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COMPARISON OF THE EFFECTS OF DIFFERENT SA CONCENTRATIONS ON SENESCENCE IN EXCISED ROCKET (*ERUCA SATIVA* L.) COTYLEDONS

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Abstract

Senescence, accepted as the last phase of growth in plants, is characterized by a series of degenerative events that decrease the metabolic activity and eventually cause the death of cells, tissues and organs. In the present studies the effect of salicylic acid, a phenolic substance that recently came to be defined as plant growth regulator, especially increasing immunity of plants against infections, regulating thermogenesis in *Arum* flowers, inhibiting ethylene biosynthesis and germination, reversing the effects of abscisic acid and blocking the wound-induced response on the senescence of excised cotyledons of cultivated *Eruca sativa* L., (Rocket) was examined. Cotyledons of ten-day-seedlings of *E. sativa* were cut apart from the petiole and used as experiment materials. The fresh weight of cut cotyledons were measured and they were then incubated in 3 ml of salicylic acid at 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} M concentrations and in distilled water as control. After 4 hours of light exposure in climatie room (25±2°C), total chlorophyll amount, total protein content, peroxidase activity and protein analysis with SDS-PAGE method were assayed spectrophotometrically with biochemical methods. Furthermore, the effect of salicylic acid on fresh weights of rocket cotyledons was studied.

Introduction

Senescence is the outcome of programmed cell death in cells, tissues, organs, or in the whole plant. Many researchers are currently studying the mechanisms that regulate senescence in excised tissues or organs or in a whole plant. The effects of exogenous hormones applied to these tissues or organs were studied in many plants where low concentrations of cytokinin, gibberellin, and auxins retard senescence whereas ethylene, abscisic acid and jasmonic acid speeds it up (Mayak & Halevy, 1972; Gepstein & Thimann, 1981; Ueda *et al*., 1981; Lamattina *et al*., 1987; Parthier, 1990; Carrasco & Carbonell, 1990; Creelman & Mullet, 1997). Furthermore, it is known that some phenolic compounds like coumarin, quercetin, vanillin, and salicylic acid (SA) also retard senescence (Knypl, 1970). Of these, SA especially is accepted as a natural plant growth regulator (Raskin, 1992), which increases immunity in plants against infections (Sakhabutdinova *et al*., 2003), regulates thermogenesis in *Arum* flowers (Raskin *et al*., 1990), inhibits ethylene and jasmonic acid biosynthesis (Pena-Cortes *et al*., 1993), as well as germination (Rajasekaran *et al*., 2002), the effects of abscisic acid (Rai *et al*., 1986), and blocks the wound-induced response (Pena-Cortes *et al*., 1993). Though SA is known to inhibit senescence, it is interesting to note that its mechanism has not yet been fully explained (Raskin, 1992). The present study aims to clarify unknown issues on this subject in *E. sativa* L., (rocket) by studying the connection between applied SA and chlorophyll content, total protein amount, peroxidase activity and cotyledon fresh weight difference during the senescence of excised cotyledons of 10-day old seedlings.

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Material and Methods

Cotyledons of 10-day old seedlings of *Eruca sativa* L., (rocket) was used as experimental material. The cotyledons (6 each) excised from the petioles were weighed and incubated over night in Petri dishes containing 3 ml of 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} M concentrations of salicylic acid and distilled water used as control. Following a 4-hour light exposure in climatie room (25±2°C), cotyledon fresh weight difference (Letham, 1971), total chlorophyll amount (Arnon, 1949), total protein content (Bradford, 1976), peroxidase activity (Birecka *et al*., 1973) and protein analysis with SDS-PAGE method (Laemnli, 1970) were studied. All spectrophotometric assays were carried out with a SHIMADZU UV 160 spectrophotometer.

Cotyledon fresh weight difference: After washing with distilled water *E. sativa* seeds were lined up in Petri dishes and in the incubator at 25ºC 3-4 days. The cotyledons of germinated seeds were cut to measure their initial weights, and incubated in climatie room for 3 days in Petri dishes containing 3 ml of different concentrations of salicylic acid (10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} M). At the end of this period the cotyledons were measured for their second weights and the cotyledon fresh weight difference was calculated in mg (Letham, 1971).

