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BRIEF COMMUNICATION

Responses of the cherry rootstock to salinity *in vitro*

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Abstract

The *in vitro* response of sweet cherry rootstock Gisela 5 (*Prunus cerasus* × *Prunus canescens*) to increasing concentrations of NaCl (0, 50, 100 and 150 mM) in the Murashige and Skoog culture medium was studied. Induced salinity reduced growth and chlorophyll content in shoots but had no effect on water content. The increase in malondialdehyde content indicated that salinity induced oxidative stress which was accompanied with the visible symptoms of salt injury in the shoots. Antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase, peroxidase, catalase, and glutathione reductase were also significantly elevated. Although no change was observed in the Cl concentration, Na concentration of shoots significantly rose and NaCl treatments impaired K, Ca and Mg nutrition and induced imbalance in K:Na and Na:Ca ratios.

Additional key words: antioxidant enzymes, chlorophyll, growth, ion content, lipid peroxidation, salt injury.

Salinity is one of the major factors that limit crop productivity. Therefore, the assessment of the existing and/or newly developed germplasm of crop plants for their tolerance to salinity has become urgent. *In vitro* assays is an ideal system for characterizing the genetic potential of tested woody plants as it can be carried out under controlled conditions with limited space and time (Vijayan *et al*. 2003, Molassiotis *et al.* 2006). Such systems can provide reasonable information concerning physiological and biochemical responses of woody species, especially if the *in vitro* induced response mimics the *in vivo* plant response exposed to similar salinity (Vijayan *et al*. 2003). Gisela 5 as a newly developed cherry rootstock has become popular because of its ability to produce dwarfing and precocity. Despite its thorough testing in many different parameters, the knowledge on the responses of this rootstock to salinity remains unclear. However, cherries are classified as sensitive to salinity (Kotuby-Amacher *et al*. 2000). Rootstocks differ in the ability to exclude toxic sodium and chloride ions and appreciably influence scion tolerance to salinity (Sotiropoulos *et al*. 2006a). The objective of the present study was to investigate the effect of salt stress on growth, water and chlorophyll contents, proline accumulation, lipid peroxidation, antioxidative enzymes and nutritional status of cherry rootstock shoots cultured *in vitro*.

The explants employed were shoots of the cherry rootstock Gisela 5 (*Prunus cerasus* × *Prunus canescens*) from previous shoot-tip cultures maintained in the growth room. The shoots (about 20 mm in length) were transferred and grown in 250 cm^3 jar containing 40 cm^3 of Murashige and Skoog (MS; *SigmaChemical Co*., St. Louis, USA) medium supplemented with sucrose (30 g dm^{-3}) and solidified with agar (7 g dm^{-3}). Four different concentrations of NaCl (0, 50, 100 and 150 mM) were added to the standard MS medium. The pH of the media was adjusted to 5.8 before autoclaving (for 20 min at 121 °C). The cultures were maintained at 25 ± 1 °C under a 16-h photoperiod (white light, 51 μmol $m^{-2}s^{-1}$).

After 4 weeks, the shoots were collected and washed for 2 min with distilled water to remove medium, dried on filter paper and either used or stored at -20 ºC for later use.

The growth response of Gisela 5 to NaCl salinity was measured in terms of both dry mass and shoot length. Dry matter of explants was obtained by heating to 70 °C for 24 h. The water content (WC) of explants was calculated

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Abbreviations: APOX - ascorbate peroxidase; CAT - catalase; Chl - chlorophyll; GR - glutathione reductase; MDA - malondialdehyde; MS - Murashige and Skoog; POX - peroxidase; SI - salt injury index; SOD - superoxide dismutase.
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from the final fresh and dry mass of explants. The relative chlorophyll (Chl) content was measured with a portable leaf chlorophyll meter (*SPAD 502*, *Minolta Co*., Osaka, Japan). Explants were also scored for visible symptoms of salt injury on a 1 - 4 scale as follows: 1 - no injury, 2 - damage on shoot-tips and leaf edges, 3 - necroses on the whole leaf and/or on part of the stem, and 4 - dead. Following this, salt injury index (SI) was calculated according to the following formula: $SI = \sum(n_i \times i)/N$, where n_i is the number of explants receiving the mark "i" (from 1 to 4) and N is the total number of explants in each salt concentration.

