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BRIEF COMMUNICATION

In vitro responses of grape rootstocks to NaCl

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Abstract

An investigation was undertaken to adjudge the *in vitro* salt tolerance and biochemical changes due to NaCl on four grape rootstocks (Dogridge, SO4, H-144 and 3309C). The *in vitro* two-node micro-cuttings from the established cultures were sub-cultured on rooting medium comprising Murashige and Skoog (MS) basal medium supplemented with 0.5 μM indolebutyric acid (IBA), 200 mg dm^{-3} activated charcoal and different NaCl concentrations ranging from 0 to 125 mM. The surviving and proliferated cultures were further sub-cultured four times on respective media. Dogridge and H-144 tolerated 125 and 100 mM NaCl, respectively, while SO4 and 3309C survived only up to 75 mM NaCl. Contents of proteins, proline, K^+ and Na^+ in tissue increased in all genotypes due to NaCl supplementation, while contents of chlorophyll and total soluble sugars declined. Higher K^+/Na^+ ratio was registered in Dogridge and H-144 than in SO4 and 3309C. The relative NaCl tolerance for different grape rootstocks under study could be ranked as Dogridge > H-144 > SO4 and 3309C.

Additional key words: chlorophyll, proline, proteins, rootstocks, salinity tolerance, total soluble sugars, *Vitis* spp.

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). Tissue injury is induced not only by the osmotic effects of salts but also by specific toxic effects resulting from the accumulation of Cl^- and Na^+ (Hasegawa *et al*. 2000).

Grapes are grown in semiarid environments, where drought and salinity are common problems. There is a wide variation for salt tolerance amongst rootstock genotypes (Walker and Douglas 1982, Behboudian *et al.* 1986). *In vitro* screening for salt tolerance has been investigated in some grape cultivars (Singh *et al.* 2000, Khawale *et al.* 2003, Cavagnaro *et al.* 2006) and the technique has been reported useful for screening of some other fruit species such as mulberry (Tewary *et al.* 2000), citrus (Singh *et al.* 2004) and cherry rootstock (Erturk *et al.* 2007). In the present study, we examined the *in vitro* responses of four genetically different grape rootstock genotypes to elevated NaCl levels.

The 5-year-old field-grown mother plants of four grape rootstocks, Dogridge (*Vitis champini*), SO4 (*V. riparia* × *V. berlandieri*), H-144 (*V. vinifera* × *V. labrusca*) and 3309C (*V. riparia* × *V. rupestris*), procured from the Main Orchard, Division of Fruits and Horticultural Technology, IARI, New Delhi, were used as source of explants. *In vitro* culture was initiated following the protocol developed by Singh *et al.* (2004).

The *in vitro* proliferated shootlets from the established cultures were used as explant for screening. Two-node micro-cuttings were excised from established cultures and were sub-cultured onto Murashige and Skoog (1962; MS) medium supplemented with 2.0 mg dm^{-3} indolebutyric acid (IBA), 200 mg dm^{-3} activated charcoal and different concentrations (0 to 125 mM) of analytical-reagent grade sodium chloride (*Hi-Media*, Mumbai,

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Abbreviations: IBA - indolebutyric acid; MS - Murashige and Skoog medium.
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India). The proliferated cultures were further sub-cultured onto respective media with elevated NaCl concentrations up to four sub-cultures. Forty five days after last sub-culture, the chlorophyll *a* and *b* contents of the leaves were measured following the method as suggested by Barnes *et al*. (1992). The rapid colorimetric method of Bates *et al*. (1973) was followed to estimate proline contents. The amount of total sugars was estimated using anthrone reagent method (Thimmaiah 2004). The amount of soluble proteins was estimated following the method of Bradford (1976). Na and K content were quantified by flame photometer (*Systronics 121*, Ahmedabad, India).

