al. (2008) method with small modifications. The RNA concentration was determined by spectrophotometry. The products were also checked on an agarose gel. cDNA was synthesized from total RNA using a cDNA Kit (Fermentas) according to the manufacturer's instructions. PCR conditions were run as following protocol: initial denaturation at 95 °C for 3 min, followed by 95 °C for 30s, annealing temperature for 30 s and 28-30 cycle, extension: 72 °C for 20 s and final extension: 72 °C for 5 min. EF1 gene (Elongation Factor 1) was used as

internal reference. Forward and reverse primers sequences that were used for RT-PCR are given in Table 1. The products of RT-PCR were separated on 1.5% agarose gel containing Ethidium Bromide (0.5 µg/ml) and visualized using Gel Doc 2000 system (Bio-Rad). Gene Ruler 100 bp plus (100-1500 bp) was used as DNA ladder (Fermentas). Experiments were repeated three times. The intensity of the RT-PCR bands was then measured using Image J software.

## Statistical analysis

All statistical analyses were done using the Statistical Package for Social Sciences (SPSS) for Windows (Version 14.0). The mean values of three replicates and the "Standard Error" of the

Table1  
Forward and reverse primers sequences that are used for RT-PCR

Genes	Forward Primer (5'→ 3')	Reverse Primer (5'→ 3')
VvAquaporin PIP 2.2	TCCGCCAAGGACTATCATGAC	CGCAATCAGAGCCCTGTAGAA
VvEF1	TCTGCCTTCTCCTTGGTA	GCACCTCGATCAAAGAGGA

Table 2  
Mean values for root and shoot lengths (cm) and fresh weights of root and shoot (g) in two table grapes (*Vitis vinifera* L.) at different salinity levels (0, 25, 50 and 100 mM NaCl). Data are the means ± standard Error (n=3, One Way ANOVA). Different letters within a column indicate significant differences (P<0.05).

Genotype	Salinity (mM NaCl)	Root Length	Shoot Length	Root Fresh Weight	Shoot Fresh Weight
Shirazi	0	48.5 d	66.83 d	5.02 d	17.11 d
	25	35.83 c	41.17 c	3.53 c	15.49 c
	50	31.33 b	37.17 b	2.37 b	11.76 b
	100	25.67 a	26.67 a	1.44 a	8.41 a
Gharashani	0	56.5 d	49.33 d	7.66 d	13.14 d
	25	47.5 c	42.5 c	6.68 c	11 c
	50	33.5 b	33.36 b	5.74 b	10.17 b
	100	32.5 a	31.63 a	5.34 a	8.81 a

Table 3  
Mean values for leaf number per plant and average leaf size in two table grapes (*Vitis vinifera* L.) at different salinity levels (0, 25, 50 and 100 mM NaCl). Data are the means ± standard Error (n=3, One Way ANOVA). Different letters within a column indicate significant differences (P<0.05).

Genotype	Salinity (mM NaCl)	Leaf Number Per Plant	Average Leaf Size (cm <sup>2</sup> )
Shirazi	0	29 c	54.46 d
	25	26.33 b	50.36 c
	50	24.66 a, b	44.42 b
	100	24.33 a	42.48 a
Gharashani	0	23.67 b	50.02 d
	25	20.33 a	55.39 c
	50	19 a	51.43 b
	100	19 a	49.17 a

means were calculated. GLM (General Linear Model) was used to determine the significance between different treatments and then Tukey's multiple range tests ( $p < 0.05$ ) was employed.

## Results

### Growth responses to salt treatments

Salinity decreased root and shoot growth in both plants (Table 2). High salt treatments were deleterious for roots, especially for Shirazi. Decrease in shoot and root lengths and fresh weights was higher in Shirazi than in Gharashani genotype.

