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BRIEF COMMUNICATION

Response of the cherry rootstock to water stress induced *in vitro*

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Abstract

The *in vitro* response of sweet cherry (*Prunus cerasus* × *P. canescens*) rootstock Gisela 5 to increasing water deficit in the culture medium was studied. Water stress induced by the incorporation of 1, 2 and 4 % polyethylene glycol (PEG-8000) into the Murashige and Skoog medium was applied for 6 weeks. PEG-induced water stress reduced shoot dry mass, length, water content and relative chlorophyll content. Water stress also induced leaf necrosis without causing loss of viability in the explants. The increase in malondialdehyde content indicated oxidative stress. The activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX) and glutathione reductase (GR) were also significantly elevated. The concentrations of K, Ca, Fe and Mn of shoots were decreased.

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

Water deficit is among the most important environmental factors that limit crop productivity. Therefore, the need for water conservation and evaluation of the existing and/or newly developed germplasm of crop plants for their tolerance to drought has become urgent. *In vitro* assays of drought tolerance have been used successfully for many woody plants (Dami and Hughes 1995, Brito *et al*. 2003, Chai *et al*. 2005, Molassiotis *et al*. 2006). Polyethylene glycol (PEG) was reported as suitable osmoticum for imposing water stress *in vitro* by decreasing the water potential of medium without being taken up or being phytotoxic (Dami and Hughes 1995). PEG has been reported to be effective in reducing the *in vitro* growth of grape and date palm (Dami and Hughes 1995, Al-Khayri and Al-Bahrany 2004). In addition to visible symptoms of drought injury (physiological disorders and chlorosis), *in vitro* induced water deficit was also associated with a progressive reduction in water content of explants (Sawwan *et al*. 2000, Al-Khayri and Al-Bahrany 2004, Chai *et al*. 2005). Osmoregulation through the accumulation of proline, and inorganic ions has been proposed as a possible means for overcoming water stress in *in vitro* cultures of woody plants (Brito *et al*. 2003, Al-Khayri and Al-Bahrany 2004). Recently, an increased content of antioxidative enzymes and tolerance to water stress has been reported on the banana and apple rootstock explants (Chai *et al*. 2005, Molassiotis *et al*. 2006). Considering the lack of knowledge on the drought tolerance of Gisela 5 as a newly developed cherry rootstock, we have investigated the effects of PEG-induced water stress on growth, water and chlorophyll contents, proline accumulation, lipid peroxidation, antioxidative enzymes and nutritional status of 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) solid medium supplemented with sucrose (30 g dm^{-3}) and different concentrations of

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; Chl - chlororphyll; GR - glutathione reductase; PEG - polyethylene glycol; POX - peroxidase, SOD - superoxide dismutase; WC - water content.
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PEG-8000 (0, 1, 2 and 4 %). 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, and a 16-h photoperiod with irradiance of 51 μmol $m^{-2}s^{-1}$.

After 6 weeks, the explants 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 to water stress was measured in terms of both dry mass and shoot length. Dry mass of explants was obtained after heating at 70 °C for 24 h. The water content (WC) of explants was calculated from the fresh and dry masses 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 drought injury on a 1 - 4 scale as follows: 1 - no injury; 2 - browning on shoot-tips and leaf edges; 3 - necroses on the whole leaf and/or on part of the stem; 4 - dead. Following this, drought injury index (DI) was calculated according to the following formula: $DI = \sum (n_i \times i)/N$, 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 PEG concentration.

