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METHODS OF TISSUES CULTURE AND POLYPLOIDY in vitro DURING BIGENERIC CROSSING OF PIP ORCHARD CROPS

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S u m m a r y

With the use of biotechnological methods the hybrid plants were obtained from seeds, which arised during bigeneric crossing of pip orchard crops. The results of investigations were presented regarding the induction of polyploidy in distant hybrids of pip orchard crops. The amitotic action of acenaphthene and colchicin on a development of plants was studied in the conditions in vitro. The analysis of morphological changes of sprouts and stomatal mechanism of plant leaves after the treatment by polyploidy chemicals was made. The determination of chromosomes number in root growing points of plants, obtained on nutrient media with 0.001 and 0.01 % acenaphthene and colchicin, confirms the chance of ploidy in hybrids.

Keywords: intergeneric hybridization, seed friut, tissue culture in vitro, polyploidy induction, amitotic, cytological analysis.

Distant hybridization of plants is of interest for many famous breeders firstly owing to useful traits of wild ancestors of cultivated plants thereby involved in breeding work: immunity to diseases, outstanding drought resistance, winter hardiness, high contents of bioactive substances. All these properties can become quite important at environmental degradation and climate changes.

The greatest difficulty of interspecific and intergeneric hybridization is overcoming the reproductive isolation of different species. Hybrid embryos formed at distant crosses can die at any stages of development. Such crosses are low efficient and resulting single seeds hardly germinate in vivo. The techniques of tissue culture in vitro help to obtain viable intergeneric hybrids from seeds with underdeveloped embryos, overcome effects of negative factors on germination and reduce a dormancy period.

Embryo culture have been successfully used at interspecific and intergeneric hybridization of pip fruit crops. Cultivation of isolated embryos in sterile culture media improves their vitality and promotes germination of even degenerating embryos. Such investigation have been described in detail by M.S. Kastritskaya (1).

At the same time, many long-term works on obtaining hybrid genotypes and further using them in breeding practice are often unsuccessful since the sterility of distant hybrids resulting from numerous violations of macro- and microsporogenesis (2). The genome of distant hybrids is usually unbalanced, so amitotic polyploidization of these plants can be an effective way to involve this valuable genetic material in selection. Polyploidy effectively restores fertility in distant hybrids, it allows hybridization and introgression between species, even those completely isolated at the diploid level. Multiple increase in number of chromosomes changes manifestation of traits, nature of inheritance, plasticity of a lifeform and its adaptive capacity. Polyploidy is a genomic mutation fairly common in nature, which can provide more opportunities for survival of the organism under extreme environmental conditions (3). Since 1830ies and up to the present time, the main way to create polyploid plants is treating meristem tissues by amitotic agents (4-6). Colchicine (alkaloid of plant origin) and acenaphthene (product of processing of coal tar) are the substances most widely and successfully used for this purpose. Polyploidization of plant material in natural and laboratory conditions using seeds, growing shoots and rooted cuttings is widely used in hybridization of berry and fruit crops, but these techniques are almost not adapted for in vitro conditions and require serious modifications. Thus, there are still not developed the techniques for pre-screening of potential polyploids and cytological investigation of resulting forms considering their features.

The purpose of this research was to obtain distant hybrids of pip fruit plants through tissue culture and to develop an effective technique of polyploidization in vitro.

Technique. The object of study were in vitro cultured embryos obtained at distant crosses of pip fruit plants: cv Pamyati Yakovlevu (pear) × cv Discovery (apple), cv Pamyati Yakovlevu (pear) × form A1 (apple), cv Severyanka (pear) × cv Discovery (apple), form A1 (apple) × cv Severyanka (pear), cv Bogatyr' (apple) × cv Avgustovskaya Rosa (pear), cv Bogatyr' (apple) × cv Bere Zimnyaya Michurina (BZM) (pear), cv Antonivka (apple) × cv BZM (pear), cv Saffron Pippin (apple) × cv Rulgo (quince).

