BIOLOGIA PLANTARUM 49 (4): 609-612, 2005

BRIEF COMMUNICATION

Effects of salicylic acid on ethylene induction and antioxidant activity in peach rootstock regenerants

A. MOLASSIOTIS*, G. DIAMANTIDIS**, I. THERIOS*,[1] and K. DIMASSI*

Laboratory of Pomology, Laboratory of Agricultural Chemistry**, Department of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece*

Abstract

Ethylene concentration in the culture tubes of peach rootstock regenerants of three genotypes (Cadaman, GF-677, Myrobalan 29C) was increased by the inclusion of 20 μM salicylic acid (SA), methionine (METH) and ethephon (ETH) in the MS medium whereas it was decreased in regenerants exposed up to 20 μM $AgNO_3$. In leaves of the regenerants the increase of ethylene concentration was accompanied with an increase of non-enzymatic antioxidant activity while remarkable genotype-depended changes in the activities of catalase, peroxidase and their isoenzymes were recorded suggesting that ethylene accumulation imposes oxidative stress responses. However, the results showed that some differences could be observed in the activity of isoenzymes in regenerants exposed to SA in respect to METH and ETH-treated ones.

Additional key words: Cadaman (*Prunus persica* × *P. davidiana*), catalase, ethephon, FRAP values, GF-677 (*Prunus amygdalus* × *P. persica*), isoenzymes, methionine, Myrobolan 29C (*Prunus cerasifera*), peroxidase.

Reactive oxygen species (ROS) are produced primarily as a consequence of aerobic respiration in all living organisms. Being toxic for the cells, ROS are efficiently eliminated by non-enzymatic (α-tocopherol, β-carotene, phenolic compounds, ascorbate, glutathione) and enzymatic antioxidants including superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (GR; EC 1.11.1.7) (Mittler 2002).

Besides ROS, the gaseous plant hormone ethylene (C_2H_4) has been reported to affect several plant growth processes as well as their the responses to various stresses (Sairam *et al*. 1998, Ievinsh *et al*. 2000, Saxena *et al*. 2000, Scebba *et al*. 2001, Khan 2004). Recently, Wang *et al.* (2002) reported a relationship between the multipurpose signalling molecule of ethylene and the generation of ROS but the interaction mechanism remains unclear. In this respect, we investigated the influence of two ethylene inhibitors, $AgNO_3$ and salicylic acid (SA), and two ethylene precursors, methionine (METH) and ethephon (ETH), on the antioxidant mechanism of three commercial peach rootstock regenerants.

Shoot tips from three genotypes used as peach rootstocks, namely, GF-677 (*Prunus amygdalus* of peach rootstocks regenerants × *P. persica***)**, Cadaman (*Prunus persica* × *P. davidiana*) and Myrobolan 29C (*Prunus cerasifera*) from previous *in vitro* cultures were used as regenerants. Each explant was cultured in parafilm-closed glass test tubes (25 × 100 mm) of 10 cm^3 MS medium (Murashige and Skoog 1962) containing 30 g dm^{-3} sucrose, 7 g dm^{-3} agar, 100 mg dm^{-3}myo-inositol, 1 mg dm^{-3} thiamine-HCl, 1 mg dm^{-3} nicotinic acid, 1 mg dm^{-3} pyridoxine-HCl, 0.8 mg dm^{-3} BA and 0.1 mg dm^{-3} IBA. The pH of all media was adjusted to 5.2 and autoclaved at 121 °C for 15 min. The ethylene

Received 30 September 2003, *accepted* 3 November 2004.
Abbreviations: CAT - catalase; DMAB - 3-dimethylaminobenzoic acid; ETH - ethephon; FRAP - ferric reducing antioxidant power; MBTH - 3 methyl-2-benzothiazolinonhydrazon hydrochloride hydrate; METH - methionine; MS medium - Murashige and Skoog medium; PAGE - polyacrylamide gel electrophoresis; POD - peroxidase; ROS - reactive oxygen species; SA - salicylic acid.
Acknowledgements: A. Molassiotis is grateful to the State Scholarship's Foundation of Greece for a fellowship during this work.
[1] Corresponding author; fax: (+32) 310 402705; e-mail: amolasio@agro.auth.gr

