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THE POSSIBLE ROLE OF OXIDATIVE STRESS AND ABA IN JASMONIC ACID MEDIATED BREAKAGE OF EMBRYONIC DORMANCY IN APPLE

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SUMMARY

Embryos isolated from dormant seeds of apple were cultured in light in the presence of jasmonic acid (JA, 20 µM) for 7 days in parallel to control non-treated ones. H_2O_2 and ABA levels were quantified and antioxidant enzymes namely, superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) activities were determined in the embryos during the culture. JA treatment stimulated the germination of embryos and activities of SOD, CAT and GR. JA treatment also increased the H_2O_2 levels on day 1 of embryo culture. In contrast, JA inhibited the levels of ABA during the first 3 days of culture, after that increase in the levels of ABA was observed. Simultaneous increase in H_2O_2 and a decrease in ABA levels were also observed during the culture. The results suggest the induction of oxidative stress by JA and a signalling role of JA in the breakage of embryonic dormancy is postulated.

Key words: **Apple, embryos, germination, jasmonic acid, oxidative stress.**

INTRODUCTION

Dormant apple embryos germinate slowly and seedlings show several growth and developmental abnormalities (Bogatek and Lewak 1991). The most important are the inhibition of hypocotyls and internodes elongation, and asymmetric growth and greening of cotyledons. Dormancy and its removal are under temperature and light control and several plant growth regulators are involved in the regulation of these processes (Ranjan and Lewak 1994). Jasmonic acid (JA) has been identified in apple embryos and quantified in the course of dormancy removal (Ranjan *et al*. 1994). Moreover, stimulating effect of JA on germination of dormant embryos and its interaction with light and other growth regulators together with its involvement in the control of hydrolytic enzymes have been demonstrated (Ranjan and Lewak 1995). Studies have also indicated that JA affects dormancy through regulation of lipids and sugar catabolism in germinating apple embryos (Ranjan, 1998 and Bogatek *et al.* 2002).

The efficiency of reactive oxygen species (ROS) scavenging through antioxidant enzymes, namely superoxide dismutase (SOD), glutathione reductase (GR) and mainly catalase (CAT), has been shown to be closely related to seed vigour and germinability. Impairment or deficiency of these activities leads to accumulation of ROS and to various cellular injuries, such as lipid peroxidation, which can in turn affect seed germinability and seedling emergence (Bailly *et al.* 2002). Since, JA plays an important role in the regulatory complex controlling the germination of dormant apple embryos; the aim of the present work was to see whether JA affects antioxidant enzymes in apple embryo germination. Therefore, studies were undertaken on the effects of JA

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on changes in the reactive oxygen species (ROS) levels, activities of antioxidant enzymes and ABA levels in germinating embryos from dormant apple seeds.

MATERIALS AND METHODS

Seeds of apple (*Malus domestica* Borb. cv. Antonowka) harvested in 2000 and provided by Centre for Apple Research in Ozarow (Poland) were used in the study. Dry seeds were stored in sealed containers at 5^0C and 10% relative humidity. The embryos were prepared from imbibed seeds and cultured according to Ranjan and Lewak (1992). Lots of 30 embryos were cultured for 7 days in petri dishes on a filter disc moistened with 5 ml of distilled water or JA solution (20 µM). This concentration was found the most effective in mobilization of reserve lipids and catabolism of sugars in germinating apple embryos (Ranjan 2001 and Bogatek *et al.* 2002). The incubation conditions of the culture were $25/20^0$C, 12h/12h day/night. An embryo was considered as germinated when geotropic curvature of the axis was observed. Germinating embryos were counted daily, and they were collected for further analysis.

The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were determined according to methods described by Ginnopolitis and Ries (1977), Clairbone (1985) and Esterbauer and Grill (1978) respectively. The extraction media for enzyme activities were prepared according to Bailly *et al.* (1996). Fifty apple embryos were homogenized with 0.4 g polyvinylpyrrolidone in 20 ml of 0.1 M phosphate buffer (pH 7.8) containing 2 mM dithiothreitol, 0.1 mM EDTA and 1.25 mM PEG-4000. The homogenate was centrifuged at 16000 x g for 15 min. The resulting supernatant was filtered, desalted and used for enzyme assays.

The reaction mixture for SOD contained 1.3 µM riboflavine, 13 mM methionine, 63 µM nitroblue tetrazolium (NBT) in 0.1 M phosphate buffer (pH 7.8) and 50 µl of enzyme extract in a final volume of 3 ml. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT. Glass test tubes containing the mixture were immersed in a thermostated bath at 25^0C and illuminated with a fluorescent lamp. Identical tubes which were not illuminated served as blanks. After illumination for 15 min absorbance was measured at 560 nm.

