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Features of induction of morphogenesis *in vitro* in some species of genus *Potentilla* L.

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The optimal protocol of sterilization for the introduction *in vitro* of some *Potentilla* L. species has been found. Use of ethanol and "Dez Tab" as antiseptics allowed to get from 15 to 100% aseptic explants. Viability ranged from 0 to 75% depending on plant species. The possibility of callus differentiation from different explants, seedlings and microshoots regeneration on modified Murashige & Skoog, Quoirin & Lepoivre, Pierik and Monnier medium has been shown.

Key words: *Potentilla* L., sterilization, morphogenesis, *in vitro*

INTRODUCTION

The possibility of the secondary metabolites extraction from plants is often limited for several reasons: rare plants, endemic or endangered species, and also the poor quality of the pharmacological stock. In this regard, the *in vitro* cell and tissue culture could be an alternative source of biologically active substances (BAS) (Kitaeva, 2011).

In the accessible literature there are many examples of cultural plants which store up various valuable metabolites. The content of BAS in the *in vitro* culture is significantly higher than the one in intact plants. For example, indole alkaloids (reserpine, serpentine, etc.) have been successfully produced from tissue culture of *Rauwolfia* sp. (Butenko, 1999; Vollosovich, 1970; Kunakh, 1994). Taxol and other taxanes are synthesized in suspension culture of *Taxus canadensis* Marshall and other similar species (Dubravina, 2005; Banerjee, 1996; Raymond, 1997; Strobel, 1992; Kim, 2000). Barberry, rose periwinkle, opium poppy and Iranian poppy alkaloids are also produced in cell culture (Delui, 1995; Dong, 2000; Zenk, 1997; Kunakh, 2003, 2004). Cardiotropic glycosides of *Digitalis lanata* Ehrh. and *Digitalis purpurea* L. and triterpene saponins of *Panax* sp. are obtained from suspension and callus cultures of foxglove and ginseng (Bourgaud, 2001; Hagimori, 1980; Reinhard, 1989; Rücker, 1988). All these

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substances are widely used in medicine for treatment of various diseases as they have a high biological activity. For example, taxol has anticancer properties. Indole alkaloids are used in medicine as an antihypertensive, antiarhythmic and sedative agents. Alkaloids of barberry are widely used in gynecology (Mashkovsky, 1985).

In the past years the plants of genus *Potentilla* L., family (*Rosaceae*) have been actively investigated. Some *Potentilla* species are used as decorative forms. Moreover, the plants contain a great number of important compounds (Kitaeva, 2011; Tomczyk, 2009; Remphrey, 1993; Zhong-wei, 2007). For example, they are rich in tannins, which are important in medicine (Wu, 1990). Tannins have anti-viral and antianaphylaxis properties, they enhance immunity and help to prevent cardiovascular disease (Li, 2005; Shi, 1998; Tang, 2000; He, 2001). Most of species in genus *Potentilla* are characterized by a high content of flavonoids. The pharmacological properties of flavanol are anti-inflammatory and angioprotective activity (Andersen, 2006).

Thus, the method of cell and tissues culture *in vitro* will be a great perspective to biodiversity conservation and using as alternative sources of BAS.

In this investigation the organ and tissue morphogenetic capacity of some species of genus *Potentilla* during the *in vitro* introduction and induction of morphogenesis was found.

MATERIALS AND METHODS

The investigation objects were leaf pieces, meristem tips, shoot and root segments obtained from intact plant of *P. depressa* Willd., *P. recta* subsp. *laciniosa* (Waldst. et Kit. ex Nestler) Nyman, *P. inclinata* Vill and internodes and seeds of *P. recta* (Mosyakin, 1999). The plant material was gathered from August to November 2012 in Crimea (Ukraine) on the mountains Ai-Petri, Chatyr-Dag and Nikita Jajly.

In this investigation the conventional and specific (developed in the Nikitsky Botanical Gardens) biotechnological methods were applied (Kalinin, 1980; Mitrofanova, 2011).