Seeds were extracted from ripe fruit, pre-stored in a storage for 2-3 months, carefully pilled and the embryos were isolated along with cotyledons. Explants were sterilized with 0,1% mercuric chloride solution for 1 min, washed three times with sterile distilled water and planted in test tubes on an artificial nutrient medium. The resulting hybrid seedlings were cut and placed on a medium for propagation (each hybrid genotype was given a number.) The intergeneric hybrid rowan-pear № 136 (source - the collection of I.V. Michurin All-Russia Research and Development Institute of Fruit Crops Genetics and Selection) was introduced into a sterile culture as vegetative buds and propagated. The hybrid embryos and buds were cultured on the mineral-based media Murashige-Skoog (MS) (7) and Quoirin-Lepoivre (QL) (8). Plant growth regulators were added to the media at the stages of introduction and micropropagation of obtained seedlings: 6-benzylaminopurine (6-BAP) - 1-2 mg/l; gibberellic acid (GA) – 0,2-1 mg/l; β-indole-3-butyric acid (IBA) – 0,1-0,2 mg/l or β-indoleacetic acid(IAA) – 0,2-0,5 mg/l. At the stage of rooting contents of macro salts and sucrose in the culture medium were reduced by half. Rooting was induced by IBA used as a component of a rooting medium (0,5-1 mg/l) or in aqueous solution (50 mg/l). In the latter case, shoot bases were kept in this solution for 16-20 h and then planted on the rooting medium without growth regulators. In both cases the control were shoots cultivated on the rooting medium without hormones.

The plants were cultured at air temperature 26 ± 2 °C, illumination 2000-2500 lux and 16 h/8 h photoperiod (day/night). There were also performed the experiments on regeneration of adventitious shoots from isolated segments of cotyledons of unsprouted embryos and callus. Before it each cotyledon was cut across the central vein into 3 parts, which then were placed on the MS-based regeneration medium. Morphogenesis was induced by 6-BAP (5 mg/l) combined with one of auxins (IBA, IAA or NAA). The ratio cytokinin : auxin was taken as 10:1 and 25:1. The explants were cultured on regeneration media in the dark at 24 °C for 12 weeks (three passages of 4 weeks). Shoots regenerated from the cotyledons were cut and included into the common cycle of microclonal propagation. The plants rooted in vitro were planted in soil in May and June to grow in small-sized film greenhouses with air-drop irrigation. The plants were grown under the film cover for 1-1,5 months, then the film was removed; in autumn the survived

plants were transplanted into a nursery garden (I.V. Michurin All-Russia Research and Development Institute of Fruit Crop Genetics and Selection).

The matrocinal hybrid rowan-pear № 136 and the form № 14/4 (pear cv Pamyati Yakovlevu × apple cv Discovery) were used as a material for working through the technique of polyploidization in vitro. Apexes of shoots obtained in vitro (0,3-0,5 cm) were placed on QL-based propagation media containing 6-BAP (2 mg/l), GA (0,5 mg/l), IBA (0,2 mg/l), vitamins according to MS prescription, hydrolyzed casein (250 mg/l), agent inducing polyploidy (colchicine or acenaphthene) at contents of 0,001% (10 mg/l) and 0,01% (100 mg/l). The control were shoots cultured on the medium of a similar mineral and hormonal composition without amitotic substances. Since acenaphthene is low-soluble in water (less than 0,002%), it was pre-mixed with castor oil. The explants were cultured on the media with amitotic agents for 6 weeks, then transplanted on the propagation medium of similar composition without amitotic agents. Each shoot obtained on amitotic media with acenaphthene and colchicine was assumed as an independent genetic individual. After reaching 1,5 cm length, the shoots were rooted. Pre-selection of potential polyploids was performed using cytological study of the stomatal apparatus on leaves of experimental plants. The size and number of stomata were evaluated on each shoot starting from the 1st bottom leaf up to the top leaf. There were taken the leaves of one passage cultured on one nutrient medium. After synchronizing divisions in the dark phase (12 h) and pre-treatment with paradichlorbenzene (3 h), the account of chromosomes in cells of root meristem areas was performed according to the method of L.A. Frolova et al. (9). The data for each genotype were summarized considering location (number) of leaf on a shoot.

Statistical processing of results was performed in Microsoft Excel.

Results. The seeds formed at distant crosses were introduced in the sterile culture in October and November without prior stratification, which greatly reduced the time of obtaining hybrid sprouts. The embryos were germinated on the artificial nutrient medium missing the dormancy period which usually takes several months at positive temperatures. As a result, in 2-3 months after harvesting fruit there were obtained germinating seeds in almost all samples cultured in vitro. To increase the total number of hybrid shoots and maintain the plantlets that hadn't developed shoots with roots on the introduction medium, all shoots were separated from cotyledons and planted on the propagation medium. At an optimum ratio of auxin and cytokinin in the regeneration media there were formed additional regenerated shoots from cotyledons of unsprouted embryos of following forms: form A1 (apple) × cv Severyanka (pear), cv Antonivka (apple) × cv BZM (pear), cv Bogatyr' (apple) × cv Avgustovskaya Rosa (pear), cv Bogatyr' (apple) × cv BZM (pear) (Fig. 1, A). The most effective combinations of growth regulators were 6-BAP (5 mg/l) + IBA (0,5 mg/l) and 6-BAP (5 mg/l) + NAA (0,2 mg/l). These variants provided regeneration of shoots from explants in 12,5 – 28,4% cases. Each explant (1/3 cotyledon) formed 1-4 regenerated shoots. Microclonal propagation of hybrid pip fruit crops was carried out by proliferation of axillary shoots according to a conventional model. By the end of the 2nd passage, on the modified nutrient medium QL there were observed viable conglomerates of 2-5 buds and shoots in most of the hybrid forms (Fig. 1, B).

