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Lipoxygenase activity and sanguinarine production in cell suspension cultures of California poppy (*Eschscholtzia californica* CHAM.)

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In this study we investigated the influence of biotic elicitor (phytopathogenic fungus *Botrytis cinerea*) and abiotic elicitors (methyljasmonate [MJ] and salicylic acid [SA]) on lipoxygenase (LOX) activity and sanguinarine production in cell suspension cultures of California poppy (*Eschscholtzia californica* CHAM.). We have observed different time effects of elicitors (10, 24, 48 and 72 h) on LOX activity and production of sanguinarine in *in vitro* cultures. All elicitors used in the experiments evidently increased the LOX activity and sanguinarine production in contrast to control samples. The highest LOX activities were determined in samples elicitated by MJ after 48 h and 72 h and the lowest LOX activities (in contrast to control samples) were detected after biotic elicitation by *Botrytis cinerea*. These activities showed about 50% lower level against the activities after MJ elicitation. The maximal amount of sanguinarine was observed after 48 h in MJ treated cultures (429.91 mg/g DCW) in comparision with control samples. Although all elicitors affect the sanguinarine production, effect of SA and biotic elicitor on sanguinarine accumulation in *in vitro* cultures was not so significant than after MJ elicitation.

1. Introduction

Lipoxygenases (LOXs, *linoleate:oxygen oxidoreductases*, EC 1.13.11.12) constitute a large family of non-heme iron containing dioxygenases that occur in plants, animals and have been detected in coral, moss, fungi and in some bacteria (Andreou and Feussner 2009). LOXs catalyze regio- and stereo-specific insertion of molecular oxygen into polyunsaturated fatty acid with (1*Z*,4*Z*)-pentadiene system to yield corresponding hydroperoxides (Obložinský et al. 2011). The step of dioxygenation leads to a cascade of reactions called lipoxygense pathway in which hydroperoxides produced by the LOX reaction are converted into a number of compounds. These products, called oxylipins, play diverse roles in plants as signal molecules that trigger activation of defence related genes (jasmonic acid, its methyl ester, traumatic acid) or as antimicrobial and antifungal compounds. LOXs play an important role in growth and development, senescence and in defence systems of plants to biotic and abiotic stress (Holková et al. 2010). Plant LOXs are monomeric proteins that consist of a single polypeptide chain with relative molecular mass of 94–104 kDa. The proteins have a N-terminal β-barrel domain and a larger catalytic C-terminal domain containing the active site of enzyme. In plants, linoleic and linolenic acids are the most common substrates for LOX (Brash 1999).

Eschscholtzia californica CHAM. is an annual plant of *Papaveraceae* family and was introduced into Europe in 19th century as a decorative garden plant. The whole plant contains a mixture of tertiary and quaternary isoquinoline alkaloids with higher content in flowering plants. The main alkaloids are pavines such as californidine and escholtzine, but protoberberines, benzylisoquinolines, aporphines, benzophenanthridines and protopines are also present (Fabre et al. 2000). California poppy is used in traditional medicine for its spasmolytic, sedative and anxiolytic effect (Paul and Maurer 2003) and various pharmacological studies confirmed the sedative and anxiolytic actions of exstracts of *Eschscholtzia californica* (Fabre et al. 2000).

Sanguinarine is a quaternary benzophenanthridine alkaloid with antimicrobial activity and belongs to the group of benzylisoquinoline alkaloids. The antimicrobial activity of sanguinarine assumed its function as plant defense secondary metabolite. Besides its antimicrobial properties, sanguinarine also presents antiviral and cytotoxic activities (Villegas et al. 2000). Higher production of alkaloids was observed in cell suspension cultures of California poppy after elicitation with different elicitors. Production of sanguinarine and dihydrosanguinarine has rapidly increased after methyljasmonate, salicylic acid and yeast elicitation (Cho et al. 2008).