To obtain aseptic culture of *Potentilla* L. species the following sterilizing agents were used: 70–96% ethanol (C_2H_5OH) and 0.375–1.125% Cl_2 (Dez Tab, Ukraine). Sterilization of leaves, petioles, meristems, internodes, roots and seeds of some species of *Potentilla* was carried out in four stages. The use of different concentrations of sterilizing agents and their exposure depend on the type of the explant (Table).

The work with sterile material by using the laminar boxes Fatran (Czech Republic) and BP-4 (Ukraine) was carried out. To evaluate the morphology of the callus a binocular microscope MBS-10 (Russia) was used.

In experiments on callus induction and shoot regeneration from different explants of

Table. The types of sterilization kinds of genus *Potentilla* L.

Variant of sterilization, No.	The type of sterilization			
	C_2H_5OH		"Dez Tab" (Ukraine)	
	Concentration, %	Exposure, min	Concentration, % Cl_2	Exposure, min
I	70	1	0.375	7
II	70	1	0.375	10
III	70	1	0.375	12
IV	70	1	0.375	15
V	70	1	0.375	20
VI	70	1	0.375	25
VII	96	1	1.125	10
VIII	96	1	1.125	12
IX	96	1	1.125	15
X	96	1	1.125	20

P. depressa, P. recta, P. inclinata several culture media Murashige and Skoog (MS) (Murashige, 1962), Monnier (Monnier, 1976), Quoirin and Lepoivre (QL) (Quoirin, 1976) and Pierik (Pierik, 1976) were investigated.

Studying the possibility of callus and shoot formation from different explants of *P. depressa, P. recta, P. inclinata*, in culture media MS and Pierik added 2,4-D (8.40–8.50 μM), 6-BAP (0.44–8.90 μM), NAA (0.054–9.40 μM) IAA (0.057–11.42 μM), kinetin (0.40–0.48 μM), IBA (0.049–0.49 μM), TDZ (6–9 μM), zeatin (0.1–0.17 μM).

The media were adjusted to pH 5.5–5.7 prior to autoclaving at 115 °C for 20–30 min and depended on cultural vessels.

The tubes with seeds were placed on stratification in refrigerator without light (4 ± 1 °C). After 20–30 days of cultivation they were transferred to the culture room at temperature of 24 ± 1 °C, photoperiod 16/8 h (day / night), in the light of 2 000–3 000 lux. Culture vessels with other types of explants were placed in a culture room or in a thermostat without light at 24 ± 1 °C temperature. Subculturing is typically required every 4–8 weeks.

There were three replicates per treatment with 30 explants (for leaf pieces, meristem tips root segments) and two replicates per treatment with 30 explants (for internodes and seeds of *P. recta*) in the experiments.

RESULTS

Studying the features of installation various explants of *Potentilla* in conditions *in vitro* allowed to obtain optimal antiseptics expositions, which allowed to receive from 15 to 100% aseptic explants of different types; their viability varied from 0 to 75% with the genotype. High frequency of obtaining of *P. depressa* aseptic culture (100%) was observed. This result was shown on leaf pieces and petiole segments by using 96% ethanol (1 min) and 1.125% active chlorine (Dez Tab, 20–25 min) (type of sterilization V and VI, Table), although high concentrations of sterilizing agents and their exposure caused a total loss of viability of the explants. Using lower concentrations of antiseptics did not exempt the plant material from endogenous infection. These results indicate that selection of leaf pieces and petiole segments of *P. depressa* for culture *in vitro* during the period from August to November is not advisable. However, more sparing types of sterilization of other *Potentilla* species leaf pieces allowed to get the explants without infection and simultaneously gave a possibility to maintain their viability. For example, using of the type IV of sterilization gave 57.1% and 87.5% of aseptic explants *P. recta* and *P. inclinata* respectively, and 50% sterile explants were viable.

Roots grown in soil *in situ* are usually heavily contaminated and have problem with sterilization to provide an adequate number of uncontaminated cultures. Nevertheless, these explants have been used as an initial source for multiplication of obtained shoots.

