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Peroxidase and polyphenoloxidase polymorphism during embryogenesis in *Eucoreosma* section currants

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The polymorphism of peroxidase and polyphenoloxidase during embryogenesis was investigated over 1997–2008 in four currant species (genus *Ribes*, section *Eucoreosma*). At organogenesis stage X (according to F. Kuperman), in all currant species an intensive growth of berries and seeds proceeded. At this stage, the embryo changed from globular to cordate, its organs differentiated, in maternal plant leaves the maximum peroxidase (Px) polymorphism was observed. At organogenesis stage XI, in all currant species, when embryo growth proceeded very intensively, the content of peroxidase isoforms decreased. The increase of the number of new Px isoforms of the genus *Ribes*, *Eucoreosma* section currants at plant organogenesis stage X was directly related to embryo differentiation. No relationship was found between the PPO isoform spectrum and embryo differentiation.

Key words: embryogenesis, organogenesis, currant species, isoenzymes, peroxidase, orthodiphenoloxidase
Abbreviations: Px – peroxidase, o-DphO – orto-diphenoloxidase, Rf – electrophoretic protein movement

INTRODUCTION

After zygote formation, a new generation of sporophyte starts its development cycle on the maternal plant of the older generation. At that moment, various biochemical processes occur both in the embryo and in the mother plant (Shamrov, 2006).

In mature *Ribes nigrum* seed, the embryo rootlet and gemma are little differentiated. Embryo growth and differentiation peculiarities in other *Eucoreosma* section currants were not investigated. Modern black currant varieties are most often developed on the basis of interspecific hybrids. Thus, investigation of embryo growth and differentiation of various currant species will allow to establish both the common regularities of *Eucoreosma* section species and the specific peculiarities of individual species.

The regularities of oxidation reduction dynamics attributed to different plant organogenesis stages were established (Bobinas, Duchowski, 2001). It is believed that auxins, coumaric acid and other phenolic compounds are important in embryogenesis. Peroxidase (Px) and polyphenoloxidase (PPO) enzymes participate in the metabolism of these compounds. The metabolism of coumarin and indole-3-butyric acid at various plant differentiation stages is related to Px activity and isoenzyme spectrum (Abenavoli, Muscolo, 1996). Peroxidases are involved in various physiological processes (Gelvonauskis et al., 2005). They can be fractionated into a large number of isoenzymes in plant cells (Kärkönen, 2001). A rise in peroxidase activity indicated that somatic embryogenesis was triggered in a few habituated tissue cultures. Separated globular embryoids showed a much lower enzyme activity than did the callus from which they originated (Konieczny et al., 2008). Peroxidase activity was analysed in embryo axes of *Lupinus luteus* L. cv. *Polo.* It has been established that peroxidases may be some of the elements of the defense system that are stimulated by sucrose in yellow lupine embryo axes in response to infection caused by *F. oxysporum* (Morkunas, Vednarski, 2008).

Comprehensive investigations of Px and PPO isoenzymes in *Ribes* plants (*Eucoreosma* section) were carried out at flower and gamete initiation stages (Shikshnianiene et al., 2000). During differentiation of flower organs, the amount of Px isoforms (Rf 0.34–0.48 and 0.57–0.75) was found to increase in buds of all currant species. During gamete initiation, the number of isoforms significantly diminishes; however, specific, large-molecular isoforms (Rf 0.21–0.23) of this enzyme arise. Changes of isoenzymes Px and PPO in *Eucoreosma* section currant species at the beginning of new plant

generation formation (at zygote formation during embryogenesis) have not been investigated.

The aim of this work was to determine the dynamics of Px and PPO polymorphism in *Eucoreosma* section currant species during embryogenesis.

MATERIALS AND METHODS

At the Lithuanian Institute of Horticulture (LIH), in the period between 1997 and 2000, *Eucoreosma* section currants *Ribes nigrum* L. (cv. 'Titania', hybrid 'Lees prolific' × 'Minai Shmyriov') and wild forms of *R. pauciflorum* Turcz., *R. dikuscha* Fisch., *R. americanum* Mill. species were investigated. Plants were grown for trials in the collection plantation at a distance of 3 × 1 m. Five plants of each species were grown.

