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Development of recipient system of woody subtropical plants *in vitro*

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Abstract

The objecive of this study was to develop recipient systems of woody subtropical species and to obtain asimina (*Asimina triloba* Dun.), feijoa (*Feijoa sellowiana* Berg.), kiwi (*Actinidia deliciosa* (Chev.) Liang, Ferguson), persimmon (*Diospyros kaki* L.), and zizyphus (*Zizyphus jujuba* Mill.) plants through direct and indirect regeneration *in vitro*. The morphogenetic capacity of organs and tissues in selected woody subtropical plants was realized via five ways: activation of apical and lateral meristems; direct regeneration of plants from leaves, shoot segments and cotyledons; direct regeneration of seedlings from zygotic embryos; and direct and indirect somatic embryogenesis. High effective microshoot regeneration was induced by using direct regeneration of plants from leaves, shoot segments and cotyledons on modified Murashige and Skoog medium with 1.0 to 6.0 μM thidiazuron. The developed *in vitro* systems of woody subtropical plants allow us to use them for different purposes of plant biotechnology: multiplication, genetic transformation and conservation *in vitro* of valuable genotypes of asimina, feijoa, kiwi, persimmon and zizyphus.

Key words: *Actinidia deliciosa*, *Asimina triloba*, *Diospyros kaki*, *Feijoa sellowiana*, *Zizyphus jujuba*, *in vitro* regeneration.

Introduction

The Nikitsky Botanical Gardens – National Scientific Center (Crimea, Ukraine) has a large plant collection of asimina (*Asimina triloba* Dun.), feijoa (*Feijoa sellowiana* Berg.), kiwi (*Actinidia deliciosa* (Chev.) Liang, Ferguson), persimmon (*Diospyros kaki* L.) and zizyphus (*Zizyphus jujuba* Mill.). The climatic conditions of the Crimean region (Southern part of Ukraine, coast of the Black See) allow us to cultivate most of the woody subtropical plants. Micropropagation via axillary shoot proliferation has been applied to the commercial propagation of fruit trees since the beginning of 1980s (Zimmerman 1991). Recent advances in plant biotechnology have revealed the vast potential of tissue culture for propagation and breeding *in vitro* of fruit plants. Efficient methods for the micropropagation of important tropical fruits (banana, papaya, passion fruit, mango and pineapple) already exist (Litz, Jaiswal 1991; Grosser 1994). The skillful combination of biotechnology methods with classical methods of breeding considerably accelerates the breeding process of subtropical fruit trees (Litz, Jaiswal 1991; Oliveira, Pais 1992; Chalak, Legave 1996; Mitrofanova et al. 1997; Mitrofanova et al. 1998; Zdruikovskaya-Richter 2003).

This paper reports on several ways of plant regeneration *in vitro* in selected cultivars and hybrids of asimina, feijoa, kiwi, persimmon and zizyphus.

Materials and methods

Experiments were carried out with two-year-old field-grown plants of asimina, five to six-year-old field-grown plants of zizyphus (cvs. 'Ya-dzao', 'Kitaiskii 2A', 'Ta-yang-dzao'), feijoa (forms 1 and 2), kiwi (cvs. 'Bruno', 'Monti', 'Tomuri', 'Saanishton' and hybrids 'Monti' × 'Tomuri', 'Bruno' × 'Tomuri'), persimmon (cvs. 'Rossianka', 'Odnodomnaja', 'Korejskaja neterpkaja', 'Bordovaja', 'Meader') and *in vitro* cultivated plants of selected subtropical fruit trees. Mother plant material was collected on the strain testing plot of Nikitsky Botanical Gardens.

Explants (single nodes with bud) were surface disinfected for 1 min in ethanol (70 %), followed by 15 min in a solution containing 1 % Thimerosal (Sigma, USA), and subsequently for 3 to 5 min in a solution containing 0.08 % $AgNO_3$ plus three drops of Tween 80, then rinsed three times in sterile distilled water. In order to induce bud development and future shoot proliferation, isolated buds from field-growth plants of asimina, zizyphus, persimmon, kiwi and feijoa were placed on modified Murashige and Skoog (1962; MS) culture medium and Pierik (1976) medium, supplemented with 0.89 to 8.90 μM benzyladenin (BA), 0.93 to 9.30 μM kinetin and 0.91 to 9.12 μM zeatin.

