Antioxidant Responses of Strawberry Plants under Stress Conditions

D. Neocleous
Agricultural Research Institute
Ministry of Agriculture, Natural Resources and Environment
Nicosia
Cyprus

V. Ziogas and M. Vasilakakis
Department of Horticulture and Viticulture
Aristotle University of Thessaloniki
Thessaloniki
Greece

Keywords: *Fragaria* × *ananassa*, salinity, drought, antioxidants, hydrogen peroxide, proline

Abstract

The purpose of this study was to examine antioxidant responses of strawberry under salinity or drought conditions. Strawberry plants (*Fragaria* × *ananassa* 'Camarosa') were supplied with a solution containing 0, 10, 20, or 40 mM NaCl. Changes in ascorbic acid, total phenolics, carotenoids, antioxidant capacity, phenyl-ammonialyase (PAL) and peroxidase (POD) activities were determined. Salinity enhanced the secondary metabolism, enabling roots and leaves to respond to salinity for defense requirements (an increase in antioxidant capacity) but not accordingly to the salinity level. To examine the effect of drought, plants were cultivated in a solution supplemented with 0 or 150 g L^{-1} polyethylene glycol (PEG 4000) for 24 h. Results suggested that strawberry plants possess specific mechanisms to detoxify reactive oxygen species which include activation and cooperation of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD). Furthermore, proline may be an indicator of drought-stress or stress-tolerance and hydrogen peroxide (H_2O_2) may be correlated with the stress response.

INTRODUCTION

Strawberries are very popular fruits rich in antioxidants with redox properties (Wang and Lin, 2000; Pirker et al., 2002). Strawberry cultivation is becoming an important greenhouse crop in the Mediterranean basin, where drought and salinity are often major limiting factors in agricultural production (Neocleous and Vasilakakis, 2007; Keutgen and Pawelzik, 2008). Yet, soil and water salinity can induce the antioxidative mechanism of the plant as a protection towards oxidative stress (De Pascale et al., 2001; Vinocur and Altman, 2005; Neocleous and Vasilakakis, 2008).

Under the impact of climate change, improving the knowledge of an altered strawberry stress response might lead to redefining agronomic practices which enhance yield and nutritional value, without heavily affecting plant fitness. However, the precise antioxidant response of strawberry is not well known (Keutgen and Pawelzik, 2007; Turhan et al., 2008) and there is little information about antioxidative responses of roots exposed to stress conditions (Blokhina et al., 2003).

Therefore, this work was focused on the effects of salinity (sodium chloride (NaCl)) (experiment I) on changes in: ascorbic acid, phenolics, carotenoids, antioxidant capacity, PAL and POD activity and on the effects of drought (PEG 4000) (experiment II) on changes in: the activities of SOD, CAT, POD, proline content and hydrogen peroxide (H_2O_2) concentration in strawberry plants.

MATERIALS AND METHODS

Treatments, Plant Material and Growth Conditions

1. Experiment I. The experiment was carried out at the experimental farm of Aristotle University of Thessaloniki, from March to June 2008. Commercial frigo-strawberry plants (*Fragaria* × *ananassa* 'Camarosa'), were grown in 3-L plastic pots filled with peat

and perlite (1:1 v:v) in a non-heated greenhouse. Plants were irrigated with water and 1/3 Hoagland solution (Hoagland and Arnon, 1950) on alternate days. Salinization treatments were begun in late May and the plants were supplied for 30 days with 1/3 Hoagland nutrient solution (pH ~6.0) containing 0, 10, 20, or 40 mM NaCl equivalent to Electrical Conductivity (EC) of 1.2, 2.6, 3.9 and 6.6 mS cm^{-1} respectively. Experiment arranged in a randomized complete block design with three replications. Each replication consisted of 10 plants. Mean temperature and relative humidity were 22°C and 58% respectively. At the end of the salt treatment period (30^{th} day), the youngest fully expanded leaves were harvested and roots were removed, carefully washed, frozen and stored at -30°C until analysis.