In this study we investigated the effect of a biotic elicitor (*Botrytis cinerea*) and abiotic elicitors (methyljasmonate, salicylic acid) on LOX activity at different elicitation times in suspension cultures of *Eschscholtzia californica* CHAM. We also examined the role of LOX on production of the secondary metabolite sanguinarine in California poppy *in vitro* cultures.

2. Investigations and Results

2.1. Influence of biotic elicitor on LOX activity and sanguinarine production

The influence of the biotic elicitor *Botrytis cinerea* on LOX activity in suspension cultures of California poppy is shown in Fig. 1. LOX activity in elicitated suspension cultures were compared with LOX activity in control samples (suspension cultures

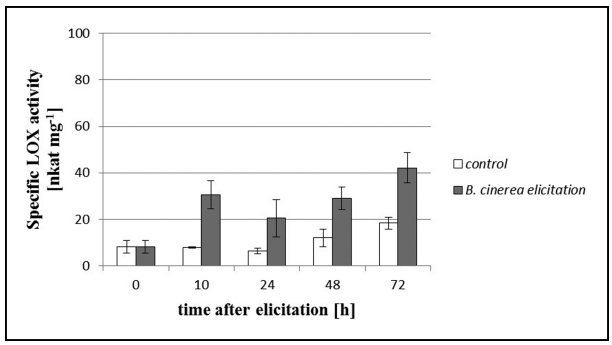

Fig. 1: Time course of LOX activity in suspension cultures of California poppy after *Botrytis cinerea* elicitation. Control samples were without the addition of elicitor. Suspension cultures were treated for 10. 24, 48 and 72 h. Values are means ± S.D from triplicate experiments. (n = 3). $p < 0{,}001$.

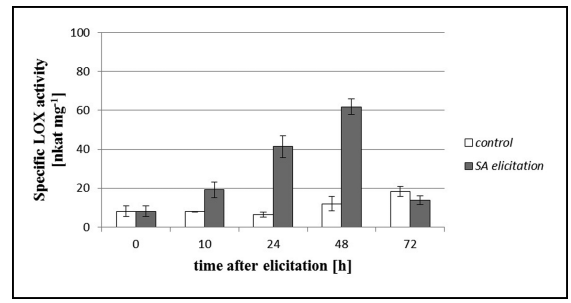

Fig. 3: Time course of LOX activity in suspension cultures of California poppy after salicylic acid (SA) elicitation. Control samples were without the addition of elicitor. Suspension cultures were treated for 10. 24, 48 and 72 h. Values are means ± S.D from triplicate experiments. (n = 3). $p < 0{,}001$.

without addition of elicitor). The LOX activity increased after treatment with biotic elicitor with the highest level after 72 h of elicitation. However, these activities showed about 50% lower level against the activities after MJ elicitation. The value of specific LOX activity after 72 h treatment with biotic elicitor was 42.20 ± 6.42 nkat/mg. The values of LOX activity in control samples were low and did not change significantly during the time course of the experiment.

The effect of fungal elicitation on the sanguinarine accumulation in California poppy *in vitro* cultures is shown in Fig. 2. The maximal amount of sanguinarine in elicited cultures was observed after 48 h (276.92 ± 10.83 mg/g DCW) which was approximately 3 times higher in comparison with control cultures (90.33 ± 20.85 mg/g DCW). The production of sanguinarine slightly decreased after 72 h of elicitor treatment to 202.86 ± 16.65 mg/g DCW, but during the whole experiments the production of alkaloid was higher than in the non-treated cultures.

2.2. Influence of salicylic acid elicitation on LOX activity and sanguinarine production

Time course of LOX activity after salicylic acid elicitation in suspension cultures of California poppy is demonstrated in Fig. 3. Treatment with SA stimulated the LOX activity in comparison with control samples and the highest LOX activities were observed between 24 and 48 h and decreased after 72 h of elicitation. The maximal specific LOX activity was observed after 48 h (61.92 ± 4.09 nkat/mg) which was approximately 5 times higher than in the control sample (12.01 ± 3.78 nkat/mg). After 72 h of SA exposure the LOX activity significantly decreased in contrast to LOX activity at 24 and 48 h of elicitation and in contrast with control samples.