Genotypes cultured on NaCl-free medium (control) exhibited good proliferation rate which was highest in H-144 followed by Dogridge, SO4 and 3309C (data not shown). However, arrested vegetative growth and explant injury owing to NaCl incorporation to the proliferation medium were observed. The number of surviving cultures, scored at days 30, 45 (data not shown) and 60, decreased as salt concentration increased (Table 1). Significant differences among salt treatments and genotypes were found for explant survival. Dogridge plantlets tolerated upto 125 mM NaCl, while H-144 upto 100 mM NaCl, and SO4 and 3309C only 75 mM NaCl. Leaf damage was not observed in any genotype cultured on medium supplemented with 25 mM NaCl suggesting some degree of salt tolerance in these genotypes. On the other hand, within a week following inoculation, leaf injury symptoms appeared on SO4 and 3309C in NaCl concentration at 50 mM and above. The examples of salt injury are presented in Fig. 1. Earlier, working also with some grape cultivars, similar results were reported by Pandey and Divate (1976).

Shoot inter-nodal length, shoot fresh and dry masses, mean vine length, root fresh and dry masses, number of leaves and leaf area of inoculated explants were drastically decreased as compared to NaCl-free medium (Fig. 1, other data not shown). Number of roots per explant was adversely affected with increasing NaCl concentration (Table 1). At 100 mM NaCl, the SO4 and 3309C genotypes failed to produce roots. The adverse effects of salinity on growth parameters may be attributed to ionic imbalance, altered availability and uptake of other ions, accumulation of ions in leaf cell vacuoles, decline in photosynthetic rate, and reduced carbon fixation as previously reported by Prior *et al.* (1992) working on Sultana grapevine. NaCl was found to inhibit the growth and development of mulberry tissue under *in vitro* conditions (Vijayan *et al.* 2003). Arrested growth due to NaCl observed in the present investigation has been also reported by Singh *et al*. (2000) while studying *in vitro* growth and leaf composition in grape cultivars.

Among the biochemical parameters affected by NaCl stress, accumulation of free proline within the tissue is the most significant one. Nevertheless, its role in combating salt stress is a matter of debate. Many studies show the important osmoprotective role of proline under stress conditions (Stewart and Lee 1974, Jain *et al*. 1987, Chandler and Thorpe, 1987). However, some workers consider an enhanced proline content simply as a stress effect, rather than a cause of stress tolerance (Moftah and Michel 1987). We observed enhanced proline accumulation with increase in *in vitro* NaCl content in all grape rootstock genotypes (Table 1). Proline accumulation in tolerant genotypes was about 1.13 times higher than in sensitive ones. Changes in soluble proteins were also found to be more or less similar to proline and high protein contents were observed in Dogridge and H-144 (tolerant genotypes). Accumulation of proteins in these genotypes grown under saline conditions may provide a storage form of nitrogen that can be re-utilized when stress is over and may play a role in osmotic adjustments. Proline and various betains can function as osmo-protectants and cryoprotectants when accumulated in cells. However, the accumulation of proline or betains under stress is species dependent (Shannon *et al*. 1994, Nolte and Hanson 1997). It has been suggested that high proline accumulation induced by NaCl correlates with growth inhibition (Perez-Alfocea *et al*. 1994, Lin and Kao 1996).

Change in soluble sugars content under salt stress has already been reported for a number of plant species, but information regarding the role of sugars in adaptation of plants to salinity is hitherto insufficient to conclude that they are universally associated with salt tolerance (Ashraf and Harris 2004). In the present study, total soluble sugars initially increased due to increasing NaCl concentrations followed by decline (Table 1). Sugar contents declined beyond 75 mM NaCl in susceptible genotypes (SO4, 3309C). The salt tolerant lines had generally greater soluble sugars than the salt sensitive ones. Similar findings were earlier reported by Ashraf and Tufail (1995), while studying total sugars content in five sunflower accessions differing in salt stress tolerance.