Castrated flowers of cv.'Lee's prolific' from the central part of inflorescence were used for investigating berry and seed growth and embryo development, while other flowers were removed. After two days, when stigmas were fully grown, the flowers were artificially pollinated with 'Minai Shmyriov' pollen in the first half of the day. Berries for investigation were collected from the 23rd till the 78th day after pollination every 2–3 days. During each analysis, the weight of five berries and the length of 20 seeds and 20 embryos were determined. In a similar way, embryo development of other *Ribes* species was investigated.

Peroxidase (Px) and polyphenoloxidase (PPO) isoenzymes were investigated in maternal plant leaves closest to inflorescences at organogenesis stages IX–XII. Plant organogenesis stages were established according to the F. Kuperman et al. methodology (1982). Samples were collected in the first half of the day. To establish isoenzymes, leaves (0.25 g), with addition of 2 ml Tris / Glyc buffer, 0.03 g ascorbic and 0.06 g glutaminic acid, were triturated in a pestle kept on ice. For establishing Px isoenzymes, 0.2 g of polyvinylpyrrolidone was added. The prepared samples were centrifuged (6500 × g) for 5 min and stained with 0.1 mg bromphenolblue.

Polyacrylamid gel (PAAG) was prepared according to Davis' (1964) methodology. 20 µl of solution was positioned on gel. Electrophoresis was carried out in a vertical gel apparatus (PROTEAN II XI Cell,) isoforms in the electric field descending from cathode (–) to anode (+). Until the dye reached the operational gel 20 mA current, 120 V voltage was applied, followed by 40 mA and 220 V. Electrophoregrams were developed according to Jaaska's (1972) methodology. At each organogenesis stage, each year (for 3 years) 5 (stage IX) to 15 (stages X–XII) biological Px and PPO analyses were carried out. In the obtained electrophoregrams, electrophoretic protein movement (Rf) of each line was measured and a standard quadratic deviation was calculated.

RESULTS

Three main periods were identified in currant berry growth. During the first period, berry weight and seed length were measured. The embryo was at the globular stage, and cotyledon differentiation was in progress. Seed linear parameters reached the maximum length (Fig. 1). The period coincided with organogenesis stage X of the maternal plant.

