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L.I. Tikhomirova, A.A. Kechaykin*, A.I. Shmakov, O.V. Alexandrova AN EFFECTIVE WAY TO CARRY OUT MASS IN VITRO PROPAGATION OF POTENTILLA ALBA L.

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Despite the plant's extensive area of distribution, *Potentilla alba* L. natural resources are scarce and cannot meet the modern needs of the pharmaceutical industry. Because of the mass preparation of medical raw materials by using *P. alba*, it entered into the list of rare and endangered species plants of the Red Data Book of the Republic of Belarus. This plant is not represented in the wild flora of Western Siberia, but there is a great need for developing a method for the mass propagation of *P. alba* using *in vitro* culture in order to obtain a high-quality planting material. At the explant stage, the technique of the *P. alba* introduction into *in vitro* culture is developed. This paper reveals the morphogenetic features of the development of *P. alba* explants of different types and the regenerative capacity of the tissue culture. At the micropropagation stage, the optimum culture media and the growth conditions for the regenerated plants are selected. At the stage of test-tube plants rooting and transferring them into *ex vitro* conditions, the most effective means of adaptation to non-sterile conditions in hydroponics are proposed.

Key words: explant, regenerated plant, growth medium, micropropagation, rooting, adaptation.

INTRODUCTION

Potentilla alba L. (Rosaceae) entered the official medicine more than 30 years ago (Kitaeva, 2014). Scientific studies of chemical composition of roots and aerial parts, as well as investigation of pharmacological activity of secondary metabolites, report the importance of P. alba in traditional and modern phytotherapy (Bashylau, 2012; Dorman et al., 2011; Shikov et al., 2011) The plant becomes suitable for harvesting in its third or fourth year of vegetation (Kosman et al., 2013); in this period, the concentration of its active substance – albinin – reaches its maximum level. It is known that the plant P. alba contains carbohydrates (starch), iridoids, saponins, phenol carbonic acids, flavonoids and tannins (Semyonova & Presnyakova, 2001; Lavrenov & Lavrenova, 1999; Gritsenko & Smyk, 1977). Medicinal products created using P. alba affect the thyroid gland (Arkhipova, 2012). Despite the fact that the plant is most commonly used in the treatment of thyroid hyperfunction (thyrotoxicosis), a positive effect on the treatment of thyroid hypofunction has been also noted (Prikhod'ko, 1976; Smyk & Krivenko, 1975). The thyrotropic activity of the P. alba rhizomes explains why, after the Chernobyl accident,

there were very few cases of endemic goiter in Belarusian Polesie in comparison with the other regions adjacent to the site of the accident, because of the widespread practice of using a *Potentilla* decoction instead of tea (Lavrenov & Lavrenova, 1999). In addition, phytotherapists recommend the use of *P. alba* in the prophylaxis and therapy of cardiovascular, gastrointestinal and liver diseases, as an antiseptic, and as a means to heal wounds (Shimko & Khishova, 2010). Despite the plant's extensive area of distribution, *P. alba*'s natural resources are scarce and cannot meet the modern needs of the pharmaceutical industry (Smyk, 1976). As a result of the mass preparation of medical raw materials using *P. alba*, it entered the list of rare and endangered species of wild animals and plants that is included in the Red Data Book of the Republic of Belarus in 2004 (Kitaeva, 2014).

Despite all the experiments in morphogenesis and regeneration of plants in tissue culture (Zaiats, 2013; He et al., 2006), the systems of *in vitro* propagation for *P. alba* are still poorly developed. It is caused by the lack of clear, well-reproducible methods, their complexity, poor knowledge on the morphogenetic potential of organs and tissues, and on the ways of morphogenetic control for this culture.

Since the plant is not represented in the wild flora of Western Siberia, but there is a great need for it there, we set the aim of developing a method for the mass propagation of *P. alba* using *in vitro* culture in order to obtain a high-quality planting material.

MATERIALS AND METHODS

Plant materials. P. alba of the collection of Siberian Research Institute of Horticulture (Barnaul) were used in the study.

For the buds of the *P. alba* vegetative shoots we used disinfectants from the different groups separately, and also combined, in five variants. The plants were washed with soapy water and rinsed with pure water. The accessory buds located at the leaf's base were isolated with a scalpel. The buds were soaked in the sterilizing agent solution for 10 minutes, taken out and washed in sterile distilled water five times. The sterile buds were transferred to a Petri dish and used for the isolation of meristems. On the slide glass, the bud was released from numerous leaf flakes using a dissecting needle and scalpel; then, we isolated the meristem tip with one or two leaf primordia with the scalpel, while holding the bud with the needle.

When the flower fragments are introduced into the *in vitro* culture, the stage of the flower development is crucial for the plant's successful regeneration. It is necessary to take unopened flowers of medium size.

The mercuric chloride solution 0.1% was used as a sterilizing agent and sterilization time was 7 min. This technique provided 85% sterile material (Table 1).

Culture media conditions. The experimental studies using tissue culture methods were carried out using conventional techniques (Vechernina, 2004; Butenko, 1999; Kalinin et al., 1980). The plant tissue was cultured in growth media MS (Murashige & Skoog, 1962). The growth media was supplemented with phytohormones of a cytokinin type of action – 6-benzylaminopurine (6-BAP) and kinetin, and an auxin type of action – indolebutyric acid (IBA), α -naphthaleneacetic acid (NAA). Gibberellic acid (GA) was also used.