Change in chlorophyll content due to salinity is the most obvious biochemical response (Rao *et al.* 1991). The decrease in chlorophyll content was not found significant upto 25 mM NaCl as compared to control. However, chlorophyll *a*+*b* in the leaves showed rapid decline as the concentration of NaCl increased (Table 1) in all rootstock including Dogridge which survived at 125 mM NaCl. Decline in chlorophyll content due to increasing salinity has been reported by Sivritepe and Eris (1999), Singh *et al.* (2000) and Khawale *et al.* (2003). In another *in vitro* study on cherry rootstock, salinity reduced growth and chlorophyll content but there was no effect on water content (Erturk *et al.* 2007). On the other hand, water stress induced *in vitro* also caused decrease in chlorophyll content in cherry rootstocks and also the activities of antioxidant enzymes, such as superoxide dismutase, catalase, ascorbate peroxidase, peroxidase and glutathione reductase were significantly

Table 1. Effect of different NaCl concentrations (0 - 125 mM) on *in vitro* explant survival [%], sodium content [mg g^{-1}(d.m.)], potassium content [mg g^{-1}(d.m.)], sodium:potassium ratio, chloride content [mg g^{-1}(d.m.)], root number, proline content [μg g^{-1}(f.m.)], total proteins [mg g^{-1}(f.m.)], total soluble sugars [mg g^{-1}(d.m.)], and chlorophyll content [mg g^{-1}(f.m.)] in four grape rootstocks grown 60 d on shoot proliferation medium supplemented with 0 to 125 mM NaCl. Mean ± SE, n = 45.