To determine the mineral composition, previously dried and ground material was dry ashed at 530 °C for 6 h. Ion extraction was achieved in 65 % HNO_3. Na, Mg, K, Ca, Cu, Zn and Fe were analyzed by *ICP-MS* instrument (*Perkin-Elmer* model *2100 DV*, Norwalk, CT, USA). Chloride was determined colorimetrically by the reaction of chloride ions and mercury thiocyanate, in the presence of the ferric ions using UV spectrophotometer (*Hewlett Packard, 8452A*, Groton, CT, USA).

Proline determination was carried out according to the method of Bates *et al*. (1973). The degree of lipid peroxidation was measured by malondialdehyde (MDA) content according to Madhava and Sresty (2000). With respect to antioxidant enzymes, superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC1.11.1.11), peroxidase (POX, EC 1.11.1.7) and glutathione reductase (GR, EC 1.6.4.2) were extracted and assayed as previously described by Bor *et al*. (2003). All enzyme activities were expressed in terms of specific enzyme activity [U mg^{-1}(protein)] and protein content was determined according to Bradford (1976), using bovine serum albumine as a standard.

The experiments were set up in a completely randomized design and repeated twice. Each treatment included three replicates (with five explants in each 250 cm^3 jar and four jars in each replicate). Analysis of variance was performed, and significant differences among treatment means were calculated by LSD test at $P < 0.05$.

The increasing NaCl concentrations decreased dry mass and shoot length (Table 1). The reduction of dry mass was between 29 and 56 % with increased NaCl concentrations whereas shoot length exhibited 18 % diminution at 150 mM NaCl. Growth reduction is an early phenomenon and a common response in woody plants to salt stress both *in vitro* and *in vivo* (Vijayan *et al*. 2003). According to Sotiropoulos *et al.* (2006b) explants are stressed in two ways under *in vitro* salinity: by the increase in osmotic potential of culture media as a result of high solute content, and by the toxic effects of high concentrations of ions. The chemical potential of the saline media initially establishes a water potential imbalance between the apoplast and symplast that leads to decrease in pressure potential, which might cause growth reduction (Bohnert *et al*. 1995). In a saline environment, leaf cells lose water, creating a lower osmotic potential. However, NaCl treatments had no effect on WC in explants of Gisela 5 (Table 1). On the other hand, the cellular response to water potential reduction is osmotic adjustment. It involves the transport, accumulation and compartmentation of organic solutes and inorganic ions (Bohnert *et al*. 1995). Although osmotic adjustment and the accumulation of organic solutes in explants exposed to salt stress have been reported for several woody plants (Singh *et al*. 2000, Molassiotis *et al.* 2006), an obvious proline accumulation in response to salinity has not been observed in this genotype (Table 1).

Salt-induced growth inhibition is accompanied by metabolic disturbances in Gisela 5. There was a marked reduction in relative Chl content in leaf tissue with increasing NaCl concentrations (Table 1). Marginal chlorosis was noted on the leaves of shoots even at the lowest NaCl concentration, which later resulted in necrosis. The severity of this injury varied depending on NaCl concentrations. 50 and 100 mM NaCl induced damage to shoot-tips and leaf edges, whereas 150 mM NaCl stimulated necroses on the whole leaf and on part of the stem (Table 1). Similarly, in addition to Chl degradation, salt-induced necroses on leaf and shoot tissues were observed in grape explants (Sivritepe and Eriş 1999).

According to MDA formation in shoots, lipid peroxidation was significantly higher under salt stress than in control plants and the rate of increase was 12, 14 and 120 % in 50, 100 and 150 mM NaCl, respectively (Table 2). However, salt stress caused significant increases in the activities of antioxidative enzymes. The constitutive and induced levels of SOD, POX and CAT

Table 1. The effects of NaCl salinity on the growth, water, proline and relative chlorophyll contents and salt injury index (SI) of Gisela 5 grown *in vitro*. Values in a column followed by the same letter are not significantly different ($P \leq 0.05$) according to LSD test, $n = 3$.

NaCl [mM]	Dry mass [% of control]	Shoot length [% of control]	Water content [%(f.m.)]	Proline content [µmol g^{-1}(f.m.)]	Chl content (SPAD)	SI
0	100.00 a	100.00 a	83.59 a	0.04 b	38.45 a	1.00 c
50	70.74 b	93.99 b	82.49 a	0.04 b	25.89 b	1.72 b
100	54.40 c	87.09 c	82.20 a	0.05 a	24.05 b	2.07 b
150	44.08 d	82.11 d	84.62 a	0.05 a	14.04 c	2.72 a

Table 2. The effects of NaCl salinity on MDA content [μmol g^{-1}(f.m.)] and activities of SOD, APOX, POX, CAT and GR [U mg^{-1}(protein)] of Gisela 5 grown *in vitro*. Values in a column followed by the same letter are not significantly different ($P \leq 0.05$) according to LSD test, $n = 3$.