Seeds were taken from mature fruits of kiwi and persimmon and surface sterilized for 20 min in 1.5 % (w/v) sodium hypochlorite followed by three rinses in sterile distilled water. Fruits of zizyphus and feijoa were submerged in 96 % ethanol and treated in a spirit-lamp flame. The embryo was separated from the surrounding tissues and placed on Monnier (1973) culture medium in a refrigerator at 5 ± 1 °C (10 to 60 days) and then transferred to cultivation room (25 ± 1 °C, 16 h photoperiod, illuminance – 25 μmol m^{-2} s^{-1}).

Leaf discs from *in vitro* cultured microshoots of kiwi were placed with abaxial and adaxial surface on modified MS medium, supplemented with different concentrations of BA (4.40 to 44.00 μM) and indol-3-acetic acid (5.71 to 57.08 μM; IAA). For direct regeneration, explants such as cotyledons (zizyphus), shoot segments (kiwi) and leaf discs (asimina, feijoa, kiwi, persimmon, zizyphus) were cultured on MS medium with different concentrations of thidiazuron (1.0 - 9.0 μM; TDZ).

To produce and cultivate of zizyphus somatic embryos, half-strength MS medium containing 1.1 to 15.8 μM 2,4-dichlorphenoxyacetic acid (2.4-D) and 2.6 to 16.1 μM α-naphthaleneacetic acid (NAA) was used (Mitrofanova et al. 1997).

Microshoots of all investigated plants were rooted on a half-strength MS culture medium, supplemented with different contents of β-indole-3-butric acid (0.98 to 4.90 μM; IBA) and NAA (1.07 to 5.37 μM). The pH of the medium was adjusted to 5.7 before sterilization (120 °C, 15 to 20 min). Plant material was subcultured onto fresh medium at 30-day intervals. Cultures of buds, shoots, germs, leaf discs and cotyledons were grown under a 16-h photoperiod (25 ± 1 °C) and photosynthetic photon flux of 25 μmol m^{-2} s^{-1} provided by Philips cool-white 36 W fluorescent tubes.

Data processing was carried out using STATISTICA for Windows, Release 5.1. Means ± SE for three replications of 50 explants used for each combination were determined.

Results and discussion

The ability of isolated asimina, feijoa, kiwi, persimmon and zizyphus buds, leaf discs and embryos to produce microshoots and plants depended on the plant genotype and the applied cytokinin and auxin combination in the culture medium. The morphogenesis of tissues and organs of selected woody subtropical plants was realized via five ways: activation of apical and lateral meristems; direct and indirect somatic embryogenesis; direct regeneration of seedlings from zygotic embryos; direct regeneration of plants from leaves, shoot segments and cotyledons.

BA, kinetin and zeatin are well known as growth regulators that can induce microshoot regeneration. We observed that kinetin in all investigated concentrations (0.93 to 9.30 μM) retarded microshoot formation and induced active callus growth. The 0.88 μM and 4.40 μM concentrations of BA were effective for microshoot development from isolated buds of zizyphus and kiwi, respectively (Table 1). The cultivation of isolated persimmon buds on MS medium containing 2.22 μM BA stimulated the active regeneration of microshoots after three weeks of cultivation (Table 1). Addition of zeatin (4.56 μM and 9.12 μM) to MS culture medium induced the primary regeneration of microshoots from isolated buds of asimina and feijoa, respectively (Table 1). High concentrations of cytokinin (9.30 μM kinetin, 9.12 μM zeatin or 8.90 μM BA) in media caused shoot vitrification in all selected species.

The results of previous experiments with kiwi explants showed that the cultivation of germs and embryos on modified MS medium supplemented with 2.22 μM BA induced the development of axillary meristems and growth of microshoots. In callus culture of kiwi, alongside with vegetative gemmagenesis, the embryogenic zones were also observed (Vijeshwar et al. 1997). During eight weeks of cultivation on MS culture medium supplemented with BA and IAA, 80 to 90 % of leaf discs of kiwi isolated from one-year

Table 1. Effect of BA and zeatin on microshoot regeneration from isolated buds of kiwi (*Actinidia deliciosa*), zizyphus (*Zizyphus jujuba*), feijoa (*Feijoa sellowiana*), persimmon (*Diospyros kaki*) and asimina (*Asimina triloba*) after three weeks of cultivation *in vitro*