For an introduction of root segments to the culture *in vitro* types IV and VI of sterilization were used (Table). When root explants *P. recta* have been sterilized by 70% ethanol (1 min) and by 0.375% concentration of active chlorine (15 min) 80% sterile and 75% of viable explants were obtained (Fig. 1 A, B).

Optimal type of sterilization of meristem tips in *P. depressa* was variant VII. 85% sterile and 17.6% of viable explants were observed (Fig. 1 A, B).

When sterilizing seeds *P. recta* by 70% ethanol (1 min) and 0.375% Cl_2 (10 min) 71.4% of aseptic explants and 40% viable seeds were obtained (Fig. 1 B).

During investigation of morphogenetic capacity of different explants in three species of *Potentilla* the culture media MS, ½ MS, Pierik, Monnier, supplemented with various growth regulators were tested.

At 50–100 days of cultivation on Monnier medium plants from seeds were developed. Thus on ½ MS medium with 1.78–3.55 μM BAP and 0.25–0.49 μM IBA at 55–96 days of cultivation the microshoots with small leaves were formed (Fig. 2 A, B). The frequency of seed germination on this medium was 16%,

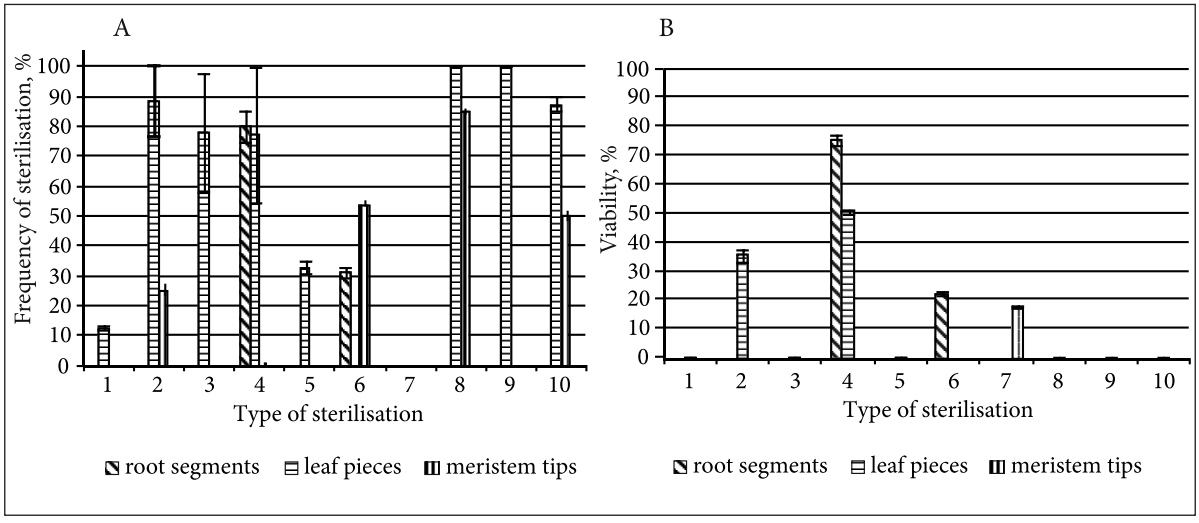

Fig. 1. Effect of different type of sterilization on obtaining the aseptic culture (A) and viability of primary explants (B)

while on Monnier medium the frequency of seed germination was more than three times higher and reached 50%.

For multiple shoot formation the microshoots with leaves were placed on MS medium supplemented with 0.80–0.90 μM BAP. Active adventitious microshoots regeneration on the second week of cultivation was observed. However, at 33–37 days the number of shoots conglomerates reached an average of 7 ± 2.7 pcs. / explant, the number of microshoots with leaves in the "rosette" averaging 3.63 ± 0.095 units (Fig. 3).

