EMBRYO RESCUE TECHNIQUES IN *Prunus* L. BREEDING

Natalia Kukharchyk and Manana Kastrickaya

Institute for Fruit Growing of National
Academy of Sciences of Belarus
Kovaleva Street 2, Samokhvalovichy, Minsk Region
223013 REPUBLIC OF BELARUS

(Received September 15, 2005/Accepted October 30, 2005)

A B S T R A C T

The greatest loss of a genetic material occurs during fruit drops in June. A large proportion of these viable germs can be raised in isolated conditions. In vitro embryo rescue techniques and inter-specific crosses in breeding programs in complex had good results. We have obtained many inter-specific hybrids of *Prunus* L. [*P. salicina* Lindl. (2n), *P. spinosa* L. (4n), *P. domestica* L. (6n)], F2 progeny of inter-specific hybrids, and hybrids of *Prunus avium* for further selection. Different methods of sterilizing fruit and embryo were studied, including $HgCl_2$, merthiolate and flaming with ethanol. Separate sterilization techniques are described for fruits with soft pits and fruits with hard pits. Embryos were cultured *in vitro* on Woody Plant Medium, Murashige and Skoog Medium, modified Murashige and Skoog Medium, and Prunus Medium with different additions: 2.0 to 3.0% sucrose; 0.35% agar; 0.25 to 1.0 mg/l GA3; 0.1 to 4.0 mg/l IBA; 0.01 to 0.02 mg/l ferulic acid; and 0.3 to 0.9 mg/l 6-BA. Techniques for breaking dormancy in embryos and for *in vitro* rooting of regenerants are also described. More than 25,000 embryos were cultivated, and more than 500 regenerants were planted to the selection orchards. Three perspective *P. avium* hybrids and twelve perspective *Prunus* inter-specific hybrids were obtained.

Keywords: embryo, *in vitro*, regeneration, dormancy, *Prunus* L.

INTRODUCTION

In fruit plant breeding, the main forms of incompatibility encountered are: fully or partially sterile male or female reproductive organs; self- or cross-incompatibility in the parental forms and post-zygotic incompatibility. In some crosses, post-zygotic barriers are expressed in the endosperm and

embryo abortion and fruit dropping occurs. The fact that the hybrid embryos abort does not mean that they are not vital. Hybrids can be raised by culturing isolated embryos (Emershad and Ramming, 1994; Misic et al., 1980; Ramming, 1986).

In stone fruit breeding programs, post-zygotic incompatibility makes it necessary to culture embryos with various degree of differentiation *in vitro.* We used embryo culture as an auxiliary method in the inter-specific hybridization of *Prunus* L. and *Prunus cerasus* to rescue genetically unbalanced hybrids.

The aim of this study was to investigate techniques for the in vitro culture of Prunus embryos.

MATERIAL AND METHODS

The studied was carried out at the Institute for Fruit Growing of the Belarusan National Academy of Sciences, near Minsk, Belarus. The vegetative growth season lasts 195 to 200 days. Mean July temperature is 17.8°C, and mean January temperature is -6.9°C. The experiments were carried out from 1990 to 2005.

Seeds and embryos of early ripening cultivars of *P. avium* and inter-specific *Prunus* hybrids were cultivated. The main early ripening cultivars of *P. avium* in Belarus are 'Gronkavaya', 'Narodnaya', 'Subarovskaya' and 'Gastinets'). Different sterilization methods were studied, including 0.1% HgCl2, 0.01% merthiolate, 3 to 4% physan, and flaming with ethanol. The initiation of *in vitro* culture was examined. Embryos were cultured *in vitro* on Woody Plant Medium, Murashige and Skoog Medium, modified Murashige and Skoog Medium, and Prunus Medium with different additions: 2.0 to 3.0 % sucrose; 0.35 % agar; 0.25 to 1.0 mg/l GA3; 0.1 to 4.0 mg/l IBA; 0.01 to 0.02 mg/l ferulic acid; and 0.3 to 0.9 mg/l 6-BA.