Cytokinin (μM)	Average number of shoots per isolated bud				
	Actinidia deliciosa	*Zizyphus jujuba*	*Feijoa sellowiana*	*Diospyros kaki*	*Asimina triloba*
Control	0	0.8 ± 0.1	0.2 ± 0.1	0	0
Zeatin 0.91	0	0.9 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.2 ± 0.1
Zeatin 1.36	0	1.1 ± 0.1	2.5 ± 0.1	3.5 ± 0.6	1.7 ± 0.1
Zeatin 2.73	0.6 ± 0.1	1.2 ± 0.2	2.9 ± 1.3	2.3 ± 0.9	1.9 ± 0.3
Zeatin 4.56	1.1 ± 0.1	0.7 ± 0.0	3.1 ± 0.8	2.7 ± 0.0	2.3 ± 0.4
Zeatin 9.12	1.6 ± 0.1	0	3.7 ± 1.2	2.9 ± 0.3	3.1 ± 0.6
BA 0.88	0	2.9 ± 1.3	0	1.4 ± 0.1	0
BA 1.33	1.5 ± 0.1	2.7 ± 1.3	0	2.3 ± 0.4	1.2 ± 0.1
BA 2.22	2.1 ± 0.3	2.4 ± 0.8	1.3 ± 0.1	3.3 ± 0.3	1.4 ± 0.1
BA 4.40	2.4 ± 0.4	1.6 ± 0.7	1.8 ± 0.1	1.5 ± 0.1	1.7 ± 0.1
BA 8.90	1.3 ± 0.1	0.7 ± 0.1	1.7 ± 0.1	0.9 ± 0.1	1.2 ± 0.1

Table 2. Effect of different concentrations of BA and IAA on regeneration rate of leaf discs of kiwi (*Actinidia deliciosa*, cv. 'Saanishton') with abaxial position on MS medium during four and eight weeks of cultivation *in vitro*

BA (μM)	IAA (μM)	Leaf discs forming shoots (%)	
		4 weeks	8 weeks
4.40	5.71	10.0 ± 2.1	27.0 ± 1.8
8.90	5.51	28.0 ± 2.4	60.0 ± 5.7
8.90	8.56	50.0 ± 4.1	90.0 ± 8.5
8.90	11.42	35.0 ± 2.5	80.0 ± 6.5
13.35	17.13	25.0 ± 0.5	40.0 ± 5.2
22.20	28.64	10.0 ± 0.8	25.0 ± 1.9
44.00	57.08	0.0	20.0 ± 2.3

Table 3. Regeneration of adventitious microshoots from leaf discs of different cultivars of kiwi (*Actinidia deliciosa*) with adaxial position on MS medium supplemented with 8.90 μM BA and 8.56 μM IAA during four and eight weeks of cultivation *in vitro*

Cultivar	Leaf discs forming shoots (%)		No. of shoots per leaf disc	
	4 weeks	8 weeks	4 weeks	8 weeks
'Monti'	14.0 ± 6.1	40.0 ± 7.0	1.2 ± 0.6	6.0 ± 0.5
'Bruno'	11.8 ± 2.1	55.6 ± 8.2	1.6 ± 0.1	6.5 ± 0.5
'Tomuri'	18.6 ± 1.6	75.0 ± 9.5	2.5 ± 0.5	7.2 ± 0.1
'Saanishton'	50.0 ± 5.5	100.0 ± 0.0	4.3 ± 0.4	10.0 ± 0.4
'Monti' × 'Tomuri'	0.0	38.0 ± 11.0	0.0	5.7 ± 1.3
'Bruno' × 'Tomuri'	45.0 ± 4.3	100.0 ± 0.0	5.7 ± 0.2	10.0 ± 0.6

Fig. 1. The plantlets of kiwi (*Actinidia deliciosa*) cv. 'Saanishton' obtained from leaf discs on MS medium with 4.40 μM BA after three month cultivation *in vitro*.

cultivated *in vitro* plants formed adventitious buds. The optimum combination of BA (8.90 μM) and IAA (8.56 μM) concentrations was established (Table 2). With adaxial position of leaf discs of kiwi cv. 'Saanishton' and hybrid 'Bruno' × 'Tomuri', the frequency of microshoot regeneration reached 100 % (Table 3). In the case of the microshoot regeneration from leaf discs of kiwi, callus was not formed. Normal plants were obtained during five month of cultivation on MS medium with 4.40 μM BA (Fig. 1). These plants can be used for future genetic transformation and other breeding investigations. The *in vitro* system via leaf discs in kiwi enabled us to create long-term shoot cultures with a high multiplication rate (8 to 10) and a 45-week subculture interval.

