In vitro culture and evaluation peculiarities of different fruit and berry plant genotypes in various periods of ontogenesis

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Development interdependence in fruits, seeds and embryos, the commencement of embryo autonomy character and the optimal period of embryo rescue were determined. The suitability of Whites, Nitch & Nitch and MS media was assessed for culturing rescued embryos, and the composition of phytohormones in a medium was optimized. It has been shown that in the embryonic stage of apple development isolated cotyledons can be successfully employed for selection of genotypes with monogenic resistance and genetic investigations on apple scab resistance. Rather pronounced differences among the strawberry genotypes occurred after freezing *in vitro* at a temperature of –11 °C when, depending on the variety, 20 to 100% of plants survived. Optimal conditions for growth and rooting *in vitro* of some *Chaenomeles* and *Prunus* genotypes were established.

Key words: *Ribes, Fragaria, Malus, Prunus, Chaenomeles, in vitro*, rooting, scab resistance, cold hardiness, seedlings, screening

INTRODUCTION

In vitro methods are beneficial in three steps of plant breeding: 1) creation of variability, 2) evaluation and selection, and 3) maintainance and cloning of valuable genotypes [1]. The advantage of *in vitro* methods in breeding is supplemented by the possibility of obtaining plants from isolated, even immature, embryos or their parts and rescue interspecific hybrids whose embryos abort in later stages of development [2, 3]. In this way we can retain the genotypes that might be lost when using ordinary breeding methods. *In vitro* methods make it possible also to screen plants in early stages of their development. Many attempts to create accelerated breeding technologies failed to determine the conditions allowing objective differentiation of genotypes in artificial conditions [4, 5]. Proper conditions of culture initiation, growth, propagation and rooting should be established for some recalcitrant species [6, 7].

Our tasks were to find optimal conditions for hybrid embryo rescue, screening of scab (*Venturia inaequalis* (Cke) Wint) resistant apple and cold-hardy strawberry seedlings *in vitro*, also for the propagation and rooting of different genotypes of *Prunus* and *Chaenomeles* plants.

MATERIALS AND METHODS

The growing media used for investigations were White [8], Murashige & Skoog [9], Nitch & Nitch [10], supplemented with sucrose (20–30 g/l) various combinations of phytohormones: kinetin, 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), gibberellic acid A3 (GA_3), 2,4-dichlorphenoxyacetic acid (2,4D). Explants were grown in a growth chamber.

Rescued apple cotyledons were cultured for 4 days on Nitch & Nitch medium and then transferred to 0.006% benzimidazole water solution environment and infected with scab fungus. Scab development on isolated apple cotyledons was assessed on a 0–4 scale after a month [11].

After 30 days of acclimation at a temperature of 0 to +2 °C strawberry plants grown on MS medium with 3% sucrose and 1 mg/l BA were frozen *in vitro* for 12 h at –11 °C and thawed for 12 h at +15 °C. The number of viable and not injured plants was established after 30 days.

Experimental data were estimated by methods of disperse analysis.

RESULTS AND DISCUSSION

Embryo rescue *in vitro*. In Table 1 data are presented on the growth of isolated embryos of black currant in Whites medium supplemented with BAP, depending on their development stage. Embryo optimal development was assessed after rescue on 43 day following pollina-

Table 1. ***In vitro*** **growth of rescued black currant embryos depending on development stage**

Days after pollination	Embryo length, mm	Planted embryos	Developed embryos[x]		Mean plant length, mm^{-1}	Differentiated plants, %
			n	%		
34	0.10	30	6	20.0d	10.8 ± 2.14[y]	16.6
36	0.32 ± 0.16[z]	29	16	55.2c	16.6 ± 2.56	50.0
41	0.35 ± 0.16	30	20	66.7c	19.0 ± 2.47	45.0
43	0.50 ± 0.15	28	28	100.0a	29.5 ± 2.00	78.6
45	0.46 ± 0.15	26	23	88.5ab	26.3 ± 3.31	82.6
48	0.47 ± 0.15	28	24	85.7b	27.8 ± 1.75	83.3
83	0.52 ± 0.15	30	19	63.3c	–	84.2

[x)] Means are significantly different at p 0.01 applying Duncan's multiple range test.
[z)] Mean ± SD for ten replications. [y)] Mean ± SE.

tion. By that time they had achieved their maximum size and full autonomy. The formation of the autonomy character in black currants coincides with the beginning of cotyledon differentiation. The differentiation of currant embryos takes place over a very short period (about two weeks), and the growing of isolated embryos from the distant hybrids that escape damage caused by genome incompatibility is possible during this period. Our investigations demonstrate that the growth of berry seeds and embryos in all the *Ribes* species studied follows the same regularities, which are characteristic also of grape and stone fruit development [12].

