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Effect of phytohormones and stratification on morphogenesis of *Paeonia lactiflora* Pall. isolated embryos

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With the aim to overcome embryo inviability, seed dormancy and related problems, the effect of 6-benzylaminopurine and 3-indolylacetic acid on *Paeonia lactiflora* Pall. cv. 'Virgilijus' isolated embryos was investigated. Torpedo-shaped embryos were isolated 60–90 days after pollination.

Exogenous growth regulators inhibited the development of roots, but stimulated the growth of leaves. Microplants that grew from unstratified embryos showed a stronger expression of this feature. Leaves of microplants from the stratified embryos grew more intensively in the nutrition media with both growth regulators.

Stratification significantly stimulated the growth of roots on the White medium, but had no influence on the growth of roots in MS medium. Cytokinin and stratification on MS nutrition medium is required for the normal development of peony plants from the isolated embryos. MS nutrition medium, enriched with major investigated concentrations of exogenous BAP and IAA, may partially replace stratification.

Key words: *Paeonia lactiflora* Pall., isolated embryos, BAP, IAA, stratification

INTRODUCTION

Morphological determination of seed dormancy is a peculiarity of the genus *Paeonia*, as different embryo components have different factors defining its dormancy. It is related with the somatic *Paeonia* sp. embryo nature [1, 2]. Asynchronic development of different embryo parts and prolonged germination of seeds are characteristic of this genus. Complex stratification is required to induce the germination of seeds. Embryo culture can help to overcome embryo inviability, seed dormancy and related problems. The first articles on the culture of *Paeonia* sp. embryos *in vitro* were published in the 1960s [3, 4]. The morphogenetic potencies of the genus *Paeonia embryo in vitro* were investigated by Batygina & Butenko [5]. Research on isolated embryo culture of *P. anomala* L., *P. mlokosewitschii* Lomakin, *P. tenuifolia* L., *P. ostii* T. Hong, *P. suffruticosa* Andr. was made [6–9]. The effect of exogenous gibberellic acid, 2,4D, BAP, NAA, abscisic acid and temperature on seeds and isolated embryos of *P. lactiflotra* Pall. sp. was investigated [10–12]. It was shown that isolated embryos of *Paeonia* L. species, depending on environmental conditions, can change their morphogenetic way [5].

The aim of the study was to investigate *in vitro* the effect of exogenous BAP and IAA and stratification on the development of the isolated embryos of *Paeonia lactiflora* Pall. cv. 'Virgilijus' developed in Lithuania.

MATERIALS AND METHODS

The growth and development of isolated *P. lactiflora* Pall. embryos of cv. 'Virgilijus' ('Pierre Reignoux' × 'Auguste Dessert') were studied in 2003–2005. The dynamic linear parameters of the embryos *in vivo* were estimated every five days from 45 to 90 days after pollination.

Seeds were excised from follicles, sterilized in 96% ethanol (2 min) and 0.1% calomel (10 min), and rinsed with sterile distilled water twice.

Torpedo-shaped embryos isolated in aseptic conditions were placed on the White nutrition medium [13] with 0.2 mgl^{-1} kinetin or 0.25 and 0.5 mg l^{-1} 6-benzylaminopurine (BAP) and on Murashige–Skoog (MS) [14] medium with 0.25 and 0.5 mgl^{-1} BAP. Half of the planted embryos were stratified at 4 °C for two months and then kept for one month in the cultivation room at 21–23 °C. A 16-h photoperiod and cool white fluorescent light of 50 µmol $m^{-2}s^{-1}$ PPFD were used. The second half of the embryos were cultivated for three months in the cultivation room. Germinated embryos were transfered to the MS nutrition medium enriched with BAP (0–2 mg l^{-1}) and 3-indolylacetic acid (IAA) (0–1.4 mgl^{-1}) growth regulators.

Thirty embryos were planted in each variant. Micro explants were measured before subcultivation every month.

Statistical analysis was performed with ANOVA software.

RESULTS AND DISCUSSION

Asynchronous development of embryos in seeds of the same follicle was observed. Embryos had a globular or heart shape 45 days after pollination. 30% of the embryos acquired a torpedo shape 50 days after pollination. Differentiation of embryos *in vivo* proceeded at days 45–60 after pollination. 90% of embryos had reached the torpedo shape by that time, but their length varied, depending on the somatic origin of the embryo. It was shown that usually only one of one-seed somatic embryoids emerged from the proembryo structure 23–30 days after pollination developed completely [2].

Torpedo-shape embryos 1.8 mm in length were used in our study. Embryos of this shape did not express the morphologic shoot apex but were already autotrophic [15].

The opening of cotyledons and primary root was observed on the White medium enriched with 0.2 mg l^{-1} kinetin, at a temperature of 21–23 °C in the first month after planting (Figure). The root reached 19.1 mm in length after 90 days of cultivation. Cotyledons didn't grow and remained etiolated. 98% of the isolated peony embryos germinated in three months.