Changes of sanguinarine production in California poppy *in vitro* cultures treated with SA are demonstrated in Fig. 4. SA stimulated sanguinarine accumulation with maximal levels at 24 h (261.76 ± 16.43 mg/g DCW) and 48 h (262.34 ± 2.17 mg/g DCW) of elicitor exposure. The production of alkaloid began to increase after elicitor addition in comparison to control cultures, but after 72 h of elicitor exposure the amount of sanguinarine decreased under values of control samples.

2.3. Influence of methyl jasmonate elicitation on LOX activity and sanguinarine production

All elicitors used in experiments have evidently increased LOX activity against control samples but treatment with MJ has the most significant effect on LOX activity. Changes of LOX activity in suspension cultures of California poppy treated with MJ are shown in Fig. 5. The increase in LOX activity was proportional to the lenght of MJ exposure. The highest specific activities of LOX were observed after 48 and 72 h of elicitation. The specific LOX activity after 48 h was 80.22 ± 13.54 nkat/mg which was approximately 6.7-fold increase of LOX activity in comparison with control sample. The highest specific LOX activity was determined after 72 h of MJ treatment (82.48 ± 13.77 nkat/mg), but the increase of LOX activity was only 4,5 higher than in control sample.

As shown in Fig. 6, the effect of MJ on sanguinarine production in California poppy *in vitro* cultures was the most significant from all elicitors. The amount of sanguinarine increased proportionally to the lenght of elicitor treatment and at 72 h slowly decreased. The highest alkaloid accumulation was observed after 48 h of MJ elicitation (429.91 ± 21.51 mg/g DCW), when it was 4.76 times higher in comparison with control culture.

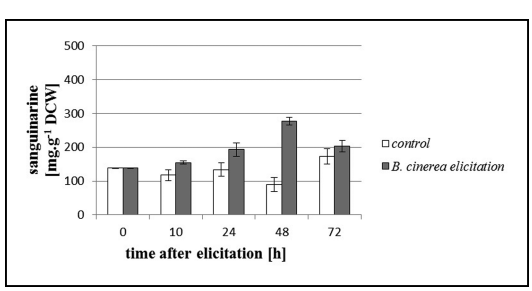

Fig. 2: Time course accumulation of sanguinarine in suspension cultures of California poppy after *Botrytis cinerea* elicitation. Control samples were without the addition of elicitor. Suspension cultures were treated for 10. 24, 48 and 72 h. Values are means ± S.D from triplicate experiments. (n = 3). $p < 0{,}001$.

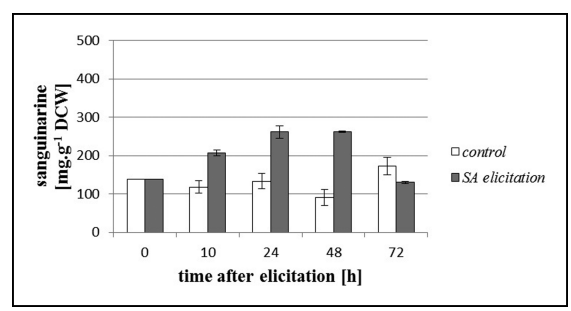

Fig. 4: Time course accumulation of sanguinarine in suspension cultures of California poppy after salicylic acid (SA) elicitation. Control samples were without the addition of elicitor. Suspension cultures were treated for 10. 24, 48 and 72 h. Values are means ± S.D from triplicate experiments. (n = 3). $p < 0{,}001$.