inhibitors $AgNO_3$ (1, 10 and 20 μM) and SA (1, 10 and 20 μM), and precursors METH (1, 10 and 100 μM) and ETH (1, 10 and 100 μM) were filter-sterilised and added to the culture medium, after autoclaving. In each treatment, 15 replicates (tubes) were included, arranged randomly in the growth chamber, and maintained at 22 ± 1 °C and 16 h irradiation, supplied by *Philips TLD 54/36W* fluorescence tubes (45 μmol m^{-2} s^{-1}), for a total period of 25 d. The ethylene concentration in the culture tubes atmosphere was analysed by withdrawing a gas sample (1 cm^3) with a hypodermic needle and injecting it into a *Varian 3300* gas chromatograph (*Varian*, Palo Alto, USA) equipped with an activated-alumina column and a flame-ionization detector. Ethylene identity was based on a retention time compared to a standard, while N_2 was used as a carrier gas. For enzymes assays leaves of regenerants were ground in 10 mM Na-phosphate buffer (pH 6.5) containing 2.5 % (m/v) insoluble poly-vinylpolypyrrolidone (PVPP) and 1 M NaCl. The suspension was centrifuged (15 000 *g*, 30 min, 4 °C) and the supernatant was used for determination of protein content, POD and CAT activities according to Bradford (1976), Ngo and Lenhoff (1980) and Wang (1995) respectively. Extracts of leaves were subjected to native polyacrylamide gel electrophoresis (PAGE) with a *Mini Protean II cell* (*Bio-Rad*, Hercules, USA) according to Laemmli (1970). Staining for POD isoenzymes was conducted according to Shimoni (1994). The non-enzymatic antioxidants of the leaves were extracted according to Kuo *et al.* (1999) and then followed FRAP assay as described by Benzie and Strain (1996). For the statistical analysis the *SPSS.11* (*SPSS*, Chicago, USA) was used.

In respect to control, ethylene concentration significantly increased in METH and ETH-treated regenerants, while it was decreased in regenerants

Table 1. Effect of different culture media on ethylene concentration in the culture tubes atmosphere, activities of non-enzymatic antioxidant (FRAP values), POD, and CAT in leaves of rootstock regenerants of different peach genotypes. Means ± SE, n = 3. Asterisks denote significant differences from control at P = 5 %.

Medium additives	Concentration [μM]	GF-677 Ethylene [μg g^{-1}]	FRAP values [μM]	Cadaman Ethylene [μg g^{-1}]	FRAP values [μM]	Myrobalan 29C Ethylene [μg g^{-1}]	FRAP values [μM]
Control		0.91 ± 0.19	1742 ± 151	0.65 ± 0.15	1955 ± 165	1.14 ± 0.15	1302 ± 155
$AgNO_3$	1	0.85 ± 0.17	1708 ± 158	0.54 ± 0.16	2017 ± 146	0.98 ± 0.20	1314 ± 175
	10	0.56 ± 0.12	1642 ± 145	0.48 ± 0.14	2103 ± 170	0.91 ± 0.16	1294 ± 169
	20	0.53 ± 0.14	1609 ± 189	0.28 ± 0.11	2095 ± 165	0.69 ± 0.17	1228 ± 165
SA	1	1.06 ± 0.17	1797 ± 175	0.69 ± 0.17	2190 ± 177	1.34 ± 0.22	1421 ± 153
	10	1.40 ± 0.15*	2055 ± 165	0.61 ± 0.17	2295 ± 167	1.29 ± 0.21	1405 ± 172
	20	1.67 ± 0.14*	2357 ± 188*	1.29 ± 0.19*	2584 ± 175*	1.95 ± 0.24*	1795 ± 160*
METH	1	1.63 ± 0.19*	2689 ± 195*	1.19 ± 0.18*	2522 ± 166*	1.89 ± 0.25*	1790 ± 185*
	10	2.08 ± 0.21*	2791 ± 185*	1.56 ± 0.24*	2685 ± 196*	2.55 ± 0.29*	1851 ± 174*
	100	2.55 ± 0.34*	2805 ± 175*	2.05 ± 0.26*	2581 ± 210*	2.98 ± 0.27*	2132 ± 184*
ETH	1	1.84 ± 0.24*	2644 ± 187*	1.14 ± 0.21*	2489 ± 191*	2.38 ± 0.25*	2005 ± 190*
	10	2.31 ± 0.28*	2515 ± 186*	1.74 ± 0.20*	2688 ± 215*	2.98 ± 0.30*	1786 ± 202*
	100	2.47 ± 0.27*	2405 ± 201*	2.21 ± 0.26*	2743 ± 190*	3.42 ± 0.32*	1898 ± 215*
		POD [U g^{-1} (f.m)]	CAT [U g^{-1} (f.m)]	POD [U g^{-1} (f.m)]	CAT [U g^{-1} (f.m)]	POD [U g^{-1} (f.m)]	CAT [U g^{-1} (f.m)]
Control		4.09 ± 0.35	16.21 ± 1.35	3.28 ± 0.30	14.52 ± 1.02	4.50 ± 0.29	18.88 ± 1.21
$AgNO_3$	1	4.17 ± 0.32	15.81 ± 1.44	3.31 ± 0.29	15.83 ± 1.10	4.41 ± 0.31	18.55 ± 1.45
	10	3.91 ± 0.39	14.93 ± 1.08	3.45 ± 0.31	16.10 ± 1.32	4.66 ± 0.33	17.37 ± 1.22
	20	3.98 ± 0.35	15.07 ± 1.11	3.18 ± 0.35	15.25 ± 1.40	4.38 ± 0.32	18.12 ± 1.12
SA	1	4.29 ± 0.37	14.82 ± 1.03	3.41 ± 0.29	14.16 ± 1.14	4.65 ± 0.34	16.83 ± 1.30
	10	4.34 ± 0.38	13.90 ± 1.12	3.32 ± 0.30	12.21 ± 1.21	5.19 ± 0.37	16.21 ± 1.42
	20	5.39 ± 0.40*	10.80 ± 1.59*	4.42 ± 0.36*	11.52 ± 0.95*	5.95 ±0.34*	15.94 ± 1.38*
METH	1	3.95 ± 0.43	17.15 ± 1.28	3.58 ± 0.29	16.90 ± 1.22*	3.91 ±0.33	18.47 ±1.47
	10	3.52 ± 0.40*	20.88 ± 1.34*	3.85 ± 0.43	17.58 ± 1.42*	3.31 ± 0.41*	17.66 ± 1.55
	100	3.42 ± 0.42*	18.57 ± 1.42*	3.73 ± 0.41	21.27 ± 1.33*	4.02 ± 0.36*	14.33 ± 1.68*
ETH	1	3.46 ± 0.42*	19.84 ± 1.44*	3.65 ± 0.38	18.42 ± 1.41*	3.25 ± 0.34*	15.12 ± 1.70*
	10	3.39 ± 0.40*	22.51 ± 1.40*	3.96 ± 0.41	19.28 ± 1.47*	3.17 ± 0.39*	15.03 ± 1.35*
	100	3.31 ± 0.43*	19.76 ± 1.31*	3.92 ± 0.38	17.86 ± 1.30*	3.09 ± 0.33*	14.22 ±1.28*