Along with the identification of morphogenetic capacity of seeds under *in vitro* conditions the ability of various organs and tissues in *Potentilla* intact plants to callus formation was investigated. MS and Pierik media supplemented with 0.93–2.79 μM kinetin and 4.30–5.88 μM NAA, 1.78–2.66 μM BAP and 0.40–1.14 μM IAA, 5.88–7.68 μM 2,4-D induced callusogenesis at leaf pieces, petiole segments and roots of *P. depressa, P. recta* and *P. inclinata* (Fig. 4). The use of the modified MS medium with 5.88–7.68 μM 2,4-D increased the frequency of callus formation (up to 100%)

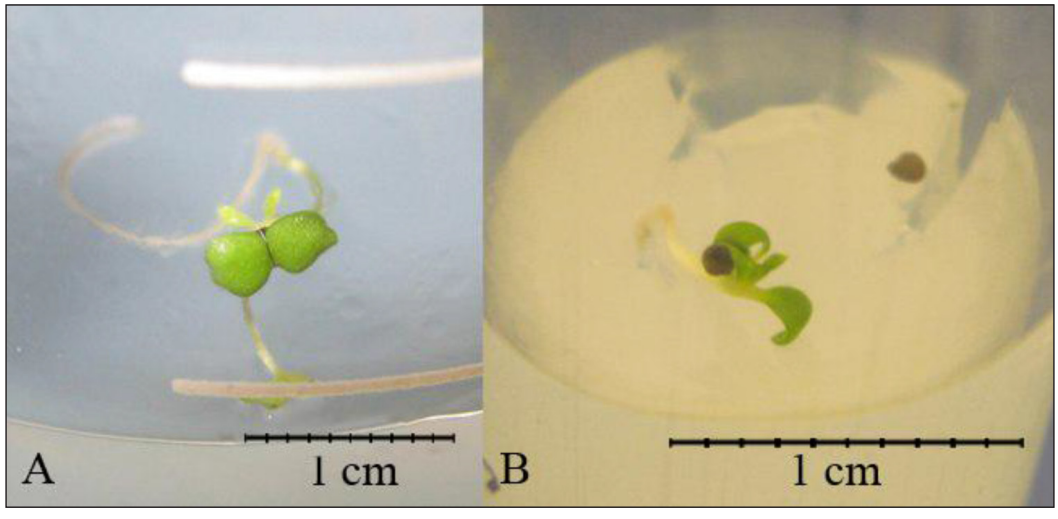

Fig. 2. *P. recta* seeds development on different culture media: A) Monnier medium B) ½ MS with 1.78–3.55 μM BAP and 0.25–0.49 μM IBA

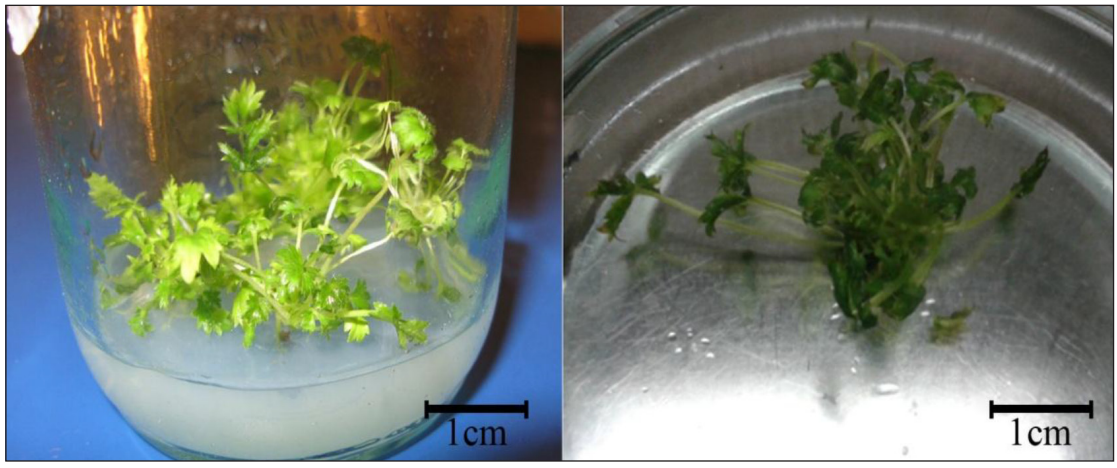

Fig. 3. Multiple *P. recta* shoot formation on modified MS medium with 0.80–0.90 μM BAP