2. Experiment II. The experiment was carried out at the School of Agriculture of Aristotle University of Thessaloniki, from January to February 2009. Freshly dug strawberry plants (*Fragaria* × *ananassa* 'Camarosa') with one well-developed crown 8-10 mm in diameter were selected for hydroponic culture in a culture room at 22±2°C temperature with 16 h photoperiod under a light density of 150 μmole m^{-2} s^{-1}. Initially, plants were cultivated in aerated distilled water and then in aerated Hoagland's nutrient solution for 15 d. After this period, plants were supplemented with 0 or 150 g L^{-1} PEG 4000 for 24 h and harvested for analysis. Experiment arranged in a randomized complete block design with three replications. Each replication consisted of 10 plants. Leaves and roots were carefully washed with distilled water, frozen and stored at -30°C until analysis.

Analyses

Ascorbic acid content was determined with the Reflequant system (Merck) in combination with a standard solution (Ascorbic Acid in Plant Material, Merck). For determining total phenolics and antioxidant capacity, samples were extracted with acetone, water and acetic acid (70:29.5:0.5 by volume) (Kähkönen et al., 2001). Total phenolic content was determined with the Folin-Ciocalteu phenol reagent according to Scalbert et al. (1989) and expressed from a standard curve as mg gallic acid per g fresh weight (FW). The antioxidant capacity was measured using ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996) and results were expressed in μmol ascorbic acid per g FW. Total carotenoids in root samples were extracted with hexane/acetone/ethanol (2:1:1 by volume), and the total carotenoids concentration in hexane layer was determined according to the method described by Kuti (2004), using the molar extinction coefficient of β carotene $E^{1}\%=2592$ (Rodriguez-Amaya, 1999) and expressed as μg carotenoids per g FW.

For determination of PAL (phenylalanine ammonialyase) activity the method described by Jouili and El Ferjani (2003) with minor modifications was followed and results expressed as change in OD290 nm h^{-1} per g FW. One Unit of activity is defined as an increase in absorbance at 290 nm of 0.01.

For POD and CAT assays, enzymes were extracted from 300 mg of tissue by grinding them in a cold mortar and pestle with 1 ml 50 mM K-phosphate buffer (pH 6.5) containing 1 mM EDTA, 2.0% (w/v) PVPP, 1 M NaCl, 0.05% Triton X-100 and 1 mM PMSF. For SOD extraction a mixture of 50 mM K-phosphate buffer (pH 7.6) containing 0.1 mM EDTA, 2% (w/v) PVPP, 0.05% Triton X-100 and 1 mM PMSF, was used. The homogenate was centrifuged (15,000 × 20 min at 4°C) and the supernatant was used for the antioxidant enzymes assays. Peroxidase activity (U) (soluble and ionically bound to cell wall) was determined according to Ngo and Lenhoff (1980); CAT activity (U) was determined by the decomposition of H_2O_2 at 240 nm, pH 7.0 and temperature of 25°C according to Cakmak et al. (1993); and SOD activity was assayed photochemically based on the photoreduction of nitro blue tetrazolium (NBT) by light in the presence of riboflavine and methionine (Beauchamp and Fridovich, 1971). Activity (U) was defined by the range of change in absorbance at 590 nm (POD) and 240 nm (CAT), and 50% inhibition of the reduction of NBT (SOD).

Hydrogen peroxide was determined using the DMAB-MBTH system according to Ngo and Lenhoff (1980) and proline was measured as described by Bates et al. (1973).

The protein content was estimated by the method of Bradford (1976), using BSA as a standard.

Statistical Analysis

The statistical analysis included analysis of variance (ANOVA) and means separation with the help of the SAS/ASSIST 9.1 software package (SAS Institute, Cary, NC, USA).