Chlorophyll determination: Plant material from harvested seedlings was homogenized with the addition of 10 mg $CaCO_3$ and 3 ml 80% acetone (Merck) after they were measured for their fresh weights. The volume of the supernatant was measured after 15-minute centrifugation at 3000 g. The absorption values of raw chlorophyll extract at wavelengths of 645 and 663 nm were used to determine the total chlorophyll amount was calculated as mg/g fresh weight Arnon (1949).

as follows:

$$\text{Total chlorophyll Amount} = (20.2 \times D_{645}) + (8.02 \times D_{663})$$

Total protein determination: Dye-binding method was employed in the determination of total proteins (Bradford, 1976). The experimental materials were homogenized in 0.1 M phosphate buffer (pH 7.0) with the proportion of 100 mg fresh weight/ml, and then the extracts were centrifugated for 45 minutes at 13000 rpm. 0.1 ml of supernatants was added to 5 ml of ¼ diluted Coomassie Brillant Blue G-250 (Merck) (Bio-Rad) and vigorously mixed. After keeping it in the dark for 15 minutes, the absorption of the protein in the extract against blank at 595 nm was spectrophotometrically measured and calculated as μg protein/ml. Bovine Serum Albumin (BSA) was used as standard.

Peroxidase determination: The activity of peroxidase enzyme was determined by employing the method of Birecka *et al*., (1973). With this method, the experimental materials were homogenized in 0.1 M pH 7.0 phosphate buffer, using the proportion of 100 mg fresh weight/ml, and then the extracts were centrifugated for 45 minutes at 13000 rpm. Supernatants were treated with 15 mM guaiacol (Merck) and 5 mM H_2O_2 (Merck) in 0.1 M phosphate buffer (pH 7.0). The absorbance of the coloured product in the extract was recorded every 10 seconds for 2 minutes at 470 nm, and the peroxidase activity was quantitatively provided as ΔA/g fresh weight/minutes with the spectral method.

Protein analysis with SDS-PAGE method: SDS-PAGE method of Laemmli (1970) with some modifications was employed for the analysis of soluble protein bands. 5% bulk gel and 12% separation gel were prepared from a stock solution containing 30% acrylamide (Merck), 0.8% N,Ń-bis methylene acrylamide (Merck), 10% ammonium persulfate and add TEMED at the end. Gently swirl the flask to mix, being careful not to generate bubbles. Pipette the solution to a level of 4cm of the top. Add 0.3ml of n-buthanol. A very sharp liquid interface will be visible within 10-20min. Let polymerize the gel for another hour at least. Rinse the surface of the gel with watter before pouring the stacking gel. Fill each sandwich with stacking gel solution and insert a comb into each place taking care not to trap any bubbles bellow the teeth. Following polymerization, the sample buffer containing equal proportions of protein samples was charged after being kept in boiling water for 5 minutes. Electrophoresis continued with an increasing current from 15 mA to 45 mA, until bromo-phenol blue (marker) reached the edge of the gel. The gel containing protein bands was kept in staining solution containing 15% acetic acid, 25% methanol, deionized water and 0.05% Coomassie Brilliant Blue R 250 at room temperature for 24 hours. Then, the gel was washed in a washing solution (destaining solution) containing 7.5% acetic acid (Merck), 5% methanol (Merck), and 87.5% deionized water.

Results

Biochemical markers: A decrease of some 15% in chlorophyll contain was observed in the control level compared to the initial level of chlorophyll content in cotyledons cut from 10-day seedlings incubated under light for 4 h in both control and different SA (10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} M) concentrations (Fig. 1). Upon comparison of experimental groups with the control group, a 15 % increase of chlorophyll was observed at 10^{-9} M, chlorophyll losses of 65% and 30% were determined at 10^{-3} M and 10^{-5} M, respectively.

Total amount of protein in all experimental groups and control group was less than the amount at initial level (Fig. 2). The lowest total protein content was found at 10^{-3} M, and the highest at 10^{-9} M respectively, when compared with the control group. PO activity of the control significantly increased as a reaction to the stress of injury due to cutting (Fig. 3). The PO activity of the cotyledons from experiment groups (10^{-5}, 10^{-7}, 10^{-9} M) gradually increased, compared to the initial level. The fact that PO activity of rocket cotyledons was zero only in 10^{-3} M SA concentration among the experimental group, made us think that this concentration was toxic to the plant.

Weight of cotyledons excised from 10-day seedlings showed that SA had a negative effect on the cotyledon fresh weight but it became close to the control value at decreasing SA concentrations, and that cotyledons maintained their survival (Table 1).

Fig. 4 shows the bands specific to soluble proteins of cotyledons after SDS polyacrylamide gel electrophoresis. Upon inspection of all protein bands belonging to the initial level, control group and all groups treated with SA, two bands different from the standard protein bands were determined between 66 kDa and 45 kDa, and under 14 kDa. The protein band of 45 kDa was seen to be more evident especially in all samples except 10^{-3} M SA treatment. The existence of a weak band of 45 kDa at 10^{-3} M and absence of any band apart from this are consistent with the biochemical results.