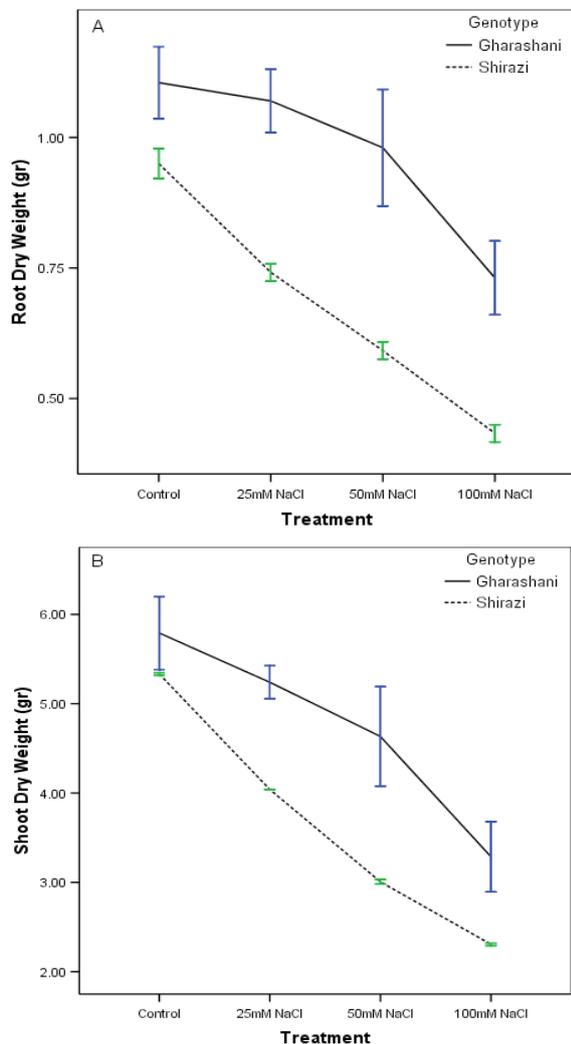


Fig. I. Dry weights of root (A) and shoot (B) (g) in two table grapes (*Vitis vinifera* L.) at different salinity levels (0, 25, 50 and 100 mM NaCl). Data are the means  $\pm$  standard Error (n=3, One Way ANOVA)

Leaf numbers and average leaf size decreased under salinity. Shirazi had also higher decrease in leaf size (23.5%) than Gharashani (15.1%). It seems that in comparison with Shirazi genotype, Gharashani could better protect its leaves under severe salinity (Table 3).

Dry weights of all parts were significantly ( $p < 0.05$ ) reduced at all salinity levels (Fig. I). Gharashani showed a higher root dry weight decrease than Shirazi (66% and 45% control, respectively) at 100 mM NaCl. Gharashani had 57% control shoot dry weight at severe salinity and Shirazi had 43%.

Salinity decreased leaf growth rate and inversely increased water saturation deficit.