exposed up to 20 μM $AgNO_3$ (Table 1). Unexpectedly, ethylene concentration was also increased in all tested regenerants exposed to 20 μM SA as well as in 10 μM SA-treated GF-677 regenerants. POD activity was stimulated in 20 μM SA-treated leaves of regenerants, whereas it was diminished in leaves of GF-677 and Myrobolan 29C exposed to METH (10 and 100 μM) and ETH (Table 1). The culture medium with 20 μM SA caused a significant decrease in CAT activity in leaves of regenerants. Furthermore, CAT activity was increased in leaves of GF-677 and Cadaman by the inclusion of METH (at 10 and 100 μM) and ETH in the MS medium as well as in 1 μM METH-treated Cadaman regenerants. In contrast, a significant decrease in CAT activity was recorded in Myrobolan 29C leaves exposed to ETH and 100 μM METH. Meanwhile, the addition of 20 μM SA, METH and ETH in the MS medium resulted in an increase of the non-enzymatic antioxidant activity (FRAP values) of regenerants. Although the highest ethylene concentrations were recorded in Myrobolan 29C, these regenerants had the lowest FRAP values (Table 1). From analysis of the anionic POD isoenzymes pattern in leaves of Cadaman no differences were found, since two POD isoenzymes were exactly the same in the different culture media used (Fig. 1*B*). However, after the exposure of regenerants to 20 μM SA one more isoenzyme in leaves of GF-677 (Fig. 1*A*, *arrow 1*, *lane 7*) and two in Myrobolan 29C (Fig. 1*C*, *arrows 4* and *5*, *lane 7*) were expressed. In leaves of GF-677 treated with 100 μM METH two isoenzymes at low molecular mass disappeared (Fig. 1*A*, *arrows 2* and *3*, *lane 10*). Furthermore, two fast migrating isoenzymes were not detected in leaves of Myrobolan 29C exposed to 1 and 100 μM ETH (Fig. 1*C*, *arrow 6*, *lanes 11* and *13*), and 20 μM SA, 10 μM METH and ETH (Fig. 1*C*, *arrow 7*, *lanes 7*, *9*, *11*, *12* and *13*) respectively. Although we applied the same amount of POD units in each lane, POD activity in Fig. 1*A*,*C* (*lane 7*) appear to have less intensity, probably due to the effect of SA in the half-life and stability of POD isoenzymes.