The embryos became swollen, slightly increased; their cotyledons opened during stratification (Figure). After stratification for two months at 4 °C, the isolated embryos were transferred to a 21–23 °C cultivation room, roots started to grow more intensively, and the length of the stratified and the unstratified embryos became analogous in one month. The stratified embryos within one month developed almost the same as the unstratified embryos within three months. The microplants had

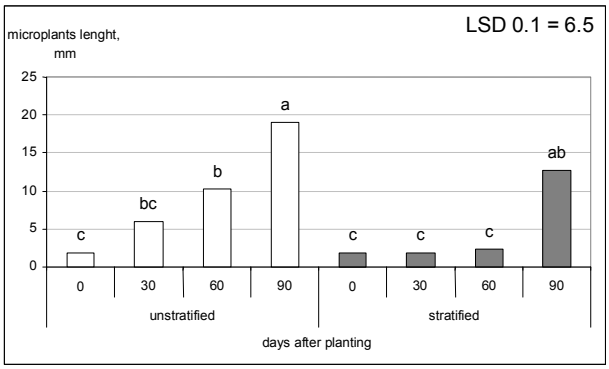

Figure. Impact of stratification on microplant length. White nutrition medium with 0.2 mg l^{-1} kinetin

etiolated cotyledons and a well developed primary root. That confirms data of different physiological mechanisms of epycotyl and hypocotyl stratification [1].

The microplants were transferred to MS nutrition medium with addition of BAP and IAA in different proportions (1.4–2.5) (Table 1). The growth of roots was weaker under the influence of exogenous BAP and IAA. These tendencies were more pronounced for microplants developed from unstratified embryos. Root necrosis was observed in microplants that grew on medium with BAP and IAA (1.6–2.5). Callus was formed on the roots of microplants developed from stratified embryos grown on the nutrition media enriched with growth regulators.

Leaves on the microplants obtained from the stratified embryos began to grow earlier. Only microplants of the stratified embryos grew leaves on MS medium without hormones and with addition of BAP, implying that changes of endogenous auxin and its physiologically active forms in the isolated embryos had occurred during the period of stratification, while the program of leaf growth starts functioning in a nutrition medium with BAP addition. Therefore, the stratified embryos could develop in the MS environment without phytohormones.

Table 1. **Effect of growth regulators on peony microplant leaf development during three subcultivations**

Nutrition medium MS with different BAP and IAA concentration	BAP and IAA ratios	% of microplants with leaves					
		After first subcultivation		After second subcultivation		After third subcultivation	
		Unstratified	Stratified	Unstartified	Stratified	Unstartified	Stratified
MS phytohormones free		0	0	0	40.0 ^{c}	0	0
MS BAP 0.25 mgl^{-1}; IAA 0 mgl^{-1}		0	33.3 ^{c}	0	50.0 ^{c}	0	100 ^{a}
MS BAP 0,5 mgl^{-1}; IAA 0,2 mgl^{-1}	2.5	0	33.3 ^{c}	66.7 ^{b}	50.0 ^{c}	66.7 ^{b}	80.0 ^{b}
MS BAP 0.75 mgl^{-1}; IAA 0.4 mgl^{-1}	1.9	0	50.0 ^{ab}	50.0 ^{c}	50.0 ^{c}	66.7 ^{b}	63.3 ^{c}
MS BAP 1.0 mgl^{-1}; IAA 0.6 mgl^{-1}	1.7	0	60.0 ^{a}	100 ^{a}	86.7 ^{a}	100 ^{a}	86.7 ^{b}
MS BAP 1.25 mgl^{-1}; IAA 0,8 mgl^{-1}	1.6	0	63.3 ^{a}	100 ^{a}	73.3 ^{b}	100 ^{a}	100 ^{a}
MS BAP 2.0 mgl^{-1}; IAA 1.4 mgl^{-1}	1.4	66.7 ^{a}	43.3 ^{bc}	100 ^{a}	66.7 ^{b}	100 ^{a}	100 ^{a}
LSD 0.1		4.6	11.6	6.7	12,1	6.5	7.0

Means in a column marked with the same letter do not differ significantly at P = 0.01 according to Duncan's multiple range test.

Table 2. **Quality of microplants after cultivation on different nutrition media for five months**