The use of zygotic embryos has opened the possibility of obtaining woody subtropical plants via direct and indirect somatic embryogenesis, direct regeneration of seedlings from zygotic embryos, and direct microshoot regeneration from cotyledons. We were the first to induce direct somatic embryogenesis from cotyledons of zygotic embryos and to produce zizyphus plants, when one culture medium (half-strength MS medium supplemented with 2.4-D) was substituted for another (modified Pierik medium without growth regulators). The largest number of somatic embryos per explant (7.2 ± 1.3 in cv. 'Ya-dzao', 15.5 ± 3.5 in cv. 'Kitaiskii 2A' and 3.0 ± 0.6 in cv. 'Ta-yang-dzao') was observed on ½ MS medium with 2.26 mM 2.4-D (data not shown; Mitrofanova et al. 1997). During 60 days of culture, most embryoids followed the stages of development of zygotic embryos: globular, heart-, and torpedo-shaped. The efficiency of somatic embryogenesis in zizyphus was increased by joint cultivation of primary and secondary embryoids. Within a year it was possible to obtain up to 630 somatic embryos from one cotyledon (Mitrofanova et al. 1997). Sometimes, indirect somatic embryogenesis was induced on the surface of cultured undeveloped zygotic embryos (torpedo-shape stage) of

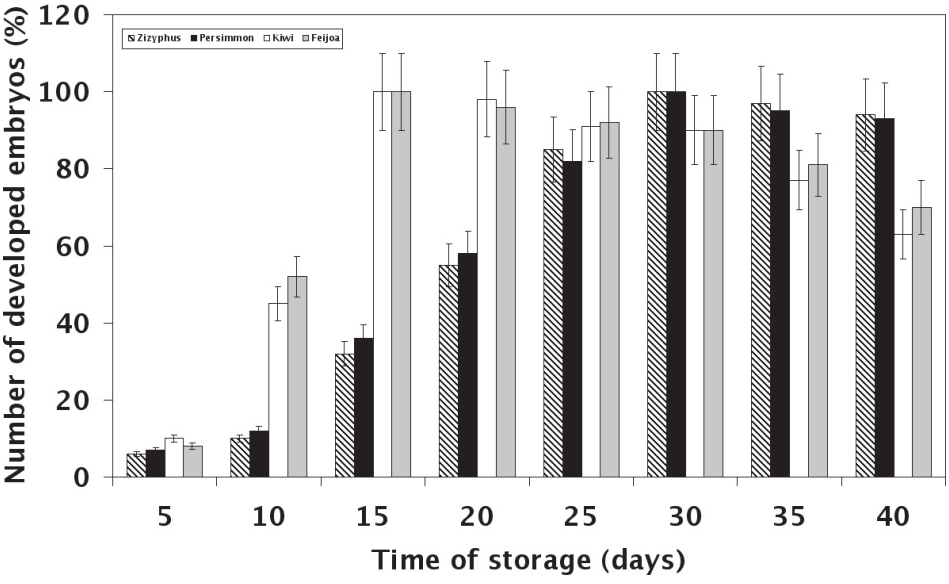

Fig. 2. Effect of the time of storage at 5 °C on development of zygotic embryos of zizyphus (*Zizyphus jujube*), persimmon (*Diospyros kaki*), kiwi (*Actinidia deliciosa*) and feijoa (*Feijoa sellowiana*) *in vitro*.

Table 4. Microshoot regeneration (no. of microshoots per explant) on culture medium with different concentrations of thidiazuron from different explants of *in vitro* cultivated zizyphus, asimina, feijoa, persimmon and kiwi

Species	Type of primary explant	Concentration of thidiazuron			
		1 μM	3 μM	6 μM	9 μM
Zizyphus	cotyledon	2.5 ± 0.1	callus	4.0 ± 0.1	7.0 ± 0.3
	leaf disc	1.2 ± 0.1	4.5 ± 0.3	2.0 ± 0.0	callus
Persimmon	cotyledon	1.4 ± 0.0	1.8 ± 0.1	2.6 ± 0.1	6.7 ± 0.4
	leaf disc	callus	0	10.0 ± 0.2	7.2 ± 0.3
Asimina	leaf disc	0	0	2.5 ± 0.1	callus
Kiwi	leaf disc	2.7 ± 0.3	callus	callus	callus
	shoot segment	0	callus	4.2 ± 0.1	callus
Feijoa	leaf disc	0	1.7 ± 0.1	3.7 ± 0.2	4.6 ± 0.1
	shoot segment	0	callus	3.7 ± 0.2	callus