RESULTS

Experiment I

Both antioxidant capacity and total phenolics in strawberry leaves were significantly higher than the control for treatments where salinity was imposed (Table 1). The rate of increase of either factor was not proportional to the salinity level (Table 1). However, the correlation (Pearson) between antioxidant capacity and total phenolics was high (r=0.94***). Salinity did not affect ascorbic acid content in leaves (Table 1). This was not the case in strawberry roots, where both ascorbic acid and phenolics significantly contributed to the increase in antioxidant capacity from higher NaCl treatments (Table 2). As in the leaves, the rate of increase of antioxidant capacity was not proportional to the salinity level applied (Table 2). Treatments with moderate NaCl concentrations (20 and 40 mM NaCl) increased PAL activity in roots (Table 2). However, total carotenoids content and peroxidase (POD) activity were negatively affected by salinity and lower values were obtained at higher salinity concentrations (Tables 1 and 2).

Experiment II

Application of PEG 4000 increased the formation of hydrogen peroxide (H_2O_2) both in leaves and roots (Fig. 1A). The increase of H_2O_2 was accompanied by corresponding increases in the key-antioxidant enzymes peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD), both in leaves and roots as shown in Figure 1B, C and D. Furthermore, PEG application increased the total protein content and the accumulation of proline (Fig. 1E and F). Leaves had higher values for the measured parameters compared with roots and generally the proportion of increase from PEG application was greater (Fig. 1).

DISCUSSION

Experiment I

The results indicated that salinity stimulated the non enzymatic antioxidant mechanism in strawberry plants. However, the increase was not proportionate to the salt concentration in the solution. Leaves were found to have higher FRAP values than roots, suggesting a higher antioxidant activity. Antioxidant biosynthesis exists mainly in leaves and chloroplasts where more precusors are available. It is not clear whether biosynthesis occurs in root mitochondria to the same extent as it does in green tissue (Blokhima et al., 2003).

A considerable body of data suggests that a higher content of total phenolics and vitamin C in strawberry enhances antioxidant activity (Whang and Zheng, 2001; Stewart et al., 2001). A relationship between salt stress and antioxidant levels in strawberry has been established (Keutgen and Pawelzik, 2007). In the current experiment, the increase of antioxidant capacity imposed by salinity was presumably the result of raised: a) total phenolics in leaves, and b) total phenolics and vitamin C in roots. Alteration of non enzymatic antioxidants in leaves and roots suggest that in different strawberry tissues different mechanisms may be involved in the protection against oxidative stress.

Interactions between ascorbic acid and phenolic compounds are well known. Phenolic compounds can be involved in the hydrogen peroxide scavenging cascade in plant cells and can improve tolerance under oxidative stress (Blokhina et al., 2003).

The negative effect of salinity on carotenoids content and POD activity may indicate a direct effect of other antioxidant mechanisms. The combined action of SOD and CAT led to efficient removal of ROS (while POD did not) in zucchini squash tissue affected by salinity (Wang, 1995).

Salinity enhanced PAL activity in roots and probably the flow of carbon atoms from proteins into phenolic compounds. PAL, a key intermediate in the phenylpropanoid pathway, is generally stimulated in plant tissues exposed to environmental stresses (Jouili and El Ferjani, 2003; Jiang and Joyce, 2003). It is suggested that in this case, strawberry roots increased PAL activity and defence processes in order to mitigate salinity damage.

In view of the above, salt stress appeared to activate the key steps of secondary metabolism and biosynthesis of antioxidant compounds and we may expect improvement in the performance of mild salt-treated strawberry plants. This is maybe of practical relevance for strawberry cultivation in hydroponics or in soils with slight salinization.

Experiment II

The increased hydrogen peroxide (H_2O_2) in the strawberry plant leaves and roots following drought stress, matches other results which showed that under normal growth conditions, the production of ROS is low and under stressful environments it is considerably higher (Mittler, 2002; Ashraf, 2009). Reactive oxygen species (ROS), like H_2O_2, are considered as cellular indicators of stresses as well as secondary messengers actively involved in the stress response signalling pathway (Jouili and El Ferjani, 2003; Ashraf, 2009). The increase in H_2O_2 was accompanied by an increase in the key-antioxidant enzymes catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD). Thus it appeared that, in strawberry leaves and roots, SOD, CAT and POD were part of a defence mechanism acting against oxidative stress to detoxify ROS. Antioxidants enzymes such as SOD, CAT and POD are known to substantially reduce the levels of superoxide and hydrogen peroxide in plants and play a vital role in plant defence against oxidative stress (Ashraf, 2009).