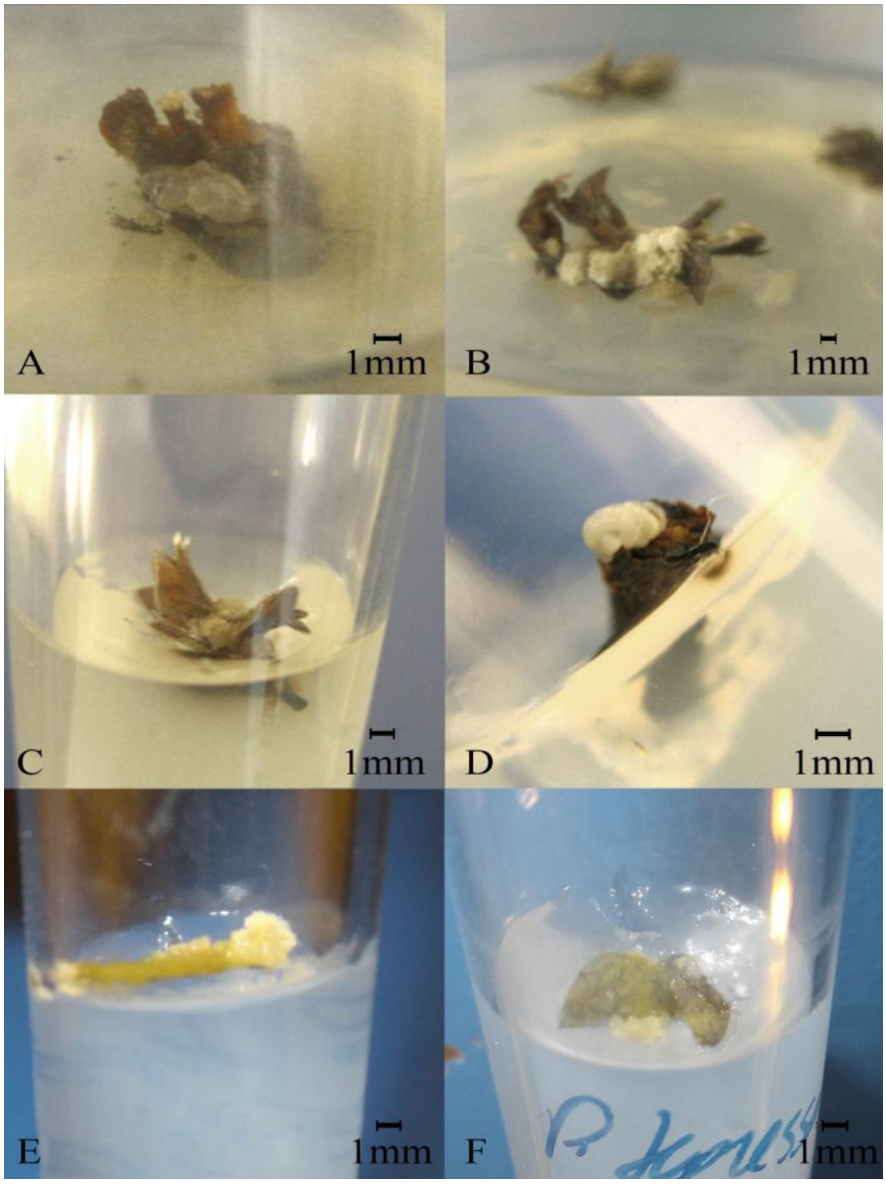

Fig. 4. Callus formation in some species of genus *Potentilla* L.: A) callus from root segments in *P. recta* on Pierik medium with 0.93–2.79 μM kinetin and 4.30–5.88 μM NAA; B) callus from leaf pieces in *P. recta* on MS medium with 5.88–7.68 μM 2,4-D; C) callus from leaf pieces in *P. inclinata* on MS medium, supplemented with 1.78–2.66 μM BAP and 0.40–1.14 μM IAA; D) callus from root segments in *P. depressa* on Pierik medium with 0.93–2.79 μM kinetin and 4.30–5.88 μM NAA; E) *P. depressa* callus from leaf pieces on Pierik medium, supplemented with 0.93–2.79 μM kinetin and 4.30–5.88 μM NAA; F) *P. depressa* callus from leaf pieces on Pierik medium with 0.93–2.79 μM kinetin and 4.30–5.88 μM NAA