feijoa on the modified MS medium with 4.56 μM and 9.12 μM zeatin. Unfortunately, even the use of different combinations of concentrations did not lead to induction of somatic embryogenesis in persimmon. Isolated zygotic embryos of three zizyphus cultivars arising from free pollination were also used for obtaining seedlings and microshoot regeneration. The size of isolated zygotic embryos differed from 4.0 mm to 10.0 mm. Zygotic embryos 6.1 to 10.0 mm in size produced well-developed plantlets. At a low positive temperature of 5 °C, the frequency of zygotic embryo development in feijoa, kiwi (15 days), persimmon and zizyphus (30 days) increased to 100 % (Fig. 2). The period of embryos storage of selected woody subtropical plants was considerably shorter than for peach, apricot, cherry plum and cherry (Zdruikovskaya-Richter 2003). Normal plants of feijoa, kiwi, zizyphus and persimmon were obtained after 1.5 to 2 months of zygotic embryos cultivation on Monnier medium.

Thidiazuron was selected among other cytokinins for its tremendous ability to stimulate *in vitro* shoot proliferation of woody species (Huetteman, Preece 1993). Today TDZ is a potent cytokinin for woody plant tissue culture. The results of our experiments (Table 4) showed that the number of regenerated microshoots per explant depended both on plant genotype and TDZ concentration in MS medium. Using 1 to 9 μM TDZ for direct and indirect regeneration of shoots allowed to induce microshoot development from cotyledons and leaf discs in zizyphus and persimmon, from leaf discs in asimina, feijoa, kiwi, persimmon and zizyphus, and from microshoot segments in feijoa, kiwi and persimmon (Fig. 3). However the number of regenerated microshoots from leaf discs of kiwi was lower in comparison with cultivation on medium containing BA and IAA.

To regenerate adventitious microshoots of selected subtropical plants, microcuttings of asimina, feijoa, kiwi, persimmon and zizyphus were placed on MS medium, supplemented with different concentrations of BA and zeatin. A high regeneration rate was obtained with cultivation of explants of kiwi and zizyphus on medium with 4.40 μM BA. After the 4th subculture on the MS medium supplemented with 1.82 μM zeatin and 0.24 μM IBA, a regeneration rate of five microshoots per bud was achieved for asimina. Elongation of microshoots also occurred on this medium. Active adventive microshoot regeneration in

Fig. 3. Microshoot regeneration from cotyledon explants of persimmon (*Diospyros kaki*) on MS culture medium with 6 μM TDZ after fore weeks of cultivation.

Fig. 4. Active regeneration of microshoots from microcuttings of feijoa (*Feijoa sellowiana*) on MS medium with 4.56 μM zeatin during two weeks of cultivation.

feijoa and persimmon was observed on media containing 4.56 μM and 3.19 μM zeatin, respectively (Fig. 4).

The obtained microshoots of selected woody subtropical plants were placed on ½ MS medium with 0.98 - 4.90 μM IBA and 1.07 - 5.37 μM NAA to promote root formation. When microshoots were cultured on ½ MS medium, supplemented with 4.90 μM IBA and 2.15 μM NAA, the rooting frequency increased and the average root number in zizyphus and asimina was about 2.3 ± 0.1 per microshoot (Fig. 5). A 0.98 μM IBA stimulated