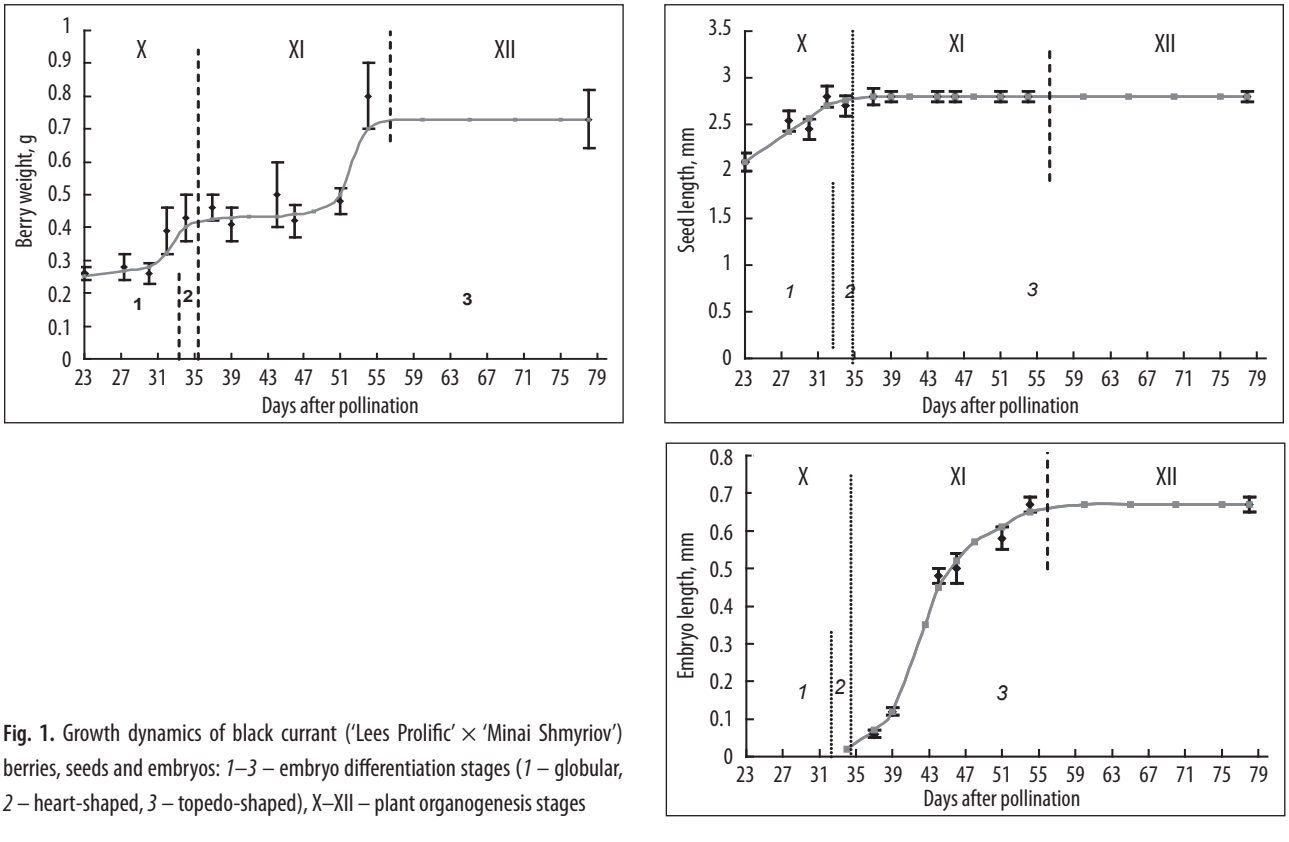

Fig. 1. Growth dynamics of black currant ('Lees Prolific' × 'Minai Shmyriov') berries, seeds and embryos: *1–3* – embryo differentiation stages (*1* – globular, *2* – heart-shaped, *3* – topedo-shaped), X–XII – plant organogenesis stages

During the second period, berry weight did not change. The embryo was growing intensively. In a short period it changed from cordate to torpedo and reached the maximum size. This period of berry, seed and embryo development coincided with organogenesis stage XI of the maternal plant.

During the third period, berry weight, seed and embryo size did not change. The period coincided with organogenesis stage XII of the maternal plant.

Thus, embryo differentiation started at organogenesis stage X of the maternal plant, while an intensive embryo growth was observed at organogenesis stage XI.

The regularities of currant embryo growth and differentiation of different *Eucoreosma* section species are shown in Fig. 2. The differentiation period of *R. nigrum* 'Titania' embryos lasted 12 days. Embryo differentiation started on the 35th day after pollination and terminated on the 46th day (Fig. 2). Embryo growth in the other currant species lasted 16–18 days. The dates of the beginning of intensive embryo growth varied. Fruits of *R. americanum* Mill. species ripened much later; also, embryo growth started only on the 48th day after pollination, and embryo differentiation lasted 18 days. The biggest embryos were found in *R. pauciflorum* Turcz and the smallest in *R. dikuscha* Fisch.

The embryo growth and differentiation of various the genus *Ribes* L. species were similar. The embryo grew most intensively when the maternal plant was at organogenesis stage XI.

The Px and PPO isoenzymes were investigated in leaves closest to inflorescences at organogenesis stages IX–XII. At stage IX (flowering and fertilization), enzymes were investigated with a view to compare the results with those obtained at organogenesis stages X–XII in which zygote formation and embryogenesis proceed.

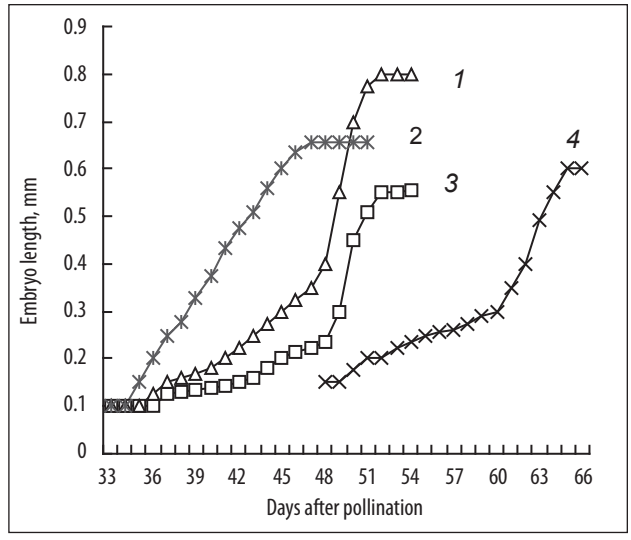

Fig. 2. Dynamics of embryo growth of different currant species: *1 – Ribes pauciflorum, 2 – R. nigrum* 'Titania', *3 – R. dikuscha, 4 – R. americanum*

In all study species, the highest number of Px isoforms was found in leaves of the maternal plant at organogenesis stage X. The embryo at that time was at the globular and heart-shaped stage. At organogenesis stages IX and XII, macromolecular (Rf 0.21–0.23) isoforms characteristic of plants during gametogenesis (Shikshnianiene et al., 2000) were not detected. Isoforms (Rf 0.54–0.56) were detected at all the organogenesis stages studied. The pattern of various Px isoforms in electrophoregrams was of different intensity. Of all species, *R. americanum* electrophoregrams were most abundant in Px isoenzymes (Table 1).