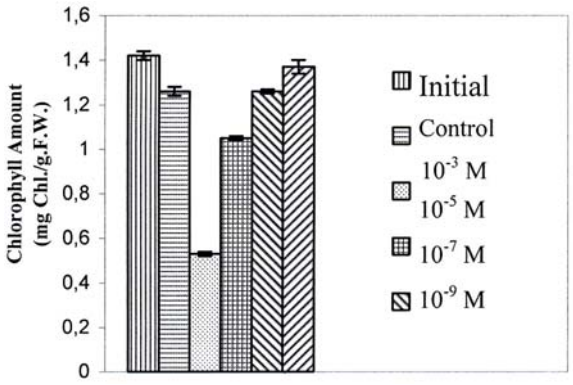

Fig. 1. The effect of different SA concentrations on the chlorophyll content of cotyledons excised from 10-day seedlings.

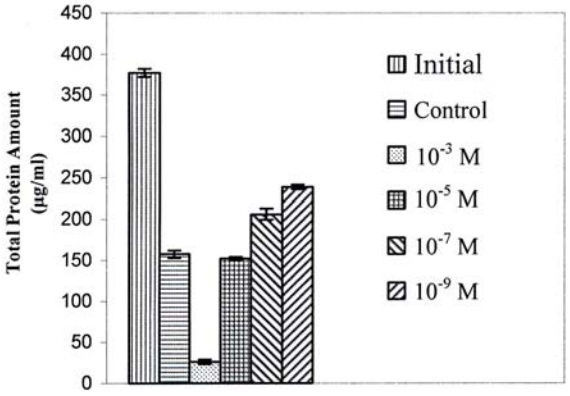

Fig. 2. The effect of different SA concentrations on the total protein amount of cotyledons excised from 10-day seedlings.

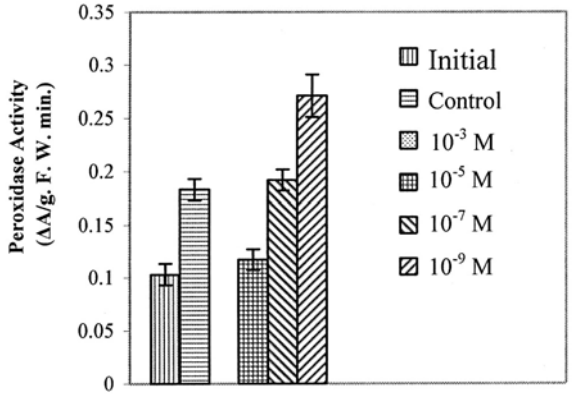

Fig. 3. The effect of different SA concentrations on the PO activity of cotyledons excised from 10-day seedlings.

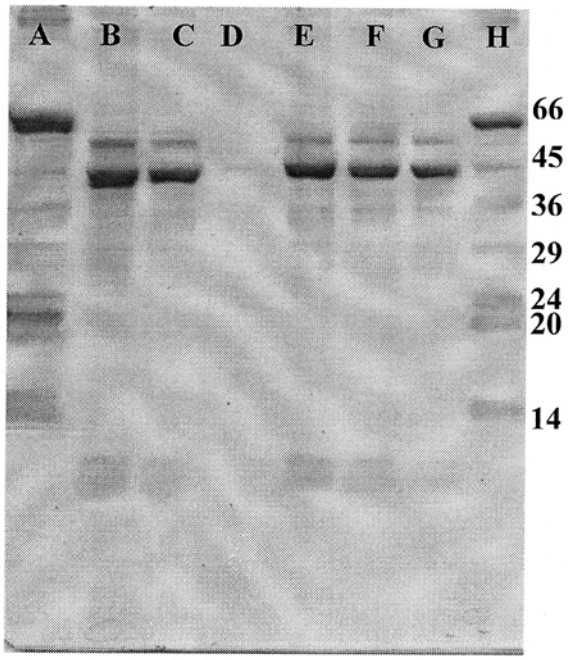

Fig. 4. SDS-PAGE analysis of soluble proteins in cotyledons cut from 10-day seedlings which were treated with different SA concentrations. Marker proteins:α-lactalbumin (14 200 MW), Trypsin inhibitor (20 100 MW), Trypsinogen (24 000 MW), Carbonic anhydrase (29000 MW), Glyceraldehyde-3-phosphate dehydrogenase (36 000 MW), Albumin, egg (45 000 MW), Albumin, bovine (66 000 MW). A, H. Standard protein mixture, B. Initial Level, C. Control, D. 10^{-3} M SA, E. 10^{-5}M SA, F. 10^{-7} M SA, G. 10^{-9} M SA.

Table 1. The effect of different SA concentrations on the fresh weight difference in cotyledons excised from 10-day seedlings.