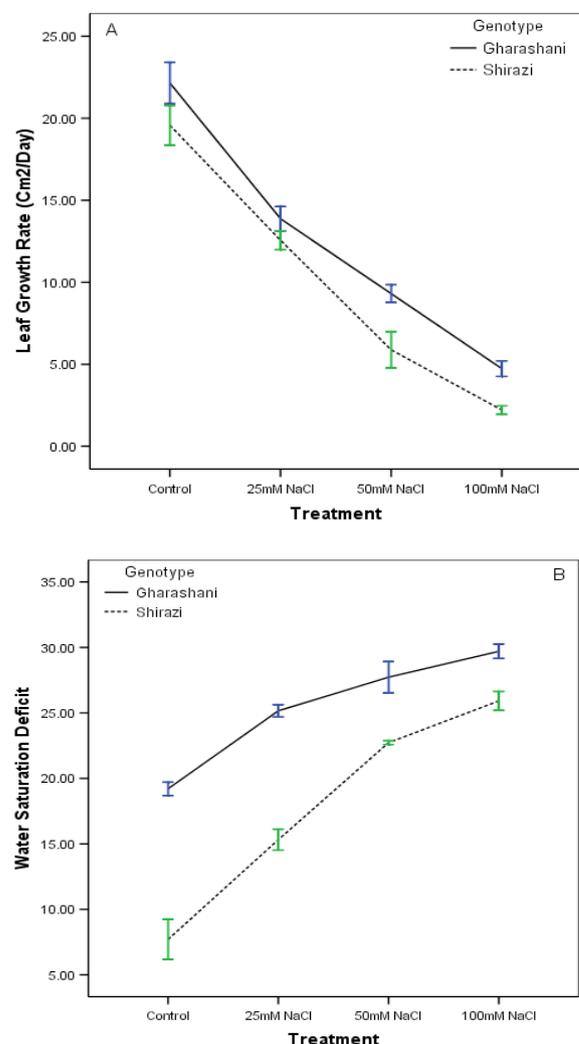


Fig. II. Leaf growth rate (Cm<sup>2</sup>/Day) [A] and water saturation deficit [B] in two table grapes (*Vitis vinifera* L.) at different salinity levels (0, 25, 50 and 100 mM NaCl). Data are the means  $\pm$  standard Error (n=3, One Way ANOVA).

Shirazi had higher decrease in leaf growth rate and higher increase in water saturation deficit than Gharashani (Fig. II). It seems that Gharashani could grow better than Shirazi genotype under salinity stress, especially at severe salinity stress (100 mM NaCl).

### Water relations and salinity

Salinity decreased water potential and relative water content in both genotypes (Fig. III). This decrease in Shirazi genotype was higher than Gharashani; Gharashani could retain better its

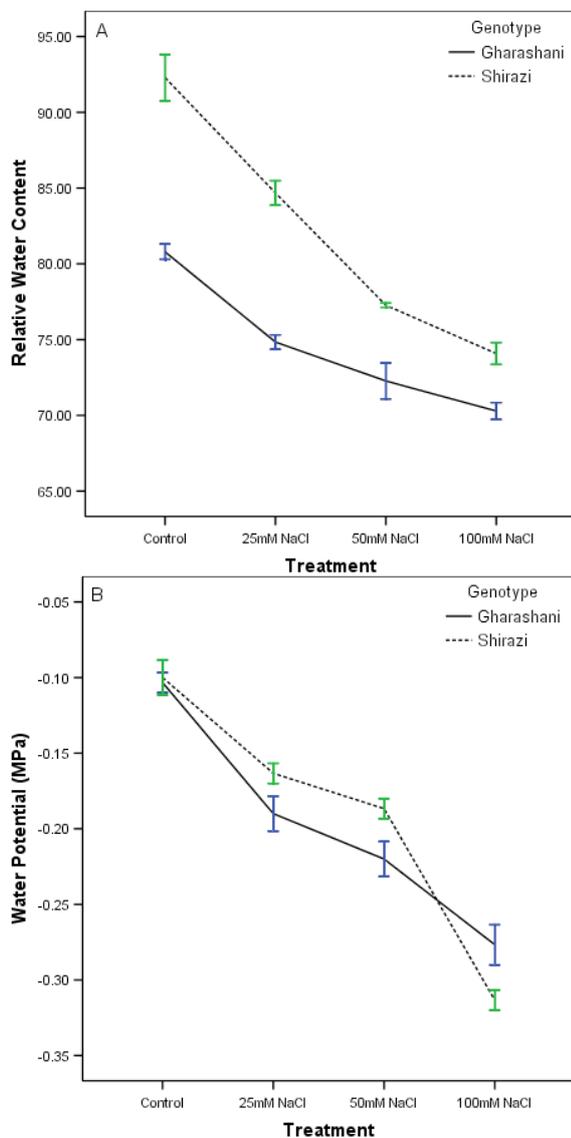


Fig. III. Relative water content [A] and water potential (MPa) [B] in two table grapes (*Vitis vinifera* L.) at different salinity levels (0, 25, 50 and 100 mM NaCl). Data are the means  $\pm$  standard Error (n=3, One Way ANOVA).

water and water potential under salinity. By comparing diagrams A and B diagrams in Fig. III, it is found that relative water content and water potential had reciprocal relations. Gharashani had lower decrease in its water content and water potential than Shirazi under salinity.