Table 1. **Peroxidase isoforms (Px) in leaves of different currant species at organogenesis stages IX–XII**

Organogenesis stage	Species			
	R. pauciflorum	*R. dikuscha*	*R. americanum*	*R. nigrum* 'Titania'
IX	0.22 ± 0.010	0.23 ± 0.006	0.23 ± 0.007	0.23 ± 0.000
		0.43 ± 0.012	0.41 ± 0.006	
			0.44 ± 0.007	
			0.48 ± 0.014	
	0.51 ± 0.007		0.52 ± 0.007	0.51 ± 0.000
		0.53 ± 0.006	0.54 ± 0.006	0.54 ± 0.006
	0.56 ± 0.013	0.57 ± 0.007		
X	0.23 ± 0.007	0.22 ± 0.010	0.22 ± 0.000	0.21 ± 0.006
			0.38 ± 0.006	
		0.43 ± 0.017	0.42 ± 0.012	
	0.46 ± 0.007		0.46 ± 0.007	
	0.49 ± 0.007	0.49 ± 0.007	0.48 ± 0.007	0.49 ± 0.000
	0.51 ± 0.000	0.52 ± 0.006	0.50 ± 0.000	0.51 ± 0.000
			0.55 ± 0.006	0.54 ± 0.006
	0.56 ± 0.006	0.56 ± 0.007		0.56 ± 0.006

Table 1 (continued)

Organo-genesis stage	Species			
	R. pauciflorum	*R. dikuscha*	*R. americanum*	*R. nigrum* 'Titania'
			0.43 ± 0.007	
	0.45 ± 0.014	0.45 ± 0.000	0.46 ± 0.000	
XI	0.52 ± 0.000		0.52 ± 0.007	0.52 ± 0.006
	0.55 ± 0.007	0.55± 0.007	0.54 ± 0.014	0.55 ± 0.007
			0.57 ± 0.007	
		0.44 ± 0.010	0.43 ± 0.007	
	0.49 ± 0.000		0.48 ± 0.000	
XII		0.52 ± 0.007	0.51 ± 0.000	0.51 ± 0.007
	0.54 ± 0.007	0.55 ± 0.008	0.55 ± 0.000	0.53 ± 0.014
		0.58 ± 0.014		

Table 2. **Polyphenoloxidase (PPO) isoforms in leaves of different currant species at organogenesis stages IX–XII**

Organo-genesis stage	Species			
	R. pauciflorum	*R. dikuscha*	*R. americanum*	*R. nigrum* 'Titania'
		0.43 ± 0.006	0.41 ± 0.000	
IX	0.50 ± 0.006	0.51 ± 0.000		0.51 ± 0.012
	0.53 ± 0.006	0.54 ± 0.000	0.53 ± 0.012	0.54 ± 0.007
			0.69 ± 0.000	
		0.43 ± 0.006	0.42 ± 0.014	
		0.46 ± 0.007	0.46 ± 0.000	
X	0.52 ± 0.007			0.52 ± 0.015
		0.53 ± 0.006	0.54 ± 0.014	0.53 ± 0.014
		0.44 ± 0.010	0.44 ± 0.021	
			0.48 ± 0.014	
XI	0.52 ± 0.000	0.52 ± 0.006		0.51 ± 0.010
	0.54 ± 0.014	0.55 ± 0.007	0.55 ± 0.006	0.54 ± 0.014
			0.42 ± 0.014	
		0.44 ± 0.012		
XII	0.51 ± 0.012	0.51 ± 0.000	0.51 ± 0.014	0.50 ± 0.007
		0.54 ± 0.010	0.54 ± 0.000	0.53 ± 0.000

The number of PPO isoforms varied insignificantly during embryogenesis. In PPO electrophoregrams, the isoforms with Rf 0.51–0.56 prevailed at all organogenesis stages (IX–XII). In PPO electrophoregrams of *R. dikuscha* and *R. pauciflorum* isoforms of smaller molecular mass, Rf 0.41–0.46 characteristic of these species were also found (Table 2).