Proline content was determined according to Bates *et al*. (1973). The degree of lipid peroxidation was measured by MDA content according to Madhava and Sresty (2000). With respect to antioxidant enzymes, explants (1 g) were homogenized with a cold mortar and pestle in 3 cm^3 extraction buffer containing 0.05 M Na phosphate buffer (pH 7.8), 1 mM EDTA and 2 % polyvinylpolypyrrolidone (PVPP). The homogenates were centrifuged (13 000 *g* for 40 min at 4 °C) and the supernatant was used for enzyme activity assays. Enzyme activity determinations were conducted using a spectrophotometer (*UV-1600*; *Shimadzu Corporation*, Kyoto, Japan). Superoxide dismutase (SOD) activity was estimated by recording the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm and the quantity of SOD required to produce a 50 % inhibition of NBT reduction was taken as one unit (Beauchamp and Fridovich 1971). Ascorbate peroxidase (APOX) activity was assayed according to Nakano and Asada (1981), by measuring the decrease in absorbance of oxidised ascorbate at 290 nm and one enzyme unit is defined as mmol cm^{-3}(oxidized ascorbate) per min. Peroxidase (POX) activity was analysed by measuring the rate of increase in absorbance of oxidized diaminobenzidine-tetrahydrochloride dihydrate (DAB) at 465 nm and one enzyme unit was defined as μmol cm^{-3}(destroyed H_2O_2) per min, according to Herzog and Fahimi (1973). Catalase (CAT) activity was assayed by measuring the decrease in absorbance of H_2O_2 at 240 nm and μmol (H_2O_2 destroyed) per min was defined as one unit CAT (Bergmeyer 1970). Glutathione reductase (GR) activity was determined by the rate of decrease in the absorbance of oxidized glutathione (GSSG) at 340 nm and one enzyme unit was defined as mmol cm^{-3}(oxidized GSSG) per min (Foyer and Halliwell 1976). All enzyme activities were expressed in terms of specific enzyme activity and protein content was determined according to Bradford (1976), using bovine serum albumin as a standard.

To determine the mineral composition, previously dried and ground material dry ashed at 530 °C for 6 h. Ion extraction was achieved in 65 % HNO_3. Cu, Zn, Mn, Fe, Mg, Na, Ca and K were analyzed by atomic absorption spectrometry (*Unicam, Model 929 AA*, Cambridge, UK). Phosphorus was determined colorimetrically by the ammonium phosphovanadomolybdate method.

The experiments were set up in a completely randomized design and repeated three times. 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 on the data, and significant differences among treatment means calculated by LSD test at $P < 0.05$.

PEG-induced water stress negatively affected dry mass and especially shoot length (Table 1). The significant decrease in the growth parameters was observed at 2 and 4 % PEG. Moreover, WC of the explants progressively decreased (Table 1). The decrease in WC limited water availability for cell expansion (Katerji *et al*. 1997). Thus, the reduced growth rate of explants could be related to decrease in WC, as has already been shown in apple rootstock MM 106 explants (Molassiotis *et al*. 2006). Osmoregulation through the accumulation of cellular solutes, such as proline, has been proposed as a possible means for overcoming water stress

Table 1. The effects of PEG treatments on the growth, water, proline and relative chlorophyll contents and drought injury 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, $n = 3$.

PEG [%]	Dry mass [mg]	Shoot length [cm]	Water content [%]	Proline content [μmol g^{-1}(f.m.)]	Chl content [SPAD]	DI
0	53.20 a*	1.92 a	80.71 a	0.04 b	44.53 a	1.00 c
1	51.82 a	1.87 a	79.24 ab	0.04 b	37.11 b	1.91 b
2	49.36 ab	1.58 b	75.30 bc	0.05 a	29.91 c	2.16 a
4	45.91 b	1.53 b	72.63 c	0.05 a	27.69 c	2.34 a

Table 2. The effects of PEG treatments on MDA content [μmol g^{-1}(f.m.)] and SOD, CAT, POX, APX and GR activities [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, $n = 3$.

PEG [%]	MDA	SOD	CAT	POX	APX	GR
0	0.05 c	64.23 d	39.30 d	29.02 d	2.77 c	0.10 c
1	0.07 b	136.72 c	90.62 c	44.42 c	5.88 b	0.15 b
2	0.07 b	140.31 b	183.10 b	65.11 b	6.34 b	0.16 b
4	0.10 a	173.99 a	360.53 a	78.27 a	8.17 a	0.24 a

Table 3. The effects of PEG treatments on mineral composition [mg g^{-1}(d.m.)] 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, $n = 3$.