### Salinity and leaf growth

Leaf area of two genotypes significantly ( $p < 0.05$ ) increased with time at all salinity levels (Fig. IV), but with increasing salinity increase in leaf area was lower than control. So, leaf area variation through 14 days in Gharashani control plants was 1.3, whereas in Shirazi the ratio was 1.27. In 25 mM NaCl, Gharashani leaf area variation through 14 days salinity was 1.2 control plants, whereas in Shirazi the ratio was 1.17. In 50 mM NaCl, Gharashani leaf area variation through 14 days was 1.18 control plants, whereas in Shirazi the ratio was 1.08. In 100 mM NaCl, Gharashani leaf area variation at 14 days salinity was 1.07 control plants, whereas in Shirazi the ratio was 1.03. Therefore, it was concluded that Gharashani showed a higher increase of leaf area in control and under all salinity levels compared to Shirazi.

### Water potential and expression of aquaporin gene under salinity

In screening experiments, it was shown that 50 mM NaCl is suitable for molecular analysis in grape, because it induces salt stress in plant without having deleterious effects. We did molecular experiments by 50 mM NaCl treatment at different time points (24 hours and 14 days). We measured water potential at the same time points by pressure chamber.

Water potential decreased with time and this decrease in Shirazi genotype was more than that in Gharashani (Fig. V).

Figs. VI and VII show the expression profile of aquaporin gene in roots and leaves of the two genotypes under salinity. While in roots of Gharashani, expression of aquaporin PIP2.2 decreased, in Shirazi expression of this gene increased. In leaves of Gharashani expression of aquaporin PIP2.2 first decreased and then increased. In Shirazi genotype this trend was