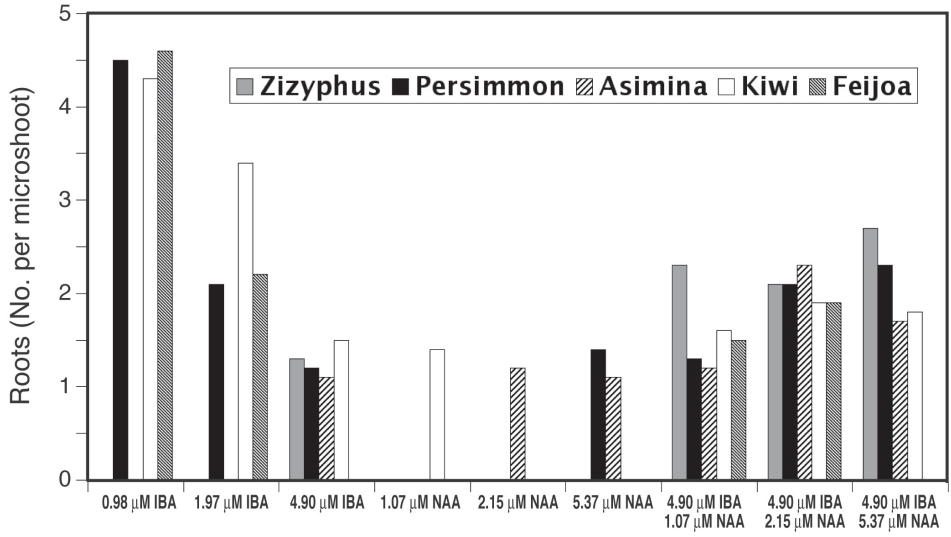

Fig. 5. Effect of IBA and NAA concentrations on root formation of microshoots in zizyphus (*Zizyphus jujube*), persimmon (*Diospyros kaki*), kiwi (*Actinidia deliciosa*), asimina (*Asimina triloba*) and feijoa (*Feijoa sellowiana*) on ½ MS medium.

in vitro rooting (100 %) of feijoa, kiwi and persimmon microshoots, and the average root number of these three cultures was about 4.5 ± 0.2 per microshoot (Fig. 5). Higher concentrations of IBA and NAA induced callus formation on the base of microshoots and reduced the rooting process.

This study showed the possibility of direct and indirect regeneration of plants in asimina, feijoa, kiwi, persimmon and zizyphus from vegetative buds, leaf discs, shoots segments, embryos and cotyledons. The developed recipient systems of woody subtropical plants allows their use for different plant biotechnology purposes: multiplication, genetic transformation and conservation *in vitro* of valuable genotypes.

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References

Chalak L., Legave J.M. 1996. Orysalin combined with adventitious regeneration for an efficient chromosome doubling of trihaploid kiwifruit. *Plant Cell Rep*. 16: 97–100.

Grosser J.W. 1994. *In vitro* culture of tropical fruits. In: Vasil I.K., Thorpe T.A. (eds.) *Plant Cell Tissue Culture*. Kluwer Academic Publishers, Dordrecht, pp. 475–496.

Huetteman C.A., Preece J.E. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tissue Organ Cult*. 33: 105–119.

Litz R.E., Jaiswal V.S. 1991. Micropropagation of tropical and subtropical fruits. In: Deberg P., Zimmerman R.H. (eds.) *Micropropagation: Technology and Applications*. Kluwer Academic Publishers, Dordrecht, pp. 247–263.

Mitrofanova I.V., Kazas A.N., Khokhlov S.Y. 1998. Features of persimmon clonal micropropagation. *Bull. State Nikita Botanical Garden* 80: 154–178.

Mitrofanova I.V., Mitrofanova O.V., Pandei D.K. 1997. Somatic embryogenesis and plant regeneration in *Zizyphus jujuba* Mill. *in vitro*. *Russ. J. Plant Physiol*. 44: 94–99.

Monnier M. 1973. Croissance et developpment des embryons globulaires de *Capsella bursa-pastoris* cultiver *in vitro* dans un milieu a la base d'une nouvelle solution minerale. *Bull. Soc. Bot. France. Memoires Coll. Morphologie*: 179–194.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant*. 15: 473–497.

Oliveira M.M., Pais M.S.S. 1992. Somatic embryogenesis in leaves and leaf-derived protoplasts of *Actinidia deliciosa* var. *deliciosa* cv. 'Hayward' (kiwifruit). *Plant Cell Rep*. 11: 314–315.

Pierik R.L.M. 1976. *Anthurium andreanum* plantlets produced from callus tissues cultivated *in vitro*. *Physiol. Plant*. 37: 80–82.

Vijeshwar P., Mitrofanova O.V., Lishchuk A.I. 1997. Clonal micropropagation of *Actinidia deliciosa* (Chev.), Liang, Ferguson. *Biotechnology Researches of Horticultural and Other Valuable Perennial Cultures: Collected Scientific Works of the State Nikita Botanical Garden*, 119: 111–126.

Zimmerman R.H. 1991. Micropropagation of temperate zone fruit and nut crops. In: Debergh P.C., Zimmerman R.H. (eds.) *Micropropagation: Technology and Application*. Kluwer Academic Publishers, Dordrecht, pp. 231-246.

Zdruikovskaya-Richter A.I. 2003. *Embryoculture of Isolated Embryos, Generative Structures and Obtaining of New Forms of Plants*. Crimea-Pharm-Trading, Simferopol. 368 p.