from leaf pieces of *P. recta*. The slow growth of the callus which had a compact structure and white coloring was observed (Fig. 4 B). Explants cultivation on Pierik medium with 0.93–2.79 μM kinetin and 4.30–5.88 μM NAA induced callus development from petiole segments and leaf pieces in *P. depressa* during 40–44 and 30–35 days, respectively (Fig. 4 E, F).

Callus, consisting of many small green globules-like-structures with diameter up to 0.5 mm, was formed on abaxial surface of the leaf pieces and on the cutting parts of explants. The active growing light brown callus was formed on the edges of petiole fragments. In other words, callus was developed from tissue of explants. The frequency of callus formation reached 85.7% for leaf explants and 100% for petiole fragments. At 16–21 days of cultivation the leaf pieces and petiole fragments in *P. recta* a light-brown and friable callus was developed. The frequency of callusogenesis was 37.5%. On this medium callus formation from the leaf pieces in *P. inclinata* was not characterized by morphogenetic response.

At 30–35 days of cultivation the MS medium supplemented with 1.78–2.66 μM BAP and 0.40–1.14 μM IAA induced the formation of light-brown callus (Fig. 4 C). On the surface of explants in *P. recta* and *P. depressa* there was formed a compact white callus at 42–49 days and 60–70 days, respectively. The frequency of callus formation was different for investigated species: *P. depressa* – 20%, *P. recta* – 41% and *P. inclinata* – 46%.

The cultivation of aseptic roots segments in *P. depressa* on Pierik medium with 0.93–2.79 μM kinetin and 4.30–5.88 μM NAA within 12–16 days was induced a slow growth of compact creamy-white callus (Fig. 4 D). Callus, consisting of small transparent globules, was produced from the explants *P. recta* at 24–28 days of culture (Fig. 4 A). The frequency of callusogenesis was 22.2% and 75% for *P. depressa* and *P. recta*, respectively.

The cultivation of meristem tips in *P. depressa* on QL medium supplemented with zeatin (6.38–7.30 μM) induced a slow growth of light-brown callus. The frequency of callus formation was 14.7%. Explants cultivation on MS medium with 0.42–0.48 μM kinetin induced callus development from the meristem tips in *P. inclinata.* On this medium callus formation from the meristem tips in *P. depressa* was not characterized by morphogenetic response.

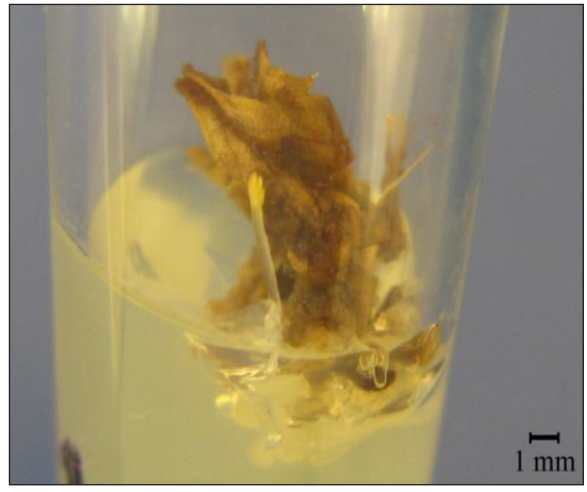

Fig. 5. Indirect microshoots regeneration from leaf explants of *P. recta* on MS medium supplemented with 1.78–2.66 μM BAP and 0.40–1.14 μM IAA

For indirect regeneration of microshoots the internodes of *P. recta* were placed on the Pierik media, supplemented with 0.93–2.79 μM kinetin and 4.3–5.88 μM NAA. Morphogenic callus, consisting of glassy transparent globules with diameter up to 1 mm, was formed at 25–30 days. Microshoots from cultivated callus were regenerated at 55–60 days (Fig. 5). Leaf pieces of *P. inclinata* placed on MS medium containing 1.78–2.66 μM BAP and 0.40–1.14 μM IAA were developed by way of indirect regeneration. Callus was formed at 30–35 days. Microshoots were regenerated from the callus at 47–54 days. In both cases the growth of microshoots was characterized by 8–10 mm / week.