The stages of *Prunus L.* embryo development *in vitro* were designated as follows:

0	– embryo dies;
0.1	– cotyledons are open;
0.2 to 0.3	– cotyledons are open and become green;
0.5	– embryoids are formed;
1 to 3	– central shoot present;
4 to 5	– central shoot and root present.

In the growth chamber, *in vitro* cultures were kept dormant for 60 to 65 days in darkness at +3 to 5°C. During the active vegetative growth phase, cultures were kept at 23 to 25°C with a 16 h photoperiod with a light intensity of 3000 Lux.

RESULTS AND DISCUSSION

The basic elements of *in vitro* embryo rescue are sterilization, breaking dormancy, isolation period, nutrient medium, and *in vitro* rooting.

Different methods of sterilizing fruits and embryos were studied, including $HgCl_2$, merthiolate and flaming with ethanol. The best procedure for sterilizing immature fruits with soft stones was the following:

- fruits are entered *in vitro* on the day they are picked;
- washed for one to two hours under flowing water;
- placed in a single layer in a Petri dish;
- washed out with vigorous stirring with one or two drops of chlorine-containing washing-up liquid;
- washed under flowing water to completely remove the washing-up liquid;
- soaked for one minute in 96 % ethanol in a Petri dish under a laminar flow hood;
- set aflame and left to burn until the alcohol burns off.

For fruits with hard stones, there are two good sterilization procedures. The first involves sterilizing the stones:

- stones are isolated;
- washed for one to two hours under flowing water;
- placed in a single layer in a Petri dish;
- washed out with vigorous stirring with one or two drops of chlorine-containing washing-up liquid;
- washed under flowing water to completely remove the washing-up liquid;
- soaked for one or two minutes in 70 & ethanol;
- rinsed in sterile water;
- treated for three or four minutes with 0.1% $HgCl_2$;
- rinsed three times in sterile water; and
- broken open with a hammer on sterile napkins, after which the embryos are transferred to in vitro culture.

The second procedure involves sterilizing the embryos:

- stones are isolated;
- washed for one to two hours under flowing water;
- placed in a single layer in a Petri dish;
- washed out with vigorous stirring with one or two drops of chlorine-containing washing-up liquid;
- washed under flowing water to remove the washing-up liquid; and broken open with a hammer or in a vice.

After which:

- seeds are isolated;
- washed with 70% ethanol;
- rinsed in sterile water;
- treated for three or four minutes with 0.1% $HgCl_2$;
- rinsed three times in sterile water; and
- transferred to in vitro culture.

Different methods of breaking dormancy were evaluated, including cold treatment and the addition of chemical compounds to the media. Plants can be directly regenerated from immature *P. avium* fruits with either method, but the most effective procedure involved *in vitro* culture with a dormancy period. Embryos were kept at +3 to 5°C for two or three months, and then grown at 23 to 25°C. To successfully culture mature embryos, the dormancy period is indispensable. To successfully culture embryos resulting from crosses in which *P. domestica* was the female component, the dormancy period is indispensable even with immature embryos.

Table 1. *In vitro* development in sour cherry embryos of various stages of differentiation degrees (2002 to 2004)

♀	Developed embryos, % from planted in vitro						
	0	Development points					all
		0.1	0.2-0.3	0.5	1.5	>1.5	
Globule stage: < 1/3 differentiation							
NS	50.0	38.8	5.6	5.6	0	0	50.0
K 11/12	59.6	2.6	27.3	10.5	0	0	40.4
K11/21	52.5	25	22.5	10.0	12.5	0	47.5
Average	56.2	9.4	19.8	9.4	5.2	0	43.8
Heart-shaped stage: 1/3 to 2/3 differentiation							
NS	46.7	13.3	13.3	20.0	0	6.7	53.3
K 11/12	20.7	17.2	24.2	20.7	17.2	0	79.3
K11/21	36.4	0	9.1	54.5	0	0	63.6
Average	30.9	12.7	18.2	27.3	9.1	1.8	69.1
Torpedo stage: 3/4 differentiation							
NS	18.1	9.1	6.1	60.6	6.1	0	81.9
K 11/12	4.6	0	9.3	72.1	9.3	4.7	95.4
Average	10.6	3.9	7.9	67.1	7.9	2.6	89.4