SA has been reported to inhibit ethylene production in pear (Leslie and Romani 1986), apple discs and mung bean hypocotyls (Romani *et al*. 1989), and in carrot cell suspension cultures (Roustan *et al*. 1990). However, ethylene concentration was increased in all regenerants exposed to 20 μM SA as well as in 10 μM SA-treated GF-677 ones. More recently, it has been reported that SA stimulates ethylene production in carrot cell suspension cultures (Nissen 1994) and aged potato tuber slices (Liang *et al*. 1997). It can be assumed that the controversial experimental data on the action of SA in the ethylene production might be attributed to the differences in the concentrations of SA used as well as in the plant material analysed.

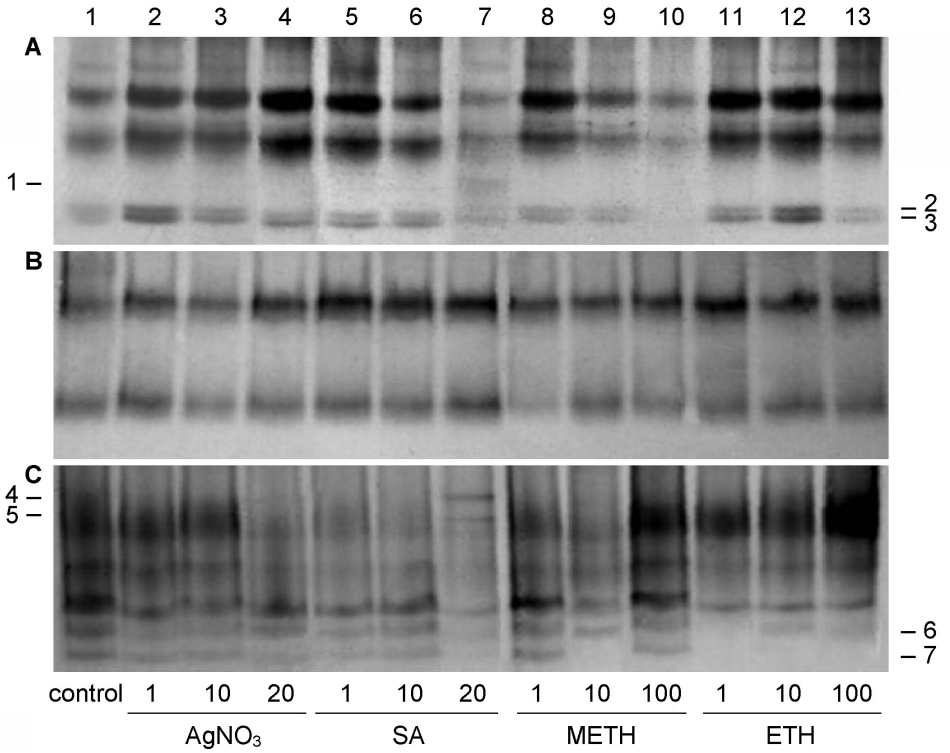

Fig. 1. Native PAGE of leaf extracts from GF-677 (*A*), Cadaman (*B*) and Myrobolan 29C (*C*) after POD activity staining. Equal amounts of protein (80 μg) were loaded onto each lane. *Lanes*: (*1*) control, (*2*) 1 μM $AgNO_3$, (*3*) 10 μM $AgNO_3$, (*4*) 20 μM $AgNO_3$, (*5*) 1 μM SA, (*6*) 10 μM SA, (*7*) 20 μM SA, (*8*) 1 μM METH, (*9*) 10 μM METH, (*10*) 100 μM METH, (*11*) 1 μM ETH, (*12*) 10 μM ETH and (*13*) 100 μM ETH. *Arrows* indicate isoenzymes as described in the text.

The induction of the antioxidant mechanism acts as a damage control system and thus provides protection against oxidative stress (Sairam *et al*. 1998). In the present study, the accumulation of ethylene was accompanied with an increase in the non-enzymatic antioxidant capacity while the regenerants had shown a different behavior in composition of isoenzymes. These results indicate that ethylene accumulation imposes oxidative stress, however, the antioxidant mechanism of regenerants seems to be genotype-depended. The differences in the POD isoenzymes patterns in leaves of regenerants could be responsible for this phenomenon, suggesting that POD isoenzymes differ in their sensitivity to ethylene. Altought all genotypes treated with 20 μM SA had highly incresead POD activity, the contents of anionic POD isoenzymes in leaf extracts of GF-677 and Myrobolan 29C were lowest (Fig. 1, *lane 7*) possibly due to additional anionic POD isoenzymes appeared while the involvement of cationic isoenzymes can not be excluded.