Drought stress was also accompanied by increasing protein content which may be explained by the production of stress related proteins which include the antioxidative enzymes. Furthermore, drought induced changes in the accumulation of proline, which is often used as an indicator of stress tolerance, osmoregulation and protection (Molassiotis et al., 2006). Nevertheless, more extensive measurements are needed to assess long-term drought effects.

CONCLUSION

The results obtained revealed that:

- Salt stress appeared to activate the key steps of secondary metabolism and the biosynthesis of antioxidant compounds.
- Under drought conditions antioxidant enzymes SOD, CAT and POD were part of a defence mechanism acting against oxidative stress to detoxify ROS.
- Proline may be an indicator of drought-stress and correlated with osmotic regulation and protection.
- Hydrogen peroxide appeared to be involved in the drought-stress response.

Alleviation of oxidative damage by the use of different antioxidants and ROS scavengers can enhance plant resistance to salt and drought (Vinocur and Altman, 2005). In our case a mild-salinity treatment or a short-term water shortage may become an important tool in strawberry stress tolerance, without heavily affecting fitness.

ACKNOWLEDGEMENTS

This work was supported by the STSM-863 program of the EU-COST office. Also, we thank the Agricultural chemistry lab of the Aristotle University of Thessaloniki.

Literature Cited

Ashraf, M. 2009. Biotechnological approach of improving plant salt tolerance using antioxidants as markers. Biotechnol. Adv. 27:84-93.

Bates, L.S., Woldren, R.P. and Teare, I.K. 1973. Rapid determination of free proline for water stress studies. Plant and Soil 39:205-208.

Beauchamp, C. and Fridovich, I. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44(1):276-287.

Benzie, F.F.I. and Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power". The FRAP assay. Anal. Biochem. 239:70-76.

Blokhina, O., Virolainen, E. and Fagerstedt, K. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann. Bot. 91:179-194.

Bradford, M.-M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72(1-2):248-254.

Cakmak, I., Strbac, D. and Marschner, H. 1993. Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. J. Exp. Botany 44(1):127-132.

De Pascale, S., Maggio, A., Fogliano, V., Ambrosino, P. and Ritieni, A. 2001. Irrigation with saline water improves carotenoids content and antioxidant activity of tomato. J. Hort. Sci. Biotechn. 76:447-453.

Hoagland, D.R. and Arnon, D.I. 1950. The water culture method for growing plants without soil. Circ. Calif. Agric. Exp. Stat. 347:1-32.

Jiang, Y. and Joyce, D.C. 2003. ABA effects on ethylene production, PAL activity, anthocyanin and phenolic contents of strawberry fruit. Plant Growth Regul. 39:171-174.

Jouili, H. and El Ferjani, E. 2003. Changes in antioxidant and lignifying enzyme activities in sunflower roots (*Helianthus annuus* L.) stressed with copper excess. C.R. Biologies 326:639-644.

Kähkönen, M.P., Hopia, A.I. and Heinonen, M. 2001. Berry phenolics and their antioxidant activity. J. Agric. Food Chem. 49:4076-4082.

Keutgen, A. and Pawelzik, E. 2007. Modifications of taste-relevant compounds in strawberry fruit under NaCl salinity. Food Chem. 105:1487-1494.

Keutgen, A. and Pawelzik, E. 2008. Quality and nutritional value of strawberry fruit under long term salt stress. Food Chem. 107:1413-1420.

Kuti, J.O. 2004. Antioxidants compounds from four *Opuntia* cactus pear fruit varieties. Food Chem. 85:527-533.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7:405-410.