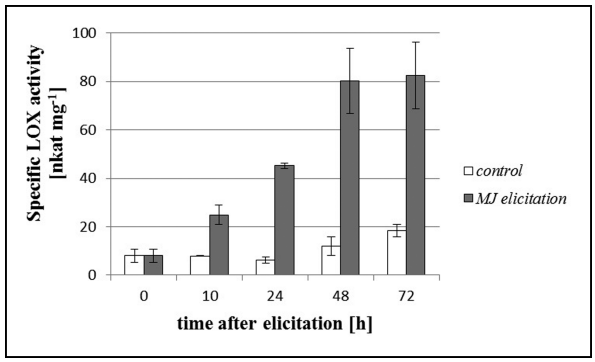

Fig. 5: Time course of LOX activity in suspension cultures of California poppy after methyl jasmonate (MJ) elicitation. Control samples were without the addition of elicitor. Suspension cultures were treated for 10. 24, 48 and 72 h. Values are means ± S.D from triplicate experiments. (n = 3). $p < 0{,}001$.

3. Discussion

Previous studies have shown that elicitation significantly changed LOX activity in various plants such as opium poppy (Holková et al. 2010), cucumber (Yang et al. 2012), potato (Mariutto et al. 2011) or grapevine (Faurie et al. 2009). In the hairy root cultures of *Silybum marianum* salicylic acid increased the activity of LOX after 24 h of elicitation and higher production of sylimarin was detected, too (Khalili et al. 2009). In cucumber fruit ten LOX genes were stimultaneously upregulated with MJ and eight genes were induced by ethylene (Yang et al. 2012). LOX activity was also stimulated in potato leaves after treatment with *Pseudomonas putida* in contrast to control samples. Furthermore the activity of enzyme increased in response to pathogen attack (*Botrytis cinerea*), remaining higher in treated plants after pathogen inoculation (Mariutto et al. 2011). The experiments with opium poppy suspension cultures showed higher LOX activities after MJ elicitation than with *Botrytis cinerea* treatment. In this study different concentrations of MJ were tested. The optimal concentration for LOX induction 100 μM was found (Holková et al. 2010). Therefore, in our experiments this concetration of MJ was used. The LOX activity had not been observed in elicited suspension cultures of California poppy yet. Cho et al. (2008) have investigated the influence of various elicitors (MJ, SA, yeast extract) on production of benzophenanthridine alkaloids in *in vitro* cultures of California poppy. The results show that sequential treatment with different elicitors was more effective than a single elicitor on sanguinarine and dihydrosanguinarine accumulation (Cho et al. 2008). Bilka et al. (2013) have studied the effect of abiotic elicitors ($AgNO_3$, $CaCl_2$) on the sanguinarine synthesis in California poppy suspension cultures. These elicitors increased the sanguinarine production after 48 h of elicitation and the amount of alkaloid was approximately 5 times higher in contrast to control samples. Because of the distinct importance of California poppy in the production of secondary metabolites, it is highly interesting to study whether LOX is involved in these processes. LOX may be involved in the regulation of secondary metabolites *via* signalling processes.

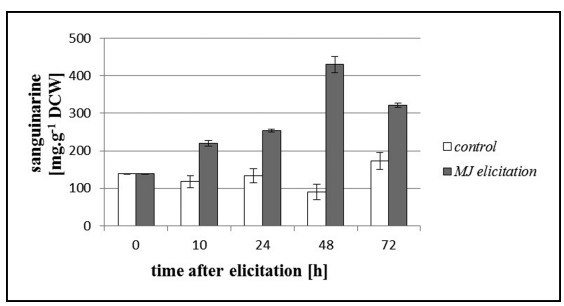

Fig. 6: Time course accumulation of sanguinarine in suspension cultures of California poppy after methyl jasmonate (MJ) elicitation. Control samples were without the addition of elicitor. Suspension cultures were treated for 10. 24, 48 and 72 h. Values are means ± S.D from triplicate experiments. (n = 3). $p < 0{,}001$.

In the present study, the influence of biotic and abiotic elicitors on LOX activity in suspension cultures of California poppy was investigated. On that account the suspension cultures of California poppy were prepared. On the 14th day of subcultivation, biotic and abiotic elicitors were individually added into the suspension cultures. The period of elicitor treatment was 10, 24, 48 or 72 h and LOX activity was determined spectrophotometrically.