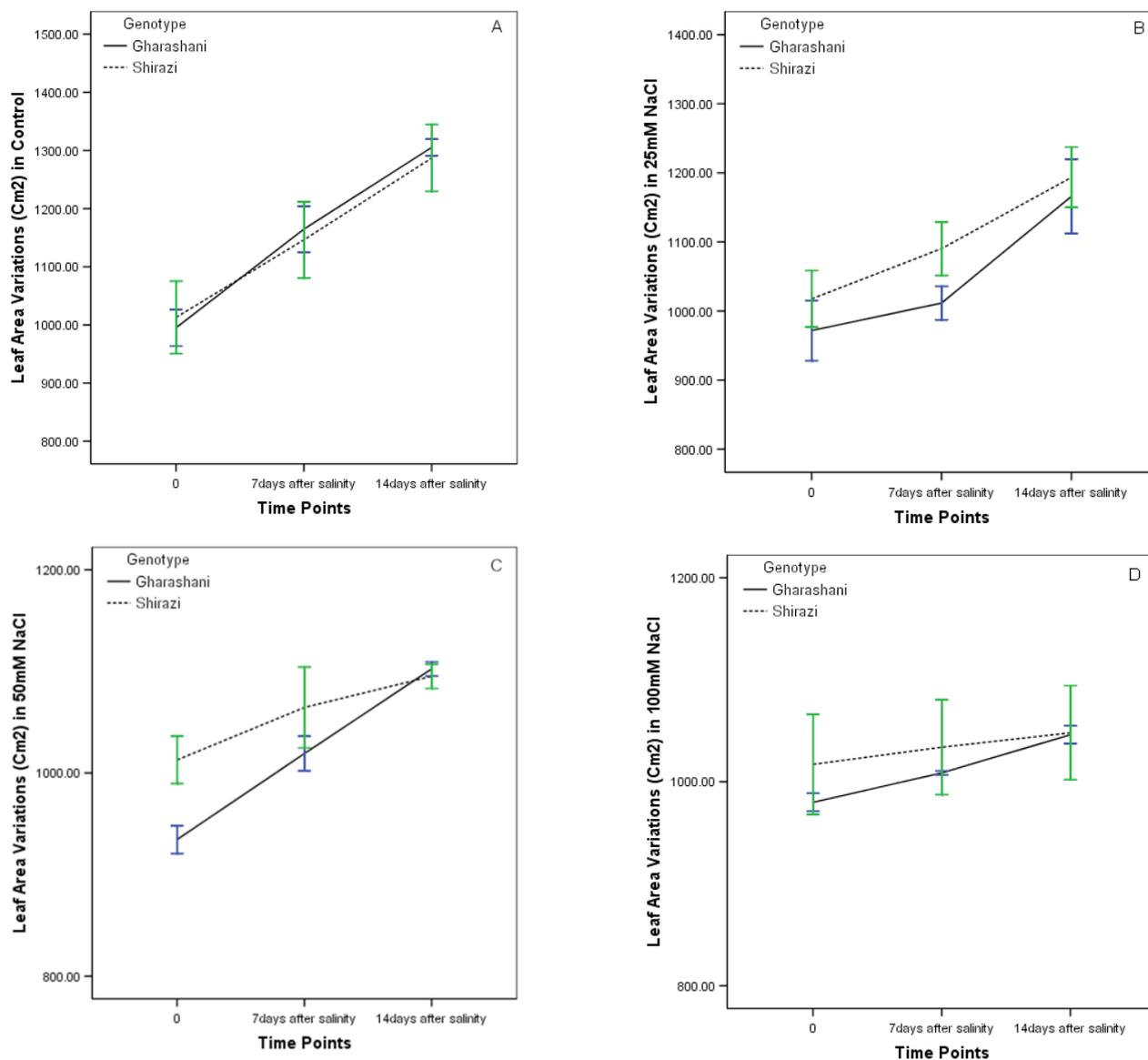


Fig. IV- Leaf area variations in two table grapes (*Vitis vinifera* L.) at Control (A), 25mM NaCl (B), 50mM NaCl (C) and 100mM NaCl (D) at different time points (0, 7days after salinity and 14days after salinity). Data are the means  $\pm$  standard Error (n=3, One Way ANOVA).

inverse. In roots and leaves of Gharashani, expression of aquaporin PIP2.2 was decreased compared to control, but in roots and leaves of Shirazi expression of this gene was partly increased compared to control.

## Discussion

Salinity is an important abiotic stress, which affects crop productivity. Unlike drought, salinity stress is an intricate phenomenon which includes osmotic stress, specific ion effect, nutrient deficiency, etc., thereby affecting various

physiological and biochemical mechanisms associated with plant growth and development (Kholova et al., 2009). The effect of salinity on non-halophytes is a reduction in growth and yield (Mass and Hoffman, 1977). According to Munns et al. (1995), there are two components of salinity that are related to salinity-induced changes in plants; one is linked to osmotic effect operating under low salinity, and the other to toxic effects due to excessive salt accumulation or imbalance in nutrient under high salinity.

Grapevines are classified as moderately sensitive to salinity (Mass and Hoffman, 1977).

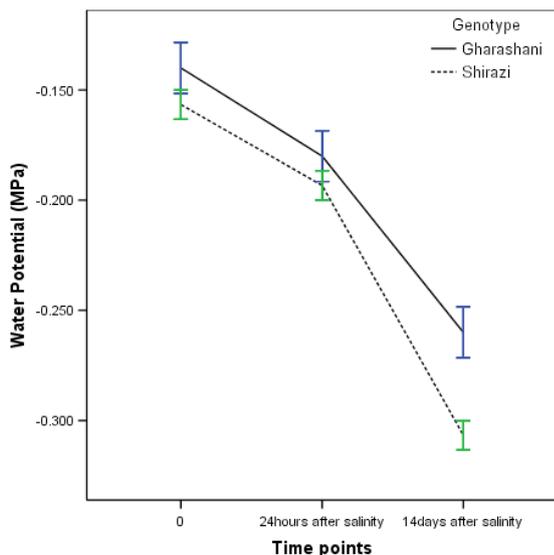


Fig. V. Water potential (MPa) of two table grapes (*Vitis vinifera* L.) at different time points (0, 24 hours after salinity and 14 days after salinity). Data are the means  $\pm$  standard Error (n=3, One Way ANOVA).