Techniques for culturing germ tissue in the early stages of differentiation have not yet been completely worked out. In stone fruit species, the number of days from pollination until the time in vitro introduction is possible varies from genotype to genotype. We cultured embryos at the following stages:

globule stage (embryos less than one third their size at full differentiation, cotyledons not yet formed, germs not visible, embryos have a vague globular form, stones are soft), heart-shaped stage (embryos between one third and two thirds their size at full differentiation, germs dense, with well developed germinal buds and cotyledons), torpedo stage (embryos three quarters their size at full differentiation, stone dense). The more advanced stage at which embryos were cultured, the fewer dead and partially developed embryos were obtained after in vitro culture, and the more embryos with germinal buds and direct microplant formation were obtained (Tab. 1).

For *in vitro* initiation, we used MS, WPM and *Prunus* media. Inorganic elements in the medium did not have a significant effect on the *in vitro* development of isolated germs in cherries and plums. The effects of organic components in the medium are presented in Table 2. Embryogenesis was successful with 6-BA at either 0.5 or 1.0 mg/l. Little effect was noted with GA_3 at 0.25 to 1.0 mg/l, or with or IBA at 0.1 mg/l.

Table 2. Development of *Prunus* L. embryos on medium containing three different cytokinins

Medium		Percentage of developed embryos from in vitro culture						
Cytokinin	mg/l	0	development points					total
			0.1	0.2-0.3	0.5	1.5	>1.5	
6-BA	0.5	39.3	6.1	15.2	21.2	12.1	6.1	51.7
6-BA	1.0	30.3	3.0	6.1	39.3	18.3	3.0	78.7
Kinetin	0.5	34.2	7.9	23.7	34.2	0	0	65.8
Kinetin	1.0	32.5	22.5	20.2	22.5	2.3	0	67.5
Zeatin	0.5	40.5	7.1	16.7	28.6	7.1	0	59.5
Zeatin	1.0	32.5	2.4	9.8	51.2	4.1	0	67.5

The best results for rooting in *Prunus* L. hybrids were on medium containing 2 mg/l IBA, on which 76.9% of the regenerants rooted (Fig. 1). Rooting in *Prunus* L. hybrids was generally worse than in cherry cultivars and rootstocks. The exception was the medium containing 2.0 mg/l NAA, on which 75.0% of the regenerants rooted. However, medium containing 2.0 or 4.0 mg/l NAA stimulated excess callus formation, and the roots which formed were often not viable. After transplantation to substrate, regenerants which had been rooted on media containing IBA developed better than regenerants on media containing IAA or NAA. Rooting in *Prunus* L. hybrid regenerants was good on medium containing 0.01 to 0.02 mg/l ferulic acid and 0.5 to 1.0 mg/l IBA. In *Prunus L.,* the size of the regenerant at the time of rooting was very important. Well-developed regenerants formed more roots per plant than smaller regenerants. For successful *in vitro* rooting, only regenerants more than 2 cm long should be used.