Parameter	Genotype	0	25	50	75	100	125 mM
Explant survival	Dogridge	92.8 ± 2.3	80.2 ± 1.4	72.6 ± 1.6	36.9 ± 1.8	26.4 ± 1.30	18.6 ± 1.01
	SO4	85.2 ± 1.8	61.4 ± 2.1	51.2 ± 1.2	26.1 ± 1.3	14.3 ± 1.01	-
	H-144	93.4 ± 1.1	83.3 ± 2.1	53.4 ± 1.4	33.7 ± 1.6	8.7 ± 0.08	-
	3309C	87.9 ± 1.2	45.7 ± 1.5	32.4 ± 1.5	12.5 ± 2.0	-	-
Sodium content	Dogridge	10.6 ± 0.15	13.8 ± 0.12	16.1 ± 0.10	18.9 ± 0.23	19.9 ± 0.23	23.2 ± 0.18
	SO4	5.7 ± 0.20	7.9 ± 0.10	12.3 ± 0.05	15.3 ± 0.12	17.2 ± 0.33	-
	H-144	5.2 ± 0.21	8.3 ± 0.06	10.7 ± 0.08	13.4 ± 0.11	15.5 ± 0.11	-
	3309C	6.9 ± 0.12	9.3 ± 0.08	11.2 ± 0.11	14.2 ± 0.09	-	-
Potassium content	Dogridge	15.4 ± 0.14	19.3 ± 0.18	24.1 ± 0.18	28.8 ± 0.20	31.1 ± 1.31	36.2 ± 1.10
	SO4	8.6 ± 0.13	12.4 ± 0.12	13.5 ± 0.12	16.2 ± 0.14	18.6 ± 0.14	-
	H-144	7.9 ± 0.08	11.9 ± 0.09	16.7 ± 0.07	21.4 ± 0.18	23.7 ± 1.01	-
	3309C	9.9 ± 0.07	11.3 ± 0.06	14.2 ± 0.06	14.8 ± 0.08	-	-
Na^+/K^+ ratio	Dogridge	1.4 ± 0.03	1.4 ± 0.02	1.5 ± 0.03	1.5 ± 0.01	1.6 ± 0.01	1.5 ± 0.01
	SO4	1.5 ± 0.01	1.6 ± 0.02	1.1 ± 0.01	1.0 ± 0.02	1.1 ± 0.00	-
	H-144	1.5 ± 0.01	1.4 ± 0.01	1.6 ± 0.02	1.6 ± 0.01	1.5 ± 0.01	-
	3309C	1.4 ± 0.01	1.2 ± 0.03	1.3 ± 0.01	1.0 ± 0.02	-	-
Chloride content	Dogridge	5.9 ± 0.08	16.9 ± 0.04	24.2 ± 1.05	31.3 ± 1.06	36.8 ± 1.08	49.6 ± 2.18
	SO4	4.1 ± 0.04	8.6 ± 0.07	16.4 ± 1.02	18.3 ± 1.02	21.4 ± 1.06	-
	H-144	3.6 ± 0.05	7.4 ± 0.01	18.2 ± 1.03	24.5 ± 1.03	26.8 ± 1.07	-
	3309C	4.9 ± 0.02	8.7 ± 0.01	13.6 ± 0.08	17.2 ± 1.02	21.5 ± 0.90	-
Root number	Dogridge	5.3 ± 0.01	4.4 ± 0.02	4.1 ± 0.02	2.3 ± 0.01	1.1 ± 0.02	-
	SO4	4.9 ± 0.01	3.8 ± 0.01	2.4 ± 0.01	2.2 ± 0.01	1.6 ± 0.01	-
	H-144	10.8 ± 0.02	9.2 ± 0.02	3.5 ± 0.01	2.4 ± 0.01	1.9 ± 0.01	-
	3309C	5.0 ± 0.02	4.8 ± 0.01	3.9 ± 0.01	3.1 ± 0.02	-	-
Proline content	Dogridge	17.8 ± 1.02	19.2 ± 1.02	22.3 ± 1.10	25.6 ± 1.09	28.1 ± 2.01	27.3 ± 1.23
	SO4	18.2 ± 1.03	21.2 ± 1.11	23.7 ± 1.08	24.2 ± 1.87	20.7 ± 1.03	-
	H-144	16.7 ± 0.97	19.4 ± 0.91	22.5 ± 1.11	26.7 ± 1.79	29.4 ± 1.13	-
	3309C	15.3 ± 0.84	17.8 ± 0.67	19.4 ± 1.13	21.9 ± 1.07	-	-
Total proteins	Dogridge	54.3 ± 3.23	75.9 ± 2.15	125.7 ± 5.14	142.7 ± 5.24	150.4 ± 4.97	168.3 ± 2.34
	SO4	65.2 ± 2.87	70.1 ± 1.19	82.7 ± 1.21	96.7 ± 2.19	108.1 ± 3.25	-
	H-144	61.3 ± 2.14	83.2 ± 1.23	96.7 ± 2.03	104.3 ± 3.04	113.2 ± 2.45	-
	3309C	57.7 ± 1.89	62.4 ± 2.11	75.2 ± 2.13	78.4 ± 2.34	-	-
Soluble sugars	Dogridge	215.9 ± 3.95	218.3 ± 1.65	230.4 ± 3.12	238.6 ± 4.35	245.2 ± 6.16	248.3 ± 6.78
	SO4	174.2 ± 2.01	182.1 ± 2.18	193.6 ± 2.11	184.3 ± 3.15	171.4 ± 5.23	-
	H-144	189.4 ± 1.79	195.4 ± 2.02	208.6 ± 3.04	219.7 ± 2.48	226.2 ± 4.15	-
	3309C	164.3 ± 1.85	170.4 ± 1.45	179.8 ± 2.15	168.3 ± 2.09	-	-
Chlorophyll *a*+*b*	Dogridge	9.1 ± 0.14	8.2 ± 0.13	7.2 ± 0.11	6.0 ± 0.14	5.6 ± 0.02	5.1 ± 0.15
	SO4	8.0 ± 0.12	6.8 ± 0.11	7.6 ± 0.08	6.0 ± 0.03	4.7 ± 0.05	-
	H-144	10.2 ± 0.23	9.0 ± 0.22	9.7 ± 0.13	8.6 ± 0.15	7.0 ± 0.95	-
	3309C	7.5 ± 0.06	7.1 ± 0.10	6.4 ± 0.12	4.7 ± 0.02	-	-

elevated (Sivritepe *et al*. 2008). The recent report on grape cultivars suggest that tolerance to moisture stress can be due to the ability of specific adjustments in the lipid composition during stress which helped to compromise stress tolerance (Toumi *et al*. 2008).