DISCUSSION

In many countries research designed on identification of morphogenetic capacity *in vitro* of organs and tissues in plants of genus *Potentilla* was carried. The most significant contribution was made by scientists from the Republic of Belarus, Ukraine and China (Kitaeva, 2011,

2012; Laskar, 2005; Jiwen, 2003; Remphrey, 1993; Chun-feng, 2008; Stupnitsky, 2007).

Obtaining of aseptic culture is one of the most difficult stages during introduction of explants to *in vitro* condition. Until now a uniform method of sterilization for plant material has not been found. The sterilization was determined experimentally for each object (Mitrofanova, 2011; Edwin, 2008). Some of the authors reported about obtaining the aseptic culture of *Potentilla alba, P. fulgens*, *P. anserina*, *P. fruticosa*, *P. glabra*, *P. rupestris*, *P. recta*, *P. potaninii*, *P. palustris* × *Fragaria* sp. China (Kitaeva, 2011, 2012; Laskar, 2005; Jiwen, 2003; Remphrey, 1993; Chun-feng, 2008; Stupnitsky, 2007).

The present research has shown the optimal protocols of sterilization for the introduction to condition *in vitro P. depressa, P. recta* subsp. *laciniosa and P. inclinata.* Antiseptics and their exposure were found which allowed to obtain from 15 to 100% aseptic explants. Their viability varied from 0 to 75% with the genotype.

Methods of plant micropropagation *in vitro* by direct regeneration for *Potentilla alba*, *P. fulgens*, *P. fruticosa*, *P. glabra*, *P. potaninii*, *P. palustris* × *Fragaria* sp. have been developed (Kitaeva, 2011, 2012; Laskar, 2005; Jiwen, 2003; Remphrey, 1993; Chun-feng, 2008; Stupnitsky, 2007). However, up-to-date other biotechnology methods for *Potentilla* plants multiplication and conservation have been studied insufficiently (Chun-feng, 2008; Kovalenko, 2004). Information about indirect regeneration of plants in *P. anserina*, *P. fruticosa* and *P.* × *Fragaria* sp. has been published by several authors (Remphrey, 1993; Chunfeng, 2008; Sutan, 2010). Nevertheless, for investigated species the culture media and cultivation conditions, inducing callus formation were selected. (Kitaeva, 2011, 2012; Laskar, 2005; Jiwen, 2003; Remphrey, 1993; Chun-feng, 2008; Stupnitsky, 2007).

It is noteworthy that during our investigations it was found that the morphology of callus was determined by the species of the plants, the type of explants and the culture medium composition. The possibility of callus formation from different explants was shown. The obtaining of microshoots in *P. recta* and *P. inclinata* through callusogenesis on Pierik and MS media with NAA, IAA, BAP and kinetin at different concentrations and combinations was demonstrated.

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Oleksii Zaiats, Irina Mitrofanova

POŽYMIŲ KAI KURIOMS *POTENTILLA* L. GENTIES RŪŠIMS SUTEIKIMAS MORFOGENEZĖS *IN VITRO* BŪDU

Santrauka

Nustatytas optimalus sterilizavimo *in vitro* protokolas kai kurioms *Potentilla* L. rūšims. Kaip antiseptikus panaudojus etanolį ir „Dez Tab" buvo gauti nuo 15 iki 100 % sterilūs eksplantai. Priklausomai nuo augalo rūšies gyvybingumas svyravo nuo 0 iki 75 %. Kaliaus diferenciacija iš skirtingų eksplantų, daigų ir mikroinjekcijų regeneracija atlikta modifikavus Murashige ir Skoog, Quoirin ir Lepoivre, Pierik ir Monnier būdus.

Raktažodžiai: *Potentilla* L., sterilizacija, morfogenezė, *in vitro*