Comparison of the effects of different salts (chloride, sulfate and carbonate salts of magnesium, calcium, potassium and sodium) on grapevine demonstrated that chloride salts caused more leaf damage than sulfate or carbonate salts at the same concentrations.

Grapevine responses to salinity include physiological and systemic disturbances leading to reductions in both growth and yield (Aydi et al., 2008). Reduction in growth in response to salinity is usually attributed to ion toxicity and low external osmotic potential. Both effects may affect plant physiological and biochemical processes (Munns and Tester, 2008).

There are reports that salinity stress reduces root extension growth and growth of vines (Aydi et al., 2008) which could imply that poor growth performance of these vines may be related to high  $Cl^-$  uptake and reduced

photosynthesis rates. Growth reductions are observed at relatively low salinities, often before the appearance of visible symptoms (Passioura, 2010). Growth is impaired by high NaCl doses which bring about the appearance of foliar (leaf curling and necrosis) and root (fishbone face and absence of rootlets) symptoms.

In our study shoot and root lengths and fresh weights of plants were drastically decreased as compared to control with increasing salinity treatments. Limited growth due to NaCl observed in the present investigation has been also reported by Alizadeh et al. (2010) while studying in vitro growth and leaf composition in grape cultivars. Growth parameters (shoot and root length and fresh weight) of Shirazi decreased more than Gharashani under NaCl stress.

Increased root zone salinity led to a progressive decline in growth of both genotypes. Salinity reduced shoot fresh weight by reducing both shoot length and total leaf area. The decline in leaf growth is the earliest response of glycophytes exposed to salt stress (Munns and Tester, 2008). Our genotypes showed decrease in leaf growth under salinity, especially in severe salinity treatments (50 and 100 mM NaCl). Decrease in leaf growth in Shirazi was higher than Gharashani. Growth factors showed negative correlations ( $p < 0.05$ ) with water saturation deficit, it means that if growth factors of a genotype highly decrease it will be countered with high water saturation deficit. Shirazi showed high reduction in its growth factors and increase in water saturation deficit.

The physiological and biochemical basis of the growth reduction are not fully understood. The visible symptoms in most cases are due to accumulation of toxic ions in leaves. In severe cases, salt causes symptoms like necrotic areas on leaves, initially at leaf margins, and progressing inwards. Such visible symptoms on

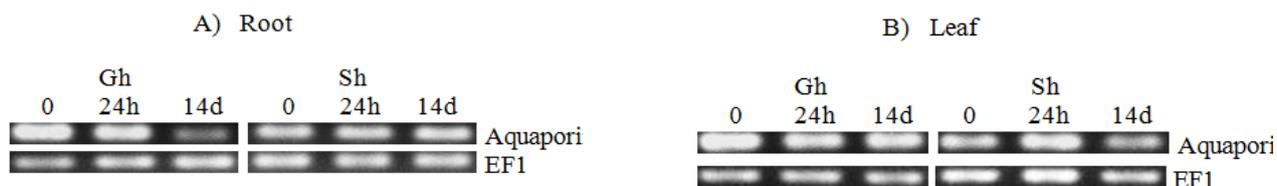


Fig. VI. Expression profile of Aquaporin gene in root and leaf of two table grape (*Vitis vinifera* L., Gh: Gharashani; Sh: Shirazi) after 0, 24 hours and 14 days treated by 50 mM salinity.