Establishment of ion homeostasis is an essential requirement for plants to survive under salt stress conditions. The sodium and potassium contents were found to be enhanced in the tissues grown under stress conditions (Table 1). The rate of Na^+ increase was more in Dogridge (17.08 %) followed by H-144 (10.62 %). K^+ content in leaves was enhanced in all the NaCl-stressed plants compared to control. The K^+ content was significantly higher in Dogridge (25.96 %) and H-144 (16.32 %) as compared to the other two genotypes. Salt tolerance requires not only adaptation to Na^+ toxicity but

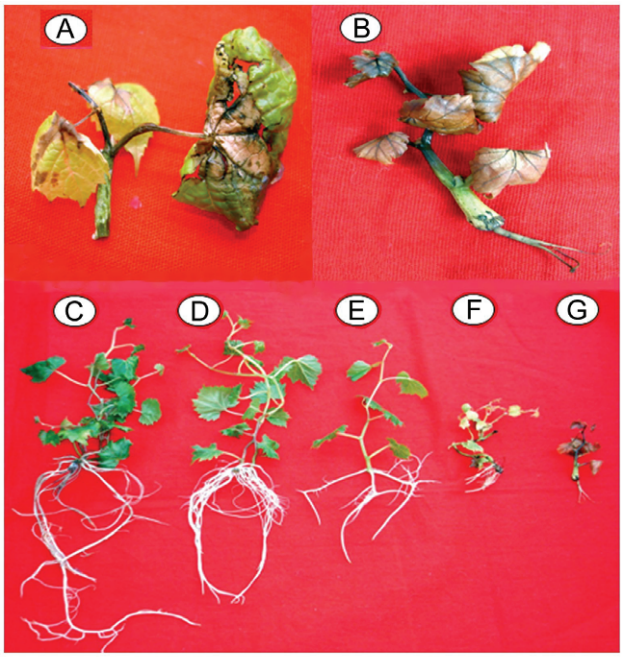

Fig. 1. Extent of explant injury due to *in vitro* salt stress. Plantlets were grown 40 d on rooting medium supplemented with 100 mM NaCl (*A* - Dogridge, *B* - H-144). Comparative vegetative growth and rooting of H-144 on medium with 0 (*C*), 25 (*D*), 50 (*E*), 75 (*F*) and 100 (*G*) mM NaCl for 60 d.

also the acquisition of K^+. The uptake of K^+ is affected by Na^+ due to the chemical similarities between both ions. K^+ is an essential nutrient in most terrestrial plants. Therefore, K^+ transport systems involving good selectivity of K^+ over Na^+ is considered as an important salt tolerant determinant (Amini and Ehsanpour 2005). The K^+/Na^+ ratio was found different in genotypes utilized in the present study (Table 1). Higher K^+/Na^+ ratio (>1.5) was registered in Dogridge and H-144 while it was found lower (< 1.3) in SO4 and 3309C. This ratio was initially high in all four genotypes, while it declined in SO4 and 3309C beyond 50 mM NaCl but remained almost constant in Dogridge and H-144. Corresponding results have been earlier reported by Gorham *et al.* (1990), Singh *et al.* (2000) and Khawale *et al.* (2003) in grape genotypes. Chloride content was increased due to salinity in all the genotypes.

Based on the *in vitro* NaCl response the four grape rootstock genotypes were ranked as Dogridge > H-144 > SO4 and 3309C. Furthermore, it is evident that *in vitro* screening procedure could be used as early diagnostic method in grape rootstock genotypes for salinity and also to identify salt tolerance limits for their commercial utilization in salt affected areas.

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