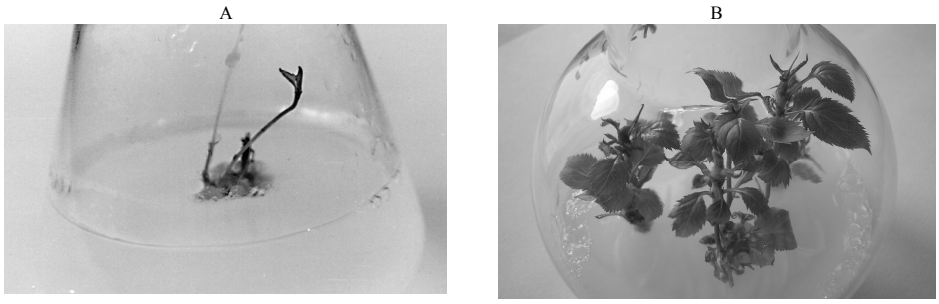

Fig. 1. Shoots developed in vitro by culture of interspecies hybrid cv Bogatyr' (apple) × cv Bere Zimnaya Michurina (pear): A — shoots regenerated from a callus on cotyledon segment; B – microclonal propagation of shoots.

Over the next three passages coefficient of shoots propagation gradually increased. Maximum values (up to 14 new shoots per passage) were observed in the forms cv Bogatyr' (apple) × cv Avgustovskaya Rosa (pear) and cv Pamyati Yakovlevu (pear) × form A1 (apple) in 6 months after introduction in culture. The studied hybrids required regular (at least once each 5-6 weeks) transplantations, otherwise there started a necrosis of shoot tips, especially in ones whose maternal form was pear.

1. Efficiency of culturing in vitro and adaptation to non-sterile conditions of growing pip fruit crops

Form	Crossed species	Proportion of explants in a culture, %		Coefficient of propagation during the 8th passage	Frequency, %	
		sterile, alive	formed shoots		rooting in vitro	adaptation in vivo
C1-8	Cv Bogatyr' (apple) × cv Avgustovskaya Rosa (pear)	68,2	10,4	7,8	60,8	23,5
K1-3	Cv Bogatyr' (apple) × cv Bere Zimnyaya Michurina (pear)	40,0	20,0	6,5	78,3	37,5
A1-4	Cv Pamyati Yakovlevu (pear) × form A1 (apple)	100	56,9	9,1	89,8	46,0
№ 14/4	Cv Pamyati Yakovlevu (pear) × cv Discovery (apple)	74,3	48,7	8,4	74,5	74,6
№ 136	Cv Titan (rowan) × mix of pear pollen	50,1	50,1	7,6	62,5	14,5

The obtained shoots demonstrated relatively low rooting capacity in vitro. Rooted shoots were obtained only through the use of auxin. In control variant cultured on media without growth regulators, rooting of shoots did not occur. The process of root formation in most variants took 1,5-2 months. The number of developed roots usually was small (1 to 4). The efficiency of adaptation to natural conditions was assessed by a share of survived plantlets from total number of planted individuals, which amounted to 14,5-74,6% (Table 1). All plants-survivors in non-sterile conditions successfully overwintered open-ground.

Cultivation of hybrids on nutrient media containing amitotic agents showed the lower effect of acenaphthene compared with colchicine. Besides, the intensity of propagation and growth of shoots were determined by genotypic characteristics of a crop. In the rowan-pear hybrid, development of shoots on the control medium and on media containing 10 mg/l amitotic was characterized by

similar parameters (Fig. 2). At 100 mg/l acenaphthene, propagation coefficient of this hybrid decreased to 4,8 vs 5,9 in control while the 2-fold reduce in average length of shoots and the proportion of shoots longer than 1,5 cm. The effect of colchicine was much more significant: its content 100 mg/l in 30-40% cases provided a complete or partial destruction of meristems leading to formation of callus instead of shoots, a sharp reduce in number of newly formed axillary shoots and their slow growth.