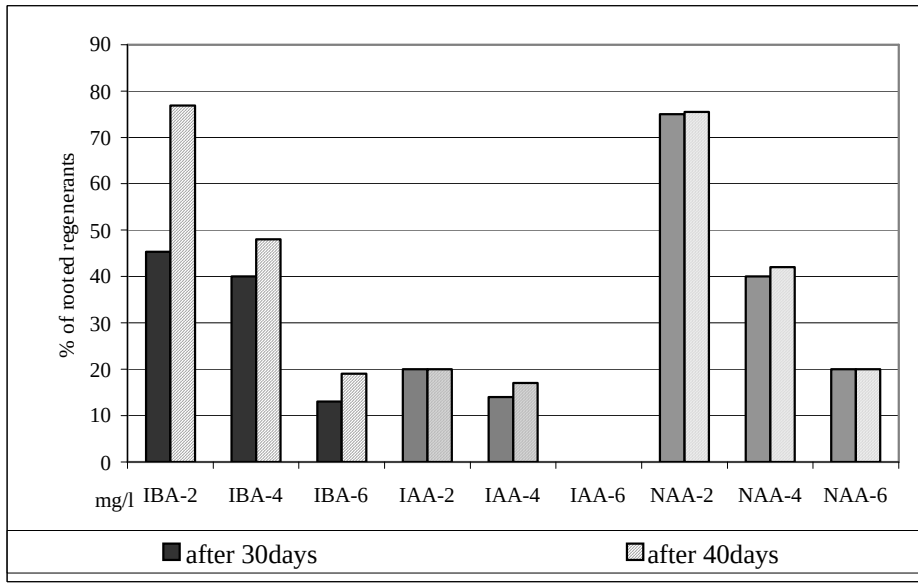

Figure 1. Rooting of cherry hybrid regenerants on media containing various auxins

More than 25,000 embryos were cultivated, and more than 500 regenerants were planted to the selection orchards. Three perspective *P. avium* hybrids and twelve perspective *Prunus* inter-specific hybrids were obtained [4n x 6n; 3n x 2n; F_2(*P. cerasifera* x *P. armeniaca*)].

REFERENCES

Emershad R.L., Ramming D.V. 1994. Effect of media on embryo enlargement, germination and plant development in early-ripening genotypes of Prunus growth in vitro. Tissue and Organ culture. PLANT CELL 37: 55-59.

Misic P.D., Vinterhaiter D.V., Todorovic R.R. 1980. Embryo culture or early cultivars of European plum (*Prunus domestica* L.). JUGOSLOVENSKO VOCARSTVO V 1-2, 51/52: 66-67.

Ramming D.V. 1986. Embryo rescue techniques in nectarine and peach breeding. N.Z. AGRONOMY SOCI. SPEC. PUBLIC. 5: 371-374.

TECHNIKA *embryo rescue* W HODOWLI ROŚLIN Z RODZAJU *Prunus*

Natalia Kukharchyk i Manana Kastrickaya

S T R E S Z C Z E N I E

Czerwcowe opadanie zawiązków owoców jest powoduje utratę dużej puli materiału hodowlanego wytworzonego po wiosennych zapyleniach. Stratom takim może zapobiec krzyżowanie międzygatunkowe i hodowla wyizolowanych zarodków w zoptymalizowanych warunkach in vitro (technika *embryo rescue*). W Instytucie Sadownictwa w Samochwałowiczach uzyskano dużą populację genotypów z krzyżowania oddalonego w obrębie rodzaju *Prunus* L. [*P. salicina* Lindl. (2n), *P. spinosa* L. (4n), *P. domestica* L. (6n)] i pokolenia F2 mieszańców międzygatunkowych i mieszańców *Prunus avium*, przeznaczonych do dalszej selekcji. Oceniono różne techniki sterylizacji oraz izolacji owoców i zarodków ($HgCl_2$ 0,1%, mertiolat 0,01%, etanol). Porównano rozwój zarodków w różnych pożywkach (klasyczna pożywka MS, zmodyfikowana pożywka MS zawierająca sacharozę (2,0-3,0 %), GA3 (0,25-1,0 mg/l), IBA (0,1-4,0 mg/l), FA (0,01-0,02 mg/l), 6-BA (0,3-0,9 mg/l) oraz opracowano system ukorzeniania zarodków w warunkach *in vitro*. Spośród 25.000 hodowanych zarodków ponad 500 adoptowano do warunków *ex vitro* i wysadzono do sadu. Wstępne obserwacje wskazują na uzyskanie 3 obiecujących mieszańców *P. avium* i 12 obiecujących mieszańców międzygatunkowych z rodzaju *Prunus*.

Słowa kluczowe: zarodek , *in vitro*, regeneracja, spoczynek, *Prunus* L.