Catalase activity was measured at 25^0C. The enzyme assay contained 3.125 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 200 µl of enzyme extract in a total volume of 3 ml. Catalase activity was estimated by the decrease in absorbance of H_2O_2 at 240 nm.

GR activity was determined at 25^0C by following the rate of NADPH oxidation at 340 nm. The assay mixture contained 0.5 mM NADPH, 10 mM oxidized glutathione, 3 mM $MgCl_2$ in 0.1 M phosphate buffer (pH 7.8) and 100 µl of enzyme extract in a total volume of 400 µl.

H_2O_2 levels were determined according to $O^{\cdot}$ Kane *et al.* (1996). Embryos of approx. 0.3 g fresh weight were homogenized in liquid nitrogen. Samples were then suspended in 1 ml of 0.2 M perchloric acid and centrifuged at 12000x g for 10 min. To remove the perchloric acid, the supernatant was neutralised to pH 7.5 with 4 M KOH and the solution was centrifuged at 1000x g to remove insoluble potassium perchlorate. A 200 µl aliquot of the supernatant was applied to column of anion-exchange resin and the column was washed with 800 µl of distilled water and the eluate was used for the assay of H_2O_2. Reaction mixture contained 1 ml of the eluate, 400 µl of 12.5 mM 3-dimethylaminobenzoic acid in 0.375 M phosphate buffer (pH 6.5), 80 µl of 1.3 mM 3-methyl-2-benzothiazolinone hydrazone and 20 µl (0.25 units) horse-radish peroxidase in a total volume of 1.5 ml. The reactions were initiated by the addition of the peroxidase and the increase in absorbance at 590 nm after 3 min was monitored and compared with increases elicited by standard samples of H_2O_2.

Extraction and purification of ABA from 200 apple embryos (10 g fresh weight) was carried out according to Grabner *et al.* (1976). The materials were homogenised in 20 ml methanol and homogenate was filtered. The solid residue was homogenised twice in 80% methanol (20 ml). The combined extracts were evaporated to dryness at 30^0 C under vacuum and residue taken up in methanol (5 ml) and purified. DEAE-Sephadex A-25 column was eluted with 50 ml of 80% methanol, next with increasing gradient of acetic acid in

80% methanol. ABA fractions were evaporated and subjected to ELISA. The properties of the antiserum were described by Weiler (1982). Immunoglobulin G fractions of this antiserum were used for enzyme immunoassay. The activity of (-) bound alkaline phosphatase were measured with p-nitrophenyl phosphate as substrate at 405 nm and concentration of ABA was calculated with the help of a standard curve (lowest reliable detection limit 0.1 ng in a sample).

All experiments were repeated 3 times with 4 replicates.

RESULTS AND DISCUSSION

Control dormant embryos showed 15% germination after 7 days of culture and developing seedlings exhibited developmental abnormalities as described earlier. Treatment of embryos with JA resulted in increased germination reaching 87% after 7 days of culture (Table 1) with normal seedling development. These results confirm our earlier data (Ranjan and Lewak, 1992). The level of ABA, which was high in dormant embryos, decreased during the culture in control but its level dropped sharply during the last days of experiment (Table 1), again this observation was consistent with the earlier data (Rudnicki 1969). There is no relationship between the decrease in ABA level and germination of embryos, suggesting that ABA is not the sole factor responsible for dormancy in apple embryos. Similar results were also obtained with seeds of other species (Walton 1980-81). In contrast, treatment with JA resulted in sharp decrease in ABA level than control during the first 3 days of culture but with days of culture, ABA trends are opposites in control and JA treated cultures (Table 1). The rise in the level of ABA in JA treated cultures (after day 3) can be attributed to free/ bound ABA interconversions and its physiological role is not clear if one takes into account the inhibitory effect of ABA on germination of apple embryo and the late rise in the level of endogenous ABA in JA treated embryo cultures. Our previous observations indicate that ABA is most effective in inhibition of germination of apple embryos when applied early during embryo incubation (Ranjan and Lewak 1994), the germination of embryos is stimulated by JA with increasing endogenous level of JA and the germinability of embryos is related to JA level (Ranjan and Lewak 1995). The lack of a good correlation between ABA level and germinability and differences in sensitivity to ABA has been observed in dormant seeds of other species, for example barley (Wang *et al*. 1995) and wheat (Corbineau *et al*. 2000).