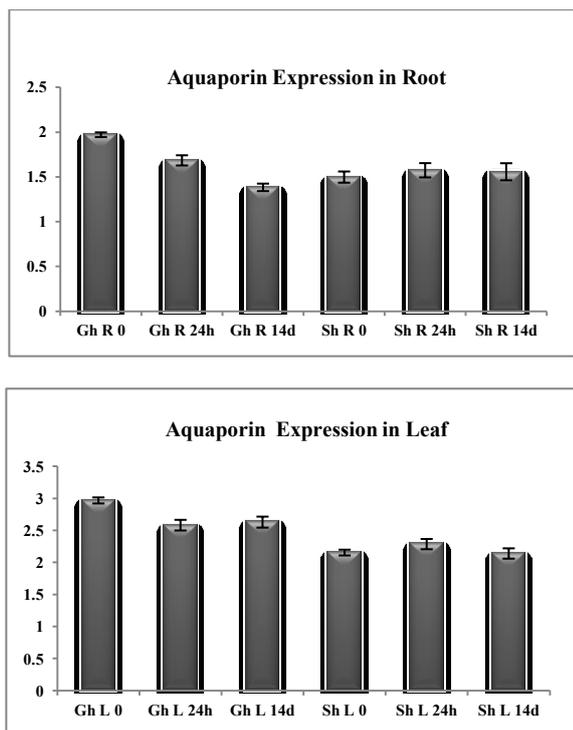


Fig. VII. Relative expression diagram of Aquaporin gene in root and leaf of two table grape (*Vitis vinifera* L., Gh: Gharashani; Sh: Shirazi) after 0, 24 hours and 14 days treated by 50 mM salinity. Bars are the means  $\pm$  Standard Deviation (n=3, One Way ANOVA, Tukey's test).

leaves are often referred to as 'leaf burn' (Aydi et al., 2008). We observed leaf burn in our genotypes at higher salinity treatments (50 and 100 mM NaCl), especially in Shirazi genotype.

Restriction of extension growth caused by salinity is the result of decreased water uptake by roots due to an imbalance between water uptake and transpiration. This, in turn, would depress xylem water potentials and collapse the water potential gradient between expanding cells and water source (xylem) that drives extension (Munns and Tester, 2008). This interpretation is supported by measurements of RWC showing a decline in leaves of treated plants. The significant changes in leaf relative water content (RWC) during the initial period of osmotic stress suggests that the dehydration may be the most important factor contributing to the decrease of osmotic potential. Under high drought stress a reduction in RWC was a common response (Pérez-Pérez et al., 2007).

In the present study in severe salinity stress (100 mM NaCl) we observed drastic

decrease in relative water content of Shirazi (20%) than in Gharashani (10%) compared to the control plants. Also, regarding RWC, Shirazi genotype showed high decrease in its water potential at 100 mM NaCl compared to the control. There were significant positive correlations ( $p < 0.05$ ) between growth factors (root and shoot length, dry weights and leaf growth rate) and water relations factors (relative water content and water potential).

Leaf area is commonly evaluated as an important variable for most physiological studies involving plant growth, photosynthetic efficiency, and response to water potential (Peksen, 2007). Leaf extensibility did not increase under salinity. The absence of an increase in leaf extensibility of NaCl-treated plants is presumed to be the main cause of slower elongation of their leaves. In our study, average leaf size decreased under salinity. In Gharashani the decrease was 16% at 100 mM NaCl compared to the control, whereas in Shirazi this ratio was 23.4%.

In long term, the number of leaves and total leaf area were affected by the presence of salt and the reduction in leaf area was more at high levels of stress than at low levels. The inhibition of leaf growth is seen as an adaptation to salinity and water deficiency, because the reduced leaf area allows plants to cut water losses by lowering transpiration and delaying the onset of more severe stress (Chaves et al., 2009).