DISCUSSION

Analysis of embryo growth and differentiation regularities as well as Px and PPO polymorphism revealed that the main embryo differentiation processes proceeded at development stages X–XI of the maternal plant (Figs. 1, 2).

At organogenesis stage X, the embryo of the genus *Ribes* species was at globular and cordate development stages. At this stage, the differentiation of the main embryo organs – buds, rootlets and cotyledons – starts. This is indicated by embryo transition into heart-shaped stage. In plants of the currant species studied, the further differentiation of embryo organs and an intensive growth took place at organogenesis stage XI. The embryo reached a torpedo stage.

The organogenesis of the genus *Ribes* species, contrary to that of other plants (Куперман и др., 1982), is characterized by the renewal of berry weight increase at stage XII (Fig. 1). This can be explained by hydrolysis of polysaccharides and other natural polymers present in berry pulp. Hydrolysis

processes are most active in seeds (aril). At that moment, the content of colloids detaining water increases. Chlorophylls in berries split, and synthesis of anthocians occurs. At organogenesis stage XII, embryo linear parameters actually do not change, implying that the differentiation of embryo organs in the maternal plant stops at organogenesis stage XI. It has been shown (Чеботарь и др., 1987) that embryo differentiation of the genus *Ribes* plants stops completely during seed stratification.

The high content of Px isoforms at organogenesis stage X is related to embryo differentiation processes and demonstrates participation of individual Px isoforms in the metabolism of phenolic compounds and growth regulators.

At organogenesis stages XI and XII, there prevailed Px isoforms whose Rf was 0.51–0.56 (Table 1). These isoforms are characteristic of all study plants at all organogenesis stages and participate in common vital processes of organisms (Shikshnianiene, 2002).

Electrophoregrams of Px isoenzymes of *R. americanum* plants during embriogenesis differed most significantly from those of other currant species plants. Plants of this species had a higher number of Px isoforms (Table 1). This can be explained by the genetic remoteness of *R. americanum* from other *Eucoreosma* section species. Meanwhile, the high number of Px isoforms was related to embryo differentiation processes.

The obtained data show that the rise of new isoforms is related to differentiation processes. The growth processes in berries, seeds and embryos are not related to the rise of new Px isoforms.

PPO isoforms changed little during embryogenesis (Table 2). It is supposed that PPO activity in oxidation–reduction reactions changes inversely to Px activity (Голышкина, 2005). No such regularities in *Ribes* species were observed during embryogenesis, though they were observed at other stages of ontogenesis (Shikshnianiene, 2002). The obtained results are insufficient to determine the role of PPO in berry, seed and embryo development.

To sum it up, the increase of the number of new Px isoforms in the genus *Ribes Eucoreosma* section currants at plant organogenesis stage X is directly related to embryo differentiation. No relationship was determined between the spectrum of PPO isoforms and embryo differentiation.

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PEROKSIDAZĖS IR POLIFENOLOKSIDAZĖS POLIMORFIZMAS EMBRIOGENEZĖS EIGOJE *EUCOREOSMA* SEKCIJOS SERBENTUOSE

S a n t r a u k a

1997–2008 m. tirtas keturių serbentų rūšių (gentis *Ribes*, sekcija *Eucoreosma*) peroksidazės ir polifenoloksidazės polimorfizmas embriogenezės eigoje. X organogenezės etape (pagal F. Kuperman) visų rūšių tirti serbentai intensyviai augina uogas ir sėklas. Šiame etape rutuliškas gemalas tampa širdišku, gemalo organai diferencijuojasi, motininio augalo lapuose nustatytas didžiausias peroksidazės polimorfizmas. XI organogenezės etape visų rūšių serbentuose, kai gemalas auga labai intensyviai, sumažėja peroksidazės izoformų kiekis. Genties *Ribes* sekcijos *Eucoreosma* serbentų naujų Px izoformų skaičiaus padidėjimas X augalo organogenezės etape yra tiesiogiai susijęs su gemalo diferenciacija. PPO izoformų spektro ir gemalo diferenciacijos ryšio neaptikta.

Raktažodžiai: embriogenezė, organogenezė, serbentų rūšys, izoenzimai, peroksidazė, polifenoloksidazė