Table 2 shows the effects of JA on H_2O_2 level and SOD activity in isolated apple embryos during culture. Maximum level of H_2O_2 was observed in one- day- old

Table 1. Effects of applied jasmonic acid (20 µM) on germination of embryos and on the levels of ABA (ng g^{-1} fw) in apple embryos after different days of culture. SE did not exceed 5 ng g^{-1} of ABA.

Days of culture	% of germination		ABA content	
	Control	JA	Control	JA
0	ND	ND	195	195
1	ND	5 ± 1	130	90
3	4 ± 1	12 ± 2	100	70
5	10 ± 1	66 ± 3	75	120
7	15 ± 2	87± 3	40	160

ND= not detected

Table 2. Effects of jasmonic acid treatment (20 µM) on H_2O_2 level and on the activity of superoxide dismutase in apple embryos after different days of culture. Values are mean ± SE.

Days of culture	H_2O_2 level (µmol/g fw)		Activity of superoxide dismutase (units/mg protein)	
	Control	JA	Control	JA
1	4.0 (± 0.32)	14.0 (± 0.95)	13.0 (± 0.80)	16.0 (± 0.86)
2	1.0 (± 0.70)	1.3 (± 0.80)	8.0 (± 1.0)	9.0 (± 1.3)
3	ND	ND	6.0 (± 0.90)	7.0 (±1.4)
5	ND	ND	5.0 (± 0.50)	6.0 (±0.80)

ND= not detected

embryo culture, after which the level dropped sharply. Treatment of embryos with JA caused a 3-fold increase in H_2O_2 level in one- day- old culture but after that there was no effect of JA, indicating an oxidative stress induction by JA. Simultaneous increase in H_2O_2 and a decrease in ABA levels were observed in control as well as in JA treated cultures but the decrease in ABA content was much higher in JA treated cultures than control (Table 1). Such a relationship between H_2O_2 and decrease in ABA levels was observed in barley seeds (Wang et al., 1998). It seems, therefore, that the observed sharp rise in H_2O_2 during the early period of embryo culture could be responsible for decrease in ABA content recorded in this study. Maximum stimulation in the activity of SOD by JA was observed in one-day-old embryo culture when H_2O_2 level was maximal (Table 2), followed by a gradual decline in its activity in control as well as in JA treated cultures. Accumulation of H_2O_2 corresponded well with maximum SOD activity both in control and in JA treated embryos on the first day of culture.

In contrast to SOD activity, the activity of CAT was relatively low until 3rd day and then increased sharply at the end of the experiment (Table 3). JA treatment resulted in about one and half-fold increase in the activity of CAT. The increase in CAT activity could probably be related to germination of apple embryos. Previous results showed that JA activates alkaline lipase, thus initiating the mobilization of reserve lipids as the first metabolic event of apple embryo germination (Ranjan and Lewak 1992). A high CAT activity is necessary to limit damaging peroxidative reactions during β-oxidation of fatty acids (Bailly *et al.* 2003). The effect of JA on the activity of GR is shown in Table 3. The embryos treated with JA resulted in two-fold increase in the activity of GR at the end of the experiment. The sharp increase in the activity of GR probably results in an increase in the levels of reduced glutathione, known to be an important factor in H_2O_2 detoxification.

The accumulation of H_2O_2, in response to oxidative stress induced by JA may play a role in supplying the growing embryo with metabolic energy and intermediates through oxidation- reduction of glutathione and of NADP which may be key step in dormancy breakage of apple embryos. Results of this work together with data from our previous studies allow us to propose that JA plays an important signalling role in events leading to breakage of embryonic dormancy in apple.

Table 3. Effects of jasmonic acid treatment (20 µM) on the changes in the activities of catalase and glutathione reductase in apple embryos after different days of culture. Values are mean ± SE.

Days of culture	Activity of catalase (nmol H_2O_2/mg protein/min)		Activity of glutathione reductase (nmol NADP/ mg protein/min)	
	Control	JA	Control	JA
1	20.0 (± 0.50)	20.0 (± 0.28)	6.0 (± 1.1)	9.0 (± 1.3)
2	24.0 (± 0.10)	26.0 (± 0.10)	8.0 (± 1.2)	12.0 (± 1.5)
3	30.0 (± 0.80)	40.0 (± 0.40)	11.0 (± 1.5)	15.0 (±1.8)
5	53.0 (± 1.0)	80.0 (± 1.2)	13.0 (± 1.8)	25.0 (±1.2)

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