Molassiotis, A., Sotiropoulos, T., Tanou, G., Diamantidis, G. and Therios, I. 2006. Boron-induced oxidative damage and antioxidant and nucleolytic responses in shoot tips culture of the apple rootstock EM 9 (*Malus domestica* Borkh). Environ. Exp. Bot. 56:54-62.

Neocleous, D. and Vasilakakis, M. 2007. Effects of NaCl stress on red raspberry (*Rubus idaeus* L. 'Autumn Bliss'). Sci. Hort. 112:282-289.

Neocleous, D. and Vasilakakis, M. 2008. Fruit quality of red raspberry as affected by salinity. Europ. J. Hort. Sci. 73(3):131-137.

Ngo, T.T. and Lenhoff, H.M. 1980. A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. Anal. Biochem. 105:389-397.

Pirker, K.F., Goodman, B.A., Pascual, E.C., Kiefer, S., Soja, G. and Reichenauer, T.G. 2002. Free radicals in the fruit of three strawberry cultivars exposed to drought stress in the field. Plant Physiol. Biochem. 40:709-717.

Rodriquez-Amaya, D.B. 1999. A guide to carotenoid analysis in foods. ILSI Press: Washington DC.

Scalbert, A., Monties, B. and Janin, G. 1989. Tannins in wood: comparison of different estimation methods. J. Agric Food Chem. 37:1324-1329.

Stewart, D., Deighton, N. and Davies, H.V. 2001. Antioxidants in soft fruit. SCRI Ann. Rep. p.94-98.

Turhan, E., Gulen, H. and Eris, A. 2008. The activity of antioxidative enzymes in three strawberry cultivars related to salt stress tolerance. Acta Physiol. Plant. 30:201-208.
Vinocur, B. and Altman, A. 2005. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr. Opin. Biotechnol. 16:123-132
Wang, C.Y. 1995. Effect of temperature preconditioning on catalase, peroxidise, and superoxide dismutase in chilled zucchini squash. Postharv.. Biol. Technol. 5:67-76.
Wang, S.Y. and Lin, H.S. 2000. Antioxidant activity in fruits and leaves of blackberry, raspberry and strawberry varies with cultivar and developmental stage. J. Agric. Food Chem. 48:140-146.

Tables

Table 1. The effect of salinity (NaCl concentration) on ascorbic acid, total phenolics, ferric reducing antioxidant power (FRAP values) and peroxidase (POD) activity in strawberry leaves.

Treatment (mM NaCl)	Ascorbic acid (mg/g FW)	Total phenolics (mg GA/g FW)	FRAP values (µmol/g FW)	POD (U/min/g FW)
0	1.42 a	32.4 a	337 c	8.13 a
10	1.45 a	38.9 b	400 b	7.32 ab
20	1.51 a	42.1 b	424 b	7.27 ab
40	1.54 a	44.2 b	440 b	6.28 b

Means within a column for each NaCl level followed by different letters are significantly different according to Duncan's multiple range test at $P<0.05$ level.

Table 2. The effect of salinity (NaCl concentration) on carotenoids, ascorbic acid, total phenolics, ferric reducing antioxidant power (FRAP values), phenyl-ammonialyase (PAL) and peroxidase (POD) activity in strawberry roots.

Treatment (mM NaCl)	Carotenoids (µg/g FW)	Ascorbic acid (mg/g FW)	Total phenolics (mg GA/ g FW)	FRAP values (µmol/ g FW)	PAL activity (Unit/ g FW)	POD (U/min/ g FW)
0	20.6 a	0.34 b	7.54 c	51.6 b	3.59 b	5.29 a
10	17.2 b	0.39 a	9.43 ab	66.5 a	4.08 b	4.28 b
20	12.4 c	0.38 a	9.20 ab	63.2 a	8.12 a	4.11 bc
40	13.7 c	0.40 a	9.80 a	66.5 a	7.92 a	3.32 c

Means within a column for each NaCl level followed by different letters are significantly different according to Duncan's multiple range test at $P<0.05$ level.