Application	Cotyledon fresh weight difference (g)
Control	6±0,0003
10^{-3} M	-1±0,0000
10^{-5} M	1±0,0003
10^{-7} M	3±0,0008
10^{-9} M	5±0,0003

Discussion

Extremely complicated events follow each other in the senescence process, which is one of the most important and interesting subjects of plant physiology. In the senescence, which is a result of programmed cell death, the accelerated catabolic reactions in the cells and consequently lipid, protein, chlorophyll, DNA, and RNA degredation were studied in detail (Thomas, 1982; Hilditch *et al*., 1989; Buchanan-Walloston, 1997; Matile 1998). Although senescence occurs in an age-dependent manner in many species, the initiation and progression of senescence can be modulated by a variety of environmental factors

such as temperature, mineral deficiency, drought conditions, and pathogen infection. It is known that internal factors such as plant growth regulators, reproduction, and cellular differentiation also influence senescence (Woo *et al*., 2001). No satisfactory information was encountered in literature with regard to the relationship between SA, a new plant regulator, and senescence.

It is known that loss of chlorophyll occurs during senescence (Noodén *et al*., 1997; Matile, 1998; Frang *et al*., 1998). The present studies showed loss of chlorophyll in excised rocket cotyledons incubated in high SA concentrations (10^{-3}, 10^{-5} M). The chlorophyll content of cotyledons increased by 15% at 10^{-9} M concentration while it was almost at the same value as the control at 10^{-7} M. The chlorophyll loss in the incubated cotyledons at 10^{-3} M concentration may be due to the toxic effect of this concentration, and it is also possibly due to the increase in the ethylene biosynthesis during cutting process. The probability that the decrease of chlorophyll amount in control compared to the initial level may be related with the increase of ethylene is similar with the findings of Abeles *et al*., (1992). Altman (1982) showed that ethylene accelerated chlorophyll loss. Furthermore, Li *et al*., (1992) established that SA inhibited the activity of ACC (l-aminocyclopropane-1-carbocyclic acid) synthase enzyme, preventing formation of ethylene and chlorophyll loss. Consequently, one can emphasize that 10^{-9} M SA concentrations applied here prevented loss of chlorophyll, and even retarded senescence by promoting chlorophyll biosynthesis.

There are reports that proteins were degraded during biochemical changes that occur in cotyledons of various plants during senescence (Hilditch *et al*., 1989; Buchanan-Walloston, 1997). In the present study we found a decrease of 80% in the total protein content compared to the control, associated with the chlorophyll loss at 10^{-3} M concentration. This decrease at 10^{-3} M concentration may be due to the toxic effect. It was, however, determined that total protein amount gradually increased at 10^{-5}, 10^{-7}, and 10^{-9} M concentrations. Especially the increase of 70% obtained in the total protein amount at 10^{-9} M concentration makes us think that some proteins, possibly newly formed, played a part in the senescence process.

Abeles *et al*., (1992) found that the ethylene formed as a result of injury increases PO activity. In the present study an increase of approximately 100% in the PO activity was observed, when compared to the initial level, contrary to the decrease in chlorophyll and proteins. This increase in PO activity might be due to the increase of ethylene that occurred during cutting process. This is similar to the reports of De Leo & Sacher (1970) and Sacher (1973). Zero PO activity at 10^{-3} M concentration may be explained by the strong toxic effect of high SA concentrations. It is possible that high SA concentrations inhibit protein synthesis and decrease PO activity. The application of 10^{-5}, 10^{-7}, and 10^{-9} M SA to excised rocket cotyledons induced PO activity. Especially the increase of 50% PO activity in rocket cotyledons treated with 10^{-9} M SA, with retarded senescence, compared to control, is not in line with the findings of Tenhaken *et al*., (2000). Furthermore, we can conclude that there is not a correlation between the PO activity and chlorophyll content.

Another parameter with this study is the effect of SA on the fresh weight difference of rocket cotyledons. Lower values than the control in all concentrations applied showed that this plant growth regulator had no direct effect on the cotyledon fresh weight. As a consequence, the decrease in chlorophyll, total protein amount and PO activity at 10^{-3} M concentration may be related with the loss of water in the plant cells at this concentration

(physiological concentration) due to osmotic effect or cell wall structure changes and thereby losing weight, and undergoing a different death than programmed cell death, with a slower metabolic activity. We can also express that the decrease here is not basically the same as the decrease in the senescence process. The increase in PO activity, parallel to the increase in chlorophyll and total protein in cotyledons, with retarded senescence, that were treated with SA in low concentrations makes it less likely for this antioxidant enzyme to be an indicator of senescence. According to the results obtained, the optimal value of SA in the senescence progress of cut rocket cotyledons is 10^{-9} M. At this concentration, we may assert that exogenous SA retards senescence of cut rocket cotyledons by inducing PO activity.

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