Nutrition media	% microplants with roots		Mean ± standard error of root length, mm		% microplants with leaves		Mean ± standard error leaf height, mm	
	Unstratified	Stratified	Unstratified	Stratified	Unstratified	Stratified	Unstratified	Stratified
White phytohormon free	53.3 [b]	100 [a]	11.67 ± 4.4	21.09 ± 3.1	0	0	0	0
White BAP 0.25 mg l^{-1}	36.7[bc]	70.0 [b]	5.41 ± 1.4	5.13 ± 0.8	0	36.7 [c**]	0	3.00 ± 1.0
White BAP 0.5 mg l^{-1}	26.7 [c]	66.7 [b]	5.53 ± 1.1	2.75 ± 0.4	0	16.7 [d**]	0	3.29 ± 0.5
MS phytohormon free	100 [a]	100 [a]	42.00 ± 5.1	47.2 ± 3.7	0	56.7 [b]	0	30.50 ± 6.0
MS BAP 0.25 mg l^{-1}	96.7 [a]	100 [a]	34.32 ± 4.5	32.5 ± 2.9	0	56.7 [b]	0	18.38 ± 4.3
MS BAP 0.5 mg l^{-1}	93.3 [a]	100 [a]	32.82 ± 2.3	26.67 ± 3.5	8 [a*]	86.7 [a]	3.6 ± 1.0	10.89 ± 2.0
LSD 0.1	10.1	7.5			2.9	11.3		

Means in a column marked with the same letter do not differ significantly at P = 0.01 according to Duncan's multiple range test.

* albino plants.

** well developed apical bud.

During the first subcultivation, plants from unstratified embryos grew leaves only on MS medium with the highest concentration of the phytohormones. Stratified plants developed leaves in all variants enriched with phytohormones. During the second subcultivation, microplants obtained from unstratified embryos formed leaves only when a combination of exogenous auxin and cytokinin had been used (Table 1). When the BAP and IAA ratio was reduced and the total concentration of hormones increased, the quantity of microplants with leaves increased. Leaves on microplants from stratified embryos developed in all variants, including the medium without hormones. The best results were obtained using medium concentrations of BAP (1.0 mg l^{-1}) and IAA (0.6 mg l^{-1}) in the nutrition medium. The number of microplants with leaves derived from unstratified embryos didn't change after the third subcultivation. 100% of microplants with leaves were received only in a variant with BAP, so it is possible to assume that during stratification the embryos had accumulated enough endogenous auxin, but exogenous cytokinin was still necessary for the normal development of microplants. All microplants developed leaves also in variants with the highest concentrations of exogenous BAP and IAA used in our study.

Development of isolated embryos *in vitro* depended on the mineral composition of the nutrition medium (Table 2). The highest number of microplants with roots in the White medium (53.3%) was obtained from unstratified embryos without BAP. The percentage of rooted microplants decreased when the concentration of BAP was increased. The development of stratified embryos was parallel. Roots of microplants from the stratified embryos were twice longer than from unstratified on the White medium without hormones.

Embryos formed roots twice more often in the MS medium. Addition of cytokinin to both nutrition media decreased root length. The stratification had no influence on the length of roots in the MS nutrition medium.

Microplants from unstratified embryos had etiolated cotyledons and not clearly developed apical buds in both media without BAP. Cotyledons became green in the nutrition medium enriched with cytokinin.

Microplants derived from stratified embryos formed a well-developed apical bud or little green leaves. Addition of BAP increased the number of microplants with leaves on MS medium, but decreased their height. After stratification, the growth of all parts of embryos became more intensive. Unlike other researchers [3, 5, 6], without stratification we did not obtain well-developed plants on the nutrition medium without addition of hormones. Photosynthesis in cotyledons and the development of leaves were abnormal without stratification.

According to our research, stratification significantly stimulates root growth in the White medium, however, it has no influence on rooting in MS nutrition medium. Growing peony microplants from isolated embryos requires the MS nutrition medium enriched with cytokinin and a two-month stratification. Supplement of the major strength exogenous BAP and IAA growth regulators to a nutrition medium may partially replace modification of exogenous hormones in isolated embryos during stratification period.

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FITOHORMONŲ IR STRATIFIKACIJOS POVEIKIS *PAEONIA LACTIFLORA* PALL. IZOLIUOTŲ GEMALŲ MORFOGENEZEI

S a n t r a u k a

Siekiant išvengti veiksnių, apsunkinančių ir sulėtinančių gemalo dygimą, buvo tirtas 6-benzilaminopurino ir 3-indolilacto rūgšties poveikis *Paeonia lactiflora* Pall. ‘Virgilijus’ izoliuotų gemalų raidai. Gemalai izoliuoti torpedos stadijoje 60–90 dieną po žiedų apdulkinimo.

Egzogeniniai augimo reguliatoriai slopino šaknų, bet skatino lapų augimą. Ši tendencija buvo stipriau išreikšta mikroaugaluose, išaugusiuose iš nestratifikuotų gemalų. Iš stratifikuotų gemalų išaugę mikroaugalai intensyviau augino lapus terpėse su abiem augimo reguliatoriais.

Nustatyta, kad stratifikacija patikimai stimuliavo šaknų augimą White terpėje, tačiau nepaveikė šaknų augimo MS terpėje. Auginant bijūnų mikroaugalus iš izoliuotų gemalų būtina MS maitinamoji terpė, praturtinta citokininu, ir jų stratifikacija. Stratifikacijos metu vykstančius endogeninius fitohormonų pakitimus iš dalies gali kompensuoti didesnis egzogeninių BAP ir IAA priedas maitinamojoje terpėje.