The results are consistent with published data showing low increase in leaf extensibility in salt-treated plants (Neumann, 1993). In the present study, the number of leaves decreased under salinity and this decrease was higher in Gharashani than in Shirazi (20% compared to 17%). Leaf area increased in all treatments with time, but the increase in high salinities was lower than the control, especially in Shirazi genotype. In control plants of Gharashani 24% increase in leaf area was observed after 14 day; this ratio in Shirazi was 23%. At 25 mM NaCl we observed 16% increase in leaf area in Gharashani and 17% in Shirazi genotype. At 50 mM NaCl 15% increase in leaf area in Gharashani and 7% in Shirazi was observed. Finally, we had 6% increase in leaf area in Gharashani and 3% in Shirazi at 100 mM NaCl. In high salinities (50 and 100 mM NaCl) Gharashani had better leaf growth (twice more)

than Shirazi. This means that Gharashani showed a capacity to accord with stress conditions better than Shirazi.

During the initial period of osmotic stress leaf growth is inhibited by a reduction of water uptake into the growing zone, as for example when the xylem water potential suddenly drops. Additionally, this reduction in the rate of leaf elongation is rapid and may be related to a reduction in turgor (Passioura, 2010). Rapid reductions in leaf expansion rates after a sudden increase in salinity have been reported in many species (Passioura, 2010). Our results confirmed them, too. In high salinities (50 and 100 mM NaCl) water potential and relative water content were suddenly dropped and there was a high decrease in leaf growth especially in Shirazi.

Results of nine representative studies on PIP expression under drought conditions showed that among the 37 PIP genes studied, 15 were down-regulated, 13 up-regulated, and nine unaltered (Aroca et al., 2007; Ruiz-Lozano et al., 2009). So, based on expression studies it is difficult to assign a role for PIP genes in drought stress. In fact, there is evidence that each PIP gene could have a specific function under specific stress circumstances. For example, Jang et al. (2007) found that the overexpression of a certain PIP aquaporin gene induced tolerance to some environmental stresses but sensitivity to others. Similarly, Aharon et al. (2003) found that the overexpression of a foreign PIP aquaporin gene in transgenic tobacco improved plant vigor under favorable growth conditions but not under drought or salt stress conditions. The highly variable response to water stress of aquaporins at the transcript level depends on species, type of water stress, degree of water stress, and the plant organ (Tyerman et al., 2002; Bramley et al., 2007).

Generally, a decrease in abundance of PIP2 proteins has been recorded (Aroca et al., 2007; Ruiz-Lozano et al., 2009), but an accumulation of PIP1 proteins under drought conditions has also been found (Aroca et al., 2007). Our results were consistent with these findings as the expression of PIP2.2 aquaporin decreased (compared to the control) significantly ( $p < 0.05$ ) in roots and leaves of Gharashani and partly increased in Shirazi. The expression of

VvPIP2.2 decreased 35% in roots of Gharashani genotype after 14 days exposure to 50 mM NaCl, but the decrease in leaves was 16% compared to the control. In Shirazi the expression of VvPIP2.2 increased 12% in roots and 8% in leaves. Based on the expression levels of VvPIP2.2 genes and water relations (water potential and relative water content) of two genotypes, since Gharashani had a better status than Shirazi considering growth factors and water relations and the lower expression of VvPIP2.2 gene in Gharashani genotype, it seems that this gene is not involved in uptake of water, especially in Gharashani.

In conclusion, salinity resulted in decreased growth factors (root and shoot lengths, fresh and dry weights, leaf area, relative water content and water potentials) in both genotypes. This decrease in Shirazi was higher than Gharashani. Under salinity stress the expression of VvPIP2.2 aquaporin gene decreased significantly ( $p < 0.05$ ) in roots and leaves of Gharashani, but it was higher in roots than in leaves. In Shirazi a partly increase was observed in expression of this gene. It is concluded that VvPIP2.2 aquaporin gene is not involved in uptake of water in these genotypes.

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