Our results showed that all elicitors used in experiments increased LOX activity and sanguinarine accumulation against control samples without the addition of elicitor. Actually, LOX activation in the treated cultures was followed by stimulation of sanguinarine production. The most significant effect on LOX activity was observed after MJ elicitation. As shown in Fig. 5, the increase in LOX activity was proportional to the lenght of MJ treatment and the highest specific LOX activities was detected after 48 h and 72 h of elicitation. Similar effect of MJ was observed on sanguinarine biosynthesis (Fig. 6) with a maximal amount after 48 h of elicitation (429.91 ± 21.51 mg/g DCW). Sanguinarine production after MJ treatment incresed almost linearly until 48 h and than slowly declined. SA also induced the increase of LOX activities, but lower induction was observed in comparison with MJ elicitation. Figure 3 demonstrates that the highest level of specific LOX activity was detected at 48 h (61.92 ± 4.09 nkat/mg) while after 72 h the LOX activity (13.83 ± 2.17 nkat/mg) has significantly decreased. The influence of SA on sanguinarine accumulation in California poppy *in vitro* cultures is shown in Fig. 4. SA elicitation resulted in the highest induction of sanguinarine production in the 24th and 48th hour in comparison to control cultures. Results shown in Fig. 1 suggest that the biotic elicitor *Botrytis cinerea* is associated with the lowest effect on LOX activity. In contrast to control samples and to the abiotic elicitation similar results were determined. The highest value of specific LOX activity (42.20 ± 6.42 nkat/mg) was obtained after 72 h of elicitor treatment. Both MJ and *Botrytis cinerea* resulted in the highest induction of LOX in 72 h, but SA significantly increased LOX activity in 48 h which was decreased after 72 h. MJ and SA were more effective inducers of LOX activity in *in vitro* cultures of California poppy than the biotic elicitor – *Botrytis cinerea*. Time course analysis of biotic elicitor stimulated sanguinarine production is shown in Fig. 6. Sanguinarine increased after 10 h and 24 h, reached a maximum after 48 h (276.92 ± 10.83 mg/g DCW) of fungal elicitor treatment and declined thereafter. However, during the whole experiments the biosynthesis of alkaloid was higher than in control cultures. Our results indicate that sanguinarine production in california poppy suspension cultures is associated with the activity of LOX.

4. Experimental

4.1. Material

California poppy seeds (*Eschscholtzia californica* CHAM., *Papaveraceae*) used in the study were obtained from SEVA-SEED, Czech Republic. The origin of chemicals used in experiments is noticed below. All other chemicals were products of the highest purity commercially available.

4.2. Preparation of suspension cultures of Eschscholtzia californica CHAM

California poppy′s suspension cultures were prepared from long-term callus cultures of *Eschscholtzia californica* CHAM. Suspension cultures were maintained in the liquid medium according to Murashige and Skoog (1962)

suplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid and 0.1 mg/l kinetin. Suspension cultures of California poppy were kept in 100 ml Erlenmeyer flasks with 50 ml of liquid in each and were placed on a shaker with shaking at 140 rpm, in diffuse light at 25 °C and relative humidity 75–80 %. The period of subcultivation was 14 days.

4.3. Fungal elicitor preparation

Fungal elicitor was prepared from *Botrytis cinerea* according to McKinley et al. (1993) of MS medium including glycine (3 mg/L) and acid casein hydrolysate (1 g/l) but lacking alfa-naphthylacetic acid and kinetin. Mycelium cultures *B. cinerea* were grown on a shaker with shaking at 140 rpm at 25 °C in diffuse light and relative humidity 75–80%, for 7–10 days. After suspension cultures filtration, mycelial walls were resuspended in redistilled water and autoclaved (0.1 MPa, 121 °C, 20 min). Mycelia (ca. 200 g fresh weight) were homogenized, hydrolyzed with trifluoracetic acid and subsequently centrifuged at 2800 x g for 15 min at 4 °C. The supernatant was used as an elicitor. Its carbohydrate concentration was determined by an anthrone method according to Yemm and Willis (1954). Fungal homogenate was diluted to concentration 15 μg of glucose/ml. Preparation was filtered through the sterile membrane filter (0.45 μm, BIO-RAD, USA) before the application. Biotic elicitor treatment was initiated by the addition of 1.0 ml of fungal homogenate to 50 ml of cultured cells.