While in all regenerants exposed to 20 μM SA ethylene production was enhanced, the different enzymatic antioxidants revealed different responses in METH and ETH-treated regenerants. In fact, in these regenerants POD activity increased while CAT activity diminished. Durner and Klessig (1996) suggested that the inhibition of CAT activity by SA probably results from peroxidative reactions. During this peroxidation reaction, SA interacts with CAT by donating an electron and converting itself into SA free radical ($SA^{\cdot}$). Furthermore, SA was found to induce ROS, such as intracellular hydrogen peroxide (H_2O_2) production and extra cellular superoxide ($O_2^{\cdot-}$) generation (Chen *et al*. 1993). Therefore, we assumed that SA, in contrast to ETH and METH, was shown to have a direct effect in the antioxidant mechanism by altering various enzymatic pathways.

References

Benzie, I.F., Strain, J.: The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. - Anal. Biochem. **239**: 70-76, 1996.

Bradford, M.M.: A rapid and sensitive method for the quantification of micrograms quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Chen, Z., Silva, H., Klessig, D.F.: Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. - Science **262**: 1883-1886, 1993.

Durner, J., Klessig, D.F.: Salicylic acid is a modulator of tobacco and mammalian catalases. - J. biol. Chem. **272**: 28492-28501, 1996.

Ievinsh, G., Kruzmane, D., Rusite, E., Arente, G., Gertnere, D.: Modulation of *Solanum tuberosum* L. morphogenesis and antioxidative status in a stem explant culture by limitation of gas exhange: putative effects of ethylene. - J. Plant Physiol. **156**: 717-723, 2000.

Khan, N.A.: NaCl-inhibited chlorophyll synthesis and associated changes in ethylene evolution and antioxidant enzyme activities in wheat. - Biol. Plant. **47**: 434-440, 2004.

Kuo, J.M., Yeh, D.B., Pan, B.S.: Rapid photometric assay evaluating antioxidative activity in edible plant material. - J. Agr. Food Chem. **47**: 3206-3209, 1999.

Laemmli, U.K.: Cleavage of the structural proteins during the assembly of the head of bacteriophage T_4. - Nature **227**: 680-685, 1970.

Leslie, C.A., Romani, R.J.: Salicylic acid: a new inhibitor of ethylene biosynthesis. - Plant Cell Rep. **5**: 144-146, 1986.

Liang, W.S., Wen, J.Q., Liang, H.G.: Stimulation of ethylene production in aged potato tuber slices by salicylic acid. - Phytochemistry **44**: 221-223, 1997.

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

Murashige, T., Skoog, F.: A revised medium for rapid growth and bio assays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962

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

Nissen, P.: Stimulation of somatic embryogenesis in carrot by ethylene - effects of modulators of ethylene biosynthesis and action. - Physiol. Plant. **92**: 397-403, 1994.

Romani, R.J., Hess, B.M., Leslie, C.A.: Salicylic acid inhibition of ethylene production by apple discs and other plant tissues. - J. Plant Growth Regul. **8**: 63-69, 1989.

Roustan, J.P., Latche, A., Fallot, J.: Inhibition of ethylene production and stimulation of carrot somatic embryogenesis by salicylic acid. - Biol. Plant. **32**: 273-276, 1990.

Sairam, R.K., Shukla, D., Saxena, D.C.: Stress induced injury and antioxidants enzymes in relation to drought tolerance in wheat genotypes. - Biol. Plant. **40**: 357-364, 1998.

Saxena, C., Samantaray, S., Rout, G.R., Das, P.: Effect of auxins on *in vitro* rooting of *Plumbago zeylanica*: peroxidase activity as a marker for rooting induction. - Biol. Plant. **43**: 121-124, 2000.

Scebba, F., Sebastiani, L., Vitagliano, C.: Activities of antioxidant enzymes during senescence of *Prunus armeniana* leaves. - Biol. Plant. **44**: 41-46, 2001.

Shimoni, M.: A method for activity staining of peroxidase and β-1,3-glucanase isozymes in polyacrylamide electrophoresis gels. - Anal. Biochem. **220**: 36-38, 1994.

Wang, C.Y.: Effect of temperature preconditioning on catalase, peroxidase and superoxide dismutase in chilled zucchini squash. - Postharvest Biol. Technol. **5**: 67-76, 1995.

Wang, K.L.C., Li, H., Ecker, J.R.: Ethylene biosynthesis and signalling networks. - Plant Cell **14**: 131-151, 2002.