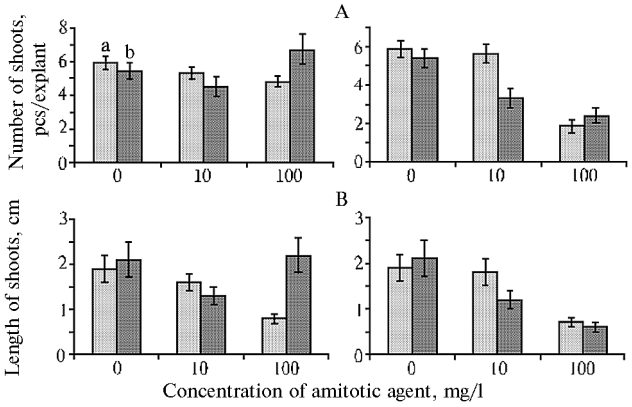

Fig. 2. Number (A) **and length** (B) **of shoots in explants developed by distant hybrids of pip fruit crops cultured in vitro on media containing acenaphthene** (left) **and colchicine** (right)**:** a — hybrid № 136, b — hybrid № 14/4.

Similar results were obtained by applying colchicine to the hybrid form № 14/4, while culturing it on the medium with acenaphthene (100 mg/l) provided greater stimulating effect – there was observed higher coefficient of propagation and longer shoots compared with control (Fig. 2).

Culturing the apexes on the media with acenaphthene and colchicine resulted in shoots of various morphology – both normal and abnormal, such as enlarged leaves, reduced leaves, modified leaf shape, light green and yellow-green color, slowed-down growth of shoots, thickened and flattened shoots. The latter abnormality was manifested by the pear-apple hybrid № 14/4. In these plantlets, the epidermis of leaves was composed by cells of a modified form and a size similar to that in control. This fact was mostly typical for individuals treated by acenaphthene. It should be noted that most of these shoots recovered normal morphology after 1-2 culturing passages on the amitotic-free propagation media. At the same time, there were selected the plantlets preserving the leaves of abnormal size and shape. Leaves and young roots of these forms were subjected to cytological analysis.

2. Number and size of stomata on leaves of distant hybrids of pip fruit crops depending on number of chromosomes and content of amitotic agent in culture medium

Form	Amitotic, mg/l	Average number of stomata, pcs/mm^2	Length of stomata, um	Number of chromosomes in somatic tissues
№ 14/4 (control)		80,3±10,8	22,8±0,4	34
№ 14/4 K_1-25	Colchicine, 10	119,0±4,2	26,1±1,0	26, 34, 38, 51,
№ 14/4 K_1-2	Colchicine, 10	69,2±3,6	27,2±0,6	56, 68
№ 136 (control)		149,6±9,8	24,3±0,6	34
№ 136 K_2-27	Colchicine, 100	70,4±4,6	36,3±1,7	68
№ 136 A_2-22	Acenaphthene, 100	80,4±9,5	31,8±2,1	68

The number and size of stomata on the leaves developed in vitro were assessed as indirect indicators of ploidy. These parameters were highly variable even within a single individual - such feature is typical to many crops including the studied hybrids. Along with it, these parameters varied up to 5-fold over a random sample of leaves formed in artificial culture, which significantly influenced the obtained values. Preliminary assessment of ploidy (Table 2) revealed the forms distinct in size of closure cells and in number of stomata.

The number of chromosomes in growing apexes of roots were accounted, which confirmed the change of ploidy in a number of studied genetic lines. Many forms derived on amitotic media were found to be mixoploid - about 80% cells contained aneuploid chromosome sets. For example, the form № 14/4 K_1-25 contained 26 chromosomes in 57,1% somatic tissues and in cells of meristem tissues - 34, 38 and 51 chromosomes. Selected lines of the rowan-pear hybrid and the hybrid form № 14/4 after adaptation to non-sterile environment were planted open-ground on experimental plot for further study.

Thus, polyploidy of meristem tissue was induced in vitro to obtain polyploid forms of distant hybrids of pip fruit crops. Up to 32% of these forms with modified ploidy developed on media containing colchicine. Acenaphthene was effective amitotic agent only at its content in a medium of 0,01% (the yield of forms with modified ploidy did not exceed 7,1%). Artificial polyploidization is a promising technique allowing to restore the fertility of distant hybrids and to overcome negative effects of various factors on seed germination. The forms whose polyploidy is confirmed by cytological tests can be successfully used at creation of new cultivars with commercially valuable properties.

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