PEG [%]	K	P	Ca	Mg	Na	Fe	Zn	Mn	Cu
0	19.53 a	4.47 a	4.64 b	1.25 a	1.19 a	0.60 a	0.16 ab	0.08 a	0.013 a
1	17.24 ab	4.55 a	5.00 a	1.17 a	1.10 a	0.57 a	0.18 a	0.07 a	0.010 a
2	16.75 ab	4.52 a	3.99 c	1.22 a	1.16 a	0.46 b	0.13 b	0.07 a	0.010 a
4	15.20 b	4.00 a	3.79 d	1.12 a	1.14 a	0.45 b	0.13 b	0.04 b	0.009 a

conditions (Jain *et al*. 2006, Sotiropoulos 2007). In the current study, the content of proline increased in response to 2 and 4 % PEG (Table 1). However, these contents of proline did not improve the WC or dry mass in the explants as previously observed in salt stressed Gisela 5 (Ertürk *et al*. 2007). On the other hand, under drought conditions, K, Na and Cl are also involved in the osmotic adjustment of leaf tissue (Patakas *et al*. 2002, Brito *et al*. 2003). However, we observed that water stress did not increase Na and K contents of this genotype (Table 3).

The relative chlorophyll content in leaf tissue of explants was significantly decreased due to increased PEG concentration (Table 1). This effect of water stress on chlorophyll content was also reported *in vitro* cultures of other plants (Hernandez-Sebastia *et al*. 2000, Brito *et al*. 2003, Molassiotis *et al*. 2006). In addition, water stress caused physiological disorders in explants and the severity of this injury increased with the elevated concentration of PEG (Table 1). However, no viability loss was observed in explants due to water deficit. Similarly, water stress induced leaf necrosis in olives without causing loss of viability in the explants (Brito *et al*. 2003).

The results regarding MDA content of the shoots indicated that water stress induced oxidative stress in PEG treated Gisela 5 (Table 2). MDA content of explants stressed with 4 % PEG increased 2-fold compared with that of the non-stressed ones. Moreover, PEG induced oxidative injury was accompanied morphologically with the visible symptoms of drought injury in the explants (Table 1) such as occurs in drought stressed *Centaurea ragusina* L. explants (Radic *et al*. 2006). According to Zlatev *et al*. (2006) the relative tolerance to drought induced oxidative stress is related with the antioxidant enzymatic activities. In the current study, water deficit caused significant increases in the activities of antioxidant enzymes (Table 2). In addition to higher basal levels of CAT, SOD and POX, their activities in explants stressed with 4 % PEG were 9, 2.7 and 2.7-fold higher, respectively, compared with the non-stressed ones. APX and GR activities also increased 3- and 2.4-fold, respectively, in water stressed (4 % PEG) explants. Recently, increased activities of antioxidant enzymes and tolerance to water stress have been reported for banana and apple rootstock MM 106 explants (Chai *et al*. 2005, Molassiotis *et al*. 2006).

At the nutrients level, the inclusion of PEG in the MS medium caused significant decreases in K, Ca, Fe and Mn contents of the explants (Table 3). However, the increase in PEG levels from 0 to 4 % did not significantly influence P, Mg, Na, Zn and Cu contents (Table 3). Brito *et al*. (2003) and Molassiotis *et al*. (2006) have also reported K, Mn and Fe decrease in response to water stress. According to Cakmak (2005), K deficiency due to water stress might be a contributory factor to drought-induced oxidative stress and enhanced activity of anti-oxidant enzymes (Cakmak 2005). As a confirmation, in the present work, we observed increased MDA production associated with elevated activities of anti-oxidant enzymes (Table 2) and decreased K content at increased PEG concentrations (Table 3). Sotiropoulos *et al*. (2006) also found that Fe deficiency in apple rootstock MM 111 under adverse environmental conditions could be a consequence of the decreased chlorophyll concentration, as was observed here in Gisela 5 (Table 1).

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