4.4. Elicitation procedure

Fungal elicitor preparation (*Botrytis cinerea*), MJ (Sigma, St. Louis, USA) or SA (Sigma, St. Louis, USA) was added individually into the suspension cultures on the 14th day of subcultivation. Suspension cultures were elicited with 10 μl of MJ to final concentration of 100 μM. SA was added in amount of 50 μl and final concentration was 1.5 mg/l. The fungal elicitor was used in an amount of 1,0 ml. The period of elicitor treatment was 10, 24, 48 or72 h.

4.5. LOX extraction

For determination of LOX activity, 5 g of California poppy suspension culture was frozen in dry ice, ground to a powder and homogenized in 5 ml of ice-cold 25 mM potassium phosphate buffer pH 6.0 containing 1 mM phenylmethyl sulfonyl fluoride, 0,5 mM ethylenediaminetetraacetic acid, 1 mM cysteine hydrochloride and 10 mM sodium thiosulfate. The homogenate was centrifuged at 12000 x g at 4 °C for 15 min. The supernatatnt was used as the enzyme extract for LOX activity determination.

4.6. LOX activity determination

The LOX activity was determined spectrophotometrically at 25 °C by measuring the increase of absorbance at 234 nm by UV/VIS Spectrophotometer Perkin Elmer, Lambda 35. Linoleic acid was used as substrate and was prepared accoridng to Chen and Whitaker (1986). The reaction mixture contained 920 μl 100 mM potassium phosphate buffer (pH 6.5), 105 μl substrate solution (10 mM) and 20 μl of enzyme crude extract. The LOX activity was expresed in katals (1 katal represents the conversion of 1 mol of substrate per 1 s). Protein concentrations were determined by method of Bradford (1976), using bovine serum albumin (Sigma, St. Louis, USA) as standard.

4.7. Sanguinarine determination

Suspension-cultured cells were collected by vacuum filtration at the 10th, 24th, 48th and 72th hours after elicitation. After separating the cells and medium using filtration, the fresh weight of each culture was determined. Dry cell weigh was calculated after drying of 1 g of culture at 105 °C in a hot air oven until a constant weight was obtained.

Sanguinarine was extracted from 5 g of fress mass. After homogenization with dry ice, sanguinarine was extracted by mixing the powdered tissue with 15 ml of methanol for 30 min at room temperature. Than the mixture was stored at 4 °C for 12 h and filtered. Extracts were evaporated to dryness under vacuum at 40 °C and the residues were extracted with 30 ml of 1 M HCl/diethylether (1/2, v/v) three times. The aqueous phase was alkalinized with ammonium hydroxide to pH 9.0 and than was extracted three times with 10 ml chloroform/isopropanol (3/1, v/v). The pooled extracts were evaporated to dryness and dissolved in 2 ml of UV-methanol. Each extract sample 10 μl was applied to Silufol chromatographic plates (Kavalier, Czech republic) and developed in a solvent system consisting of methanol/chloroform (97/3, v/v). Silufol plates were dried at 25 °C and sanguinarine was identified using the standard sample of sanguinarine (Sigma, St. Louis, USA) under UV illumination at 366 nm (Reprostar II, Camag). Spots corresponding to sanguinarine were removed and dissolved in 2 ml of 0.02 M NaOH in 50% UV methanol. After centrifugation (12000 x g for 10 min) the amounts of sanguinarine were determined using a Perkin-Elmer LS-30 luminiscence spectrophotometer at 324 nm of excitation and 408 nm of emission wavelengths. Sanguinarine contents were evaluated from a calibration curve prepared from standard sanguinarine solutions. The sanguinarine production in elicited and non-elicited cultures was expressed in mg per g of dry cell weight.

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