Rescued currant embryos rarely developed into the global stage. During the first month of cultivation they formed small undifferentiated plants. The percentage of developing embryos *in vitro* depended directly on their lifetime. After one month of cultivation, plants derived from later embryo rescue grew bigger, and a significantly greater number of them were differentiated as compared to early embryo rescue. The same tendencies can bee seen if strawberry and apple embryos are grown *in vitro*. Owing to the genetic variance of wide hybrids, the embryo development often differs from the norm that determines embryo survival. Applying the embryo rescue technology *in vitro*, several valuable interspecific *Ribes* hybrids were obtained.

Evaluation and selection *in vitro*. The possibility was studied to use isolated apple cotyledons for selection of scab *Venturia inaequalis* (Cke) Wint) resistant genotypes in an *in vitro* system. The highest number of infested cotyledons was found in seedling populations of the scab-susceptible cv. 'Noris' (70.3%), and the lowest in the immune cv. 'Priam' (7.6%) (Table 2). Both the 'Priam' and 'Liberty' apple trees are scab-immune. This trait is predetermined by the Vf gene. Research data show that scab expression on the cotyledons of these apple cultivars varied in *in vitro* conditions both in quantitative and qualitative aspects. The difference between these cultivars might be determined by the gene modifiers that affect the Vf gene. The scab pathogen affected some cotyledons by spots, some in diffuse way. The ratio of diffusely and spottily affected cotyledons varied within apple trees of different resistance. A diverse scab infestation character was observed on apple leaves under *in vivo* conditions as well [11].

Though the winterhardiness of strawberry depends on many factors, the most important is cold. After freezing at – 8 °C *in vitro*, most of plants (90%) survived, and after freezing at –12 °C more than 90% of plants died. The maximum differences among the strawberry genotypes occurred at temperatures of –9 to – 11 °C when, depending on the variety, 20 to 100% of

Table 2. ***Venturia inaequalis*** **expression on isolated apple cotyledons *in vitro* with diverse genetic control of resistance**

Origin of cotyledons	Infected cotyledons	Scab-affected cotyledons		Infestation character				Average
				Spots		Diffusive		
		Quantity	%[x]	Quantity	%[x]	Quantity	%[x]	
'Noris'	74	52	70.3a	12	23.1b	40	76.9a	1.36
'Antonovka'	76	36	47.4b	15	41.7a	21	58.3b	1.25
'Priam'	79	6	7.6d	1	16.7b	5	83.3	0.13
'Liberty'	74	22	29.7c	3	13.6b	19	86.4a	0.59
'Štaris'	86	23	26.7c	10	43.5a	13	56.5b	0.53

[x)] Means are significantly different at p 0.01 applying Duncan's multiple range test.

plants survived. By the survival rate of the plants strawberry varieties could be divided into three groups: 1) highly cold-resistant ('Melody' having the wild *Fragaria virginiana* in the progeny, 'Shchedraja'), 2) cold-resistant ('Venta', 'Dangė'), 3) cold-susceptible ('Holiday', 'Elsanta'), with the survival rates 100%, 80–83%, and 20–35%, respectively (Figure).

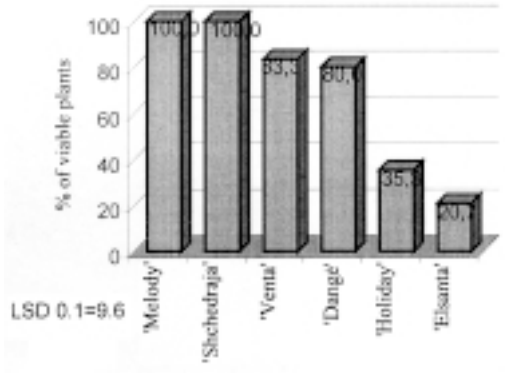

Figure. Survival rate of strawberry plants after freezing at –11 °C *in vitro*

ceeded in bypass supersensitivity reaction by keeping plants in the dark during the first 7 days. Therefore less phenolic compounds were released to the medium and more explants developed *in vitro*. After culture stabilization, the *in vitro* propagation rate was similar for all forms and reached 3–6 shoots per plant within four weeks. The most problematic step in the propagation of stone fruits *in vitro* is rooting. Plum varieties differed in their ability to form roots *in vitro*.