Figures

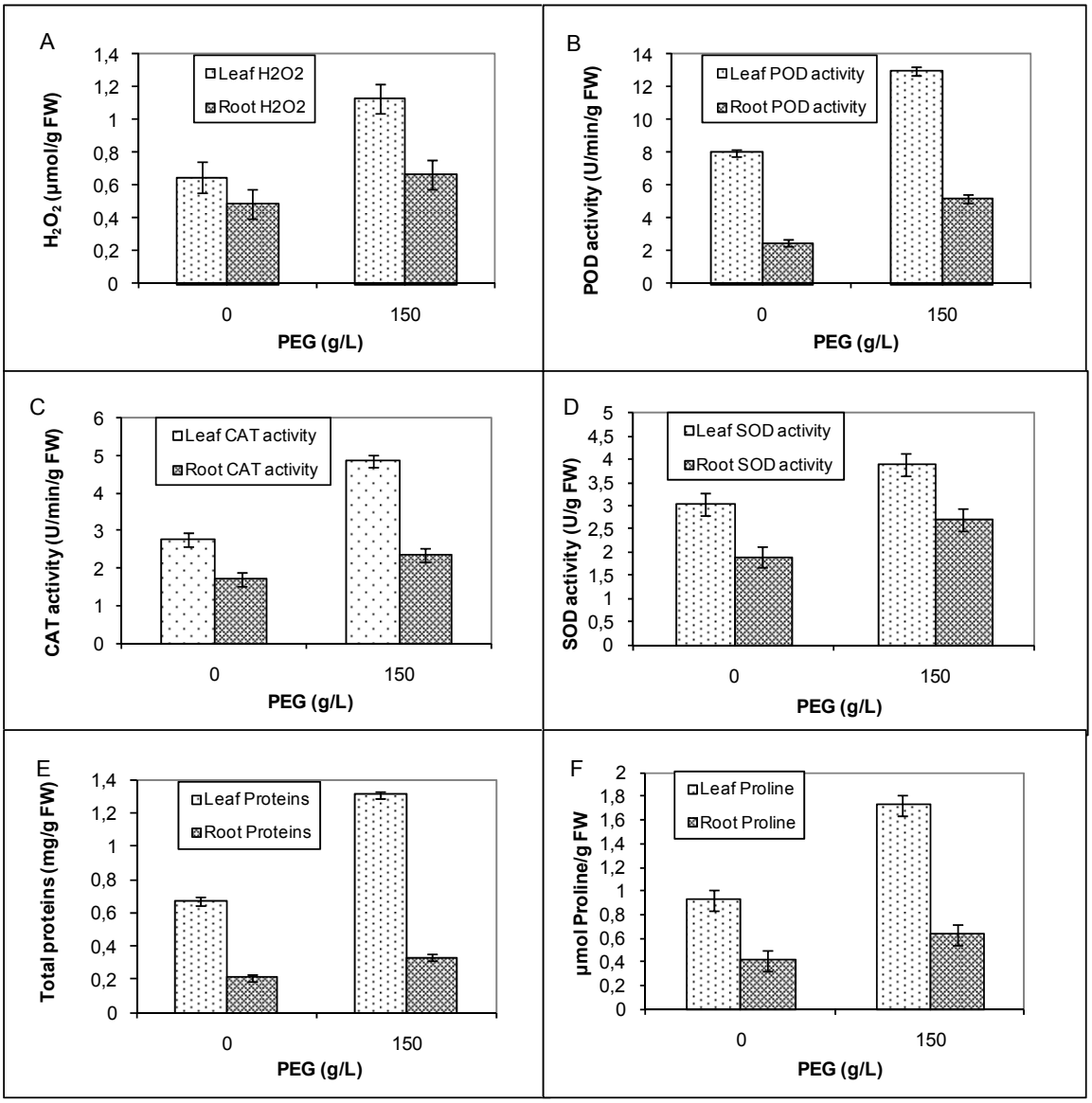

Fig. 1. The effect of drought induced by PEG on hydrogen peroxide (H_2O_2), enzymatic activity of peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), protein and proline content in strawberry leaves and roots. Bars represent ± SE values (n=3).