NaCl [mM]	MDA	APOX	CAT	GR	POX	SOD
0	0.05 c	2.28 d	32.05 d	0.09 c	27.33 d	31.33 d
50	0.06 b	7.11 c	82.31 c	0.16 b	28.30 c	41.27 c
100	0.07 b	8.32 b	179.19 b	0.18 b	48.45 b	93.92 b
150	0.11 a	10.56 a	204.53 a	0.27 a	65.50 a	138.66 a

Table 3. The effects of NaCl salinity on ion concentrations [mg g^{-1}(d.m.)] and balances of Gisela 5 grown *in vitro*. Values in a column followed by the same letter are not significantly different ($P \leq 0.05$) according to LSD test, $n = 3$.

NaCl [mM]	Cl	Na	K	Ca	K/Na	Na/Ca	Mg	Fe	Zn	Cu
0	4.96 a	2.80 c	9.01 a	8.67 a	3.54 a	0.32 c	2.07 a	0.63 a	0.20 b	0.011 a
50	3.14 b	5.90 c	8.80 a	7.73 b	1.49 b	0.79 c	1.53 b	0.62 a	0.28 ab	0.006 a
100	4.56 ab	11.18 b	8.19 ab	7.27 b	0.79 b	1.53 b	1.69 b	0.56 a	0.31 a	0.008 a
150	5.02 a	20.48 a	7.48 b	6.30 c	0.36 b	3.25 a	1.70 b	0.56 a	0.33 a	0.009 a

activities were higher than those of APOX and GR (Table 2). The increased activities of the enzymes could reflect a defence response to the cellular damage provoked by higher NaCl concentrations in the culture media, such as occurs in apple rootstock *in vitro* (Molassiotis *et al.* 2006).

Concerning the toxic elements, the concentration of Cl in shoots was unaffected by NaCl salinity with the exception of 50 mM salt treatment (Table 3). However, Na concentration significantly rose with increasing NaCl concentrations and Na content was almost 10 times higher in 150 mM NaCl in comparison with the control. The data presented here indicate that one of the main effects of salt stress on Gisela 5 could be salt-induced ion toxicity (Table 3). Moreover, the impairment of growth and development was mainly caused by Na because Cl was effectively excluded from the explants. Similar results were noticed previously when peach seedlings were exposed to salinity (Karakas *et al*. 2000).

Increased NaCl salinity caused significant decrease in the K and Ca contents of shoots (Table 3). According to Cakmak (2005), K deficiency at the cellular level might be a contributory factor to salt-induced oxidative stress and related cell damage. As a confirmation, in the present work, we also observed increased MDA production (Table 1) and decreased K concentrations occurring as a result of increased levels of NaCl salinity (Table 3). On the other hand, a higher K:Na ratio is an improvement strategy to minimize growth decreases of plants to salinity (Cakmak 2005). In contrast, salt-induced growth sensitivity of Gisela 5 could be related to the diminished K:Na ratio due to NaCl salinity (Table 3). Ca deficiency can impair the selectivity and the integrity of the cell membrane and permit the passive accumulation of Na in plant tissue (Hu and Schmidhalter 2005). The significant decreases in Ca concentration in explants of Gisela 5 indicates that the tested levels of salinity affected the membrane permeability, which is consistent with the high Na:Ca ratio (Table 3). As NaCl concentration in the culture medium increased, Mg concentration in tissues decreased too (Table 3). Lycoskoufis *et al*. (2005) also found that diminished Mg concentrations in pepper under saline conditions could be a consequence of the decreased chlorophyll concentration, as was observed here in Gisela 5. However, Fe and Cu concentrations of explants were not significantly affected by NaCl levels in the culture media (Table 3). These results are in parallel to those of Sotiropoulos and Dimassi (2004) and Shiyab *et al*. (2003) who worked with kiwifruit and sour orange shoots *in vitro*, respectively. In contrast increasing NaCl concentration in the growth medium raised Zn acquisition significantly (Table 3). Verma and Neue (1984) also showed that Zn uptake was increased in rice by salinity.

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