It was established that the rooting of the *Chaenomeles japonica* genotypes depended on medium pH (Table 3). The average rooting rate of quince hybrid explants was highest (43.1–42.8%) when the pH of the medium was less than 5.0. However, explants of hybrids N47 and N9365 rooted best at pH 5.5. It was also shown that higher amounts of BAP in proliferation media negatively affected the rooting of quince plants in peat substrate. If to keep the propagated plants for some time in the medium without cytokinins, they soon will use up the accumulated cytokinins and root well *ex vitro*. It is clear that there are no universal factors limiting the rhizogenesis of different forms.

Table 3. **Impact of medium pH on Japan quince rhyzogenesis *in vitro***

pH[1]	N43		N47		N18		N9365		Average %
	Number of shoots planted	Rooted %[x]	Number of shoots planted	Rooted %[x]	Number of shoots planted	Rooted %[x]	Number of shoots planted	Rooted %[x]	
6.2	34	0c	30	26.7d	56	19.6a	–	–	15.8
6.0	34	29.4b	30	28.1cd	30	0a	32	56.2ab	31.2
5.5	32	6.2c	30	56.7a	30	3.3a	30	66.7a	32.8
5.0	42	42.8a	42	54.8ab	28	3.6a	28	64.3a	42.1
4.5	36	47.2a	34	41.2bc	8	12.5a	34	47.0b	42.8

[1] Medium: Nitch ½ + IBA 0.75 mg/l.
[x)] Means are significantly different at p 0.01 applying Duncan's multiple range test.

It was established that the cold resistance of strawberry plants depended on hardening duration, light conditions during hardening, the size and structure of explants [13]. A correlation between cold resistance *in vitro* and winterhardiness *in situ* was rather high (r = = 0.78). A correlation between cold-hardiness *in vitro* and *in vivo* was even stronger and reached r = 0.83.

Peculiarities of *in vitro* growth and rooting of some recalcitrant genotypes. Apical meristems of six plum varieties were used for *in vitro* culture induction. It has been proved that culture induction depends on the physiological condition of the donor plant. The results were best when explants were isolated in spring, during active growth of the plant. Differences among the genotypes were manifested in the duration of culture stabilization, expression of internal infection (endophytes) and output of productive explants. We succeeded in bypass supersensitivity reaction by keeping plants in the dark during the first 7 days. Therefore less phenolic compounds were released to the medium and more explants developed *in vitro*. After culture stabilization, the *in vitro* propagation rate was similar for all forms and reached 3–6 shoots per plant within four weeks. The most problematic step in the propagation of stone fruits *in vitro* is rooting. Plum varieties differed in their ability to form roots *in vitro*.

Our experiments have shown that success in the application of *in vitro* methods in breeding and germplasm conservation depends on the genotype and development stage of the plant. Using the *in vitro* method it is possible to rescue valuable hybrids, select scab-resistant apple seedlings in the embryonic stage of development and cold-hardy strawberry seedlings 3 months after pollination and even to carry out early genetic investigations.

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SKIRTINGŲ GENOTIPŲ SODO AUGALŲ AUGINIMO IR TYRIMO *IN VITRO* YPATUMAI ĮVAIRIAIS ONTOGENEZĖS PERIODAIS

S a n t r a u k a

Įvertintas serbentų rūšių gemalo autonomiškumo formavimas priklausomai nuo vaisiaus, sėklos ir gemalo išsivystymo, nustatytas optimalus gemalų izoliacijos laikotarpis, parinktos maitinamosios augalų regeneracijos terpės. Parodyta, kad obelų sėklaskiltės gali būti panaudotos obelų genetiniams tyrimams ir sėjinukams su monogeniniu atsparumu rauplėms atrinkti. Nustatytos optimalios braškių genotipų diferencijavimo temperatūros pagal atsparumą šalčiui *in vitro*. Šaldant augalus –11°C temperatūroje išgyveno nuo 20 iki 100% augalų. Optimizuotos sąlygos *Prunus, Chaenomeles* genčių augalų atskirų formų *in vitro* kultūrai inicijuoti, dauginti ir įsišaknydinti.