The meristem, isolated in the laminar box, was transferred into the flask with the growth medium. The flask was covered with foil and put into the light climatic chamber at a temperature of +25 ° C. After four weeks, the number of formed buds was indicated, and they were used for the next stage of the micropropagation process. At this stage, the following media were used: # 1 – MS+1.0 µM kinetin, # 2 – MS+1.0 µM 6-BAP, # 3 – MS+ 2.5 µM 6-BAP, # 4 – MS+1.0 6-BAP+0.5 IBA+ 0.05 GA, # 5 – MS+0.5 6-BAP+0.5 IBA+ 0.05 GA. For the control, we used a hormone-free medium based on MS.

The plants were grown in laboratory conditions using artificial light (2000–4000 lx) with a photoperiod of 16 hrs of light followed by 8 hrs of dark and in a temperature range of 24-26 °C.

Statistical data processing was performed in Excel 2007 using standard indicators.

The sterilization technique	Chlorhexidine 0.5 mg/l	Mercuric chloride solution 0.2%	70% ethanol + 3% hydrogen peroxide (1:1)	96% ethanol 3% hydrogen peroxide (1:1)	Sulfohlorantin solution 6%
microbial contamination, %	100	60	100	90	80
Viability, %	60	50	70	40	70

Table 1. The influence of the sterilization technique on the viability and infectivity of the *Potentilla alba* vegetative shoot buds (exposure 10 min).

RESULTS AND DISCUSSION

Introduction of Potentilla alba into in vitro culture

The vitality of the explants was high under all tested sterilization methods. The microbial contamination was low during the first 7-10 days, which indicates the quality of the surface sterilization, but later it reached higher values, probably due to an internal infection (Table 1). When the apical meristems of *P. alba* are cultured on the growth media with high 6-BAP, the effect of this hormone causes the apical meristem dominance to be suppressed and the axillary meristems are activated. As a result, about two weeks after the transfer of the isolated apex (including the meristem tip and one or two leaf primordia) to the growth medium, the bases of the unfolding leaves start to turn green, increase their volume, and leaf bundles appear from them. At the bases of the new leaves, new primordia again begin germinating after some time. After one or two months, the explants become a conglomerate of buds of different ages and sizes with unfurled leaves.

The primary explant buds' development depended on the growth medium composition. When using only 2,5 μ M of 6-BAP, the mass of the buds was set, but the shoots were small, which hampered their further separation and replanting in the media. The auxins enabled the shoots to stretch out, and the gibberellic acid intensified this effect (Figure 1). After 20–30 days, the developed shoots were replanted in the propagation media.



Figure 1. The Potentilla alba explants in medium # 4

The use of buds as explants is complicated by their high infectivity and the low output of the sterile material. *P. alba* can also be introduced into tissue culture using flower fragments.

The pedicels and receptacles were used as explants. In 19–25 days, full vegetative shoots were regenerated (Figure 2). The resulting buds were easily separated from each other. After replanting into the new growth medium, each of them formed shoots and new auxiliary buds, thereby increasing the number of growth points.



Figure 2. The *Potentilla alba* explants: the pedicel (a) and the receptacle (b) in the MS medium supplemented with 20.0 μ M 6-BAP and 1.0 μ M IBA

micropropagation stage of <i>Potentilla alba</i>					
Number of the growth medium variants	The hormonal composition of the growth medium, μM	The plant's height, mm	The propagation factor	The presence of roots	
Control	hormone-free	20.1±0.1	1.0	yes	
# 1	1.0 kinetin	17.3±0.2	1.8±0.1	yes	
# 2	1.0 6-BAP	15.7±0.2	2.5±0.5	no	
# 3	2.5 6-BAP	17.2±0.8	5.5±0.1	no	
# 4	1.0 6-BAP+0.5 IBA+ 0.05 GA	12.3±0.3	9.3±0.6	no	
# 5	0.5 6-BAP+0.25 IBA+ 0.05 GA	15.3±0.1	7.3±0.2	no	

Table 2. The hormonal composition of the growth media based on MS at the micropropagation stage of *Potentilla alba*

The micropropagation of Potentilla alba

To increase the propagation factor, the resulting shoots were replanted in the propagation media (Table 2). The introduction of 1.0–2.5 μ M 6-BAP to the growth medium induced the maximum regeneration and development of the

auxiliary and adventive buds of *P. alba*. In some cases, it was possible to get up to 20 buds from one passage. However, in the case of the prolonged passaging of these media, the newly formed shoots had morphological changes, rooted poorly and died, which was probably caused by a higher than necessary accumulation of the phytohormone in the tissues. To eliminate the recurrence of this phenomenon, it is necessary to alternate the media with different concentrations of phytohormones. The use of kinetin on media # 1 resulted in the smallest quantity of auxiliary and adventive buds.

According to our data, the optimum concentration of phytohormones for the *P. alba in vitro* micropropagation is 1.0 μ M BAP + 0.5 μ M IBA+0.05 μ M GA (medium # 4). The resulting conglomerated microshoots were easily divided into single ones and transferred to the media containing 0.5 μ M BAP+0.25 μ M IBA+0.05 μ M GA (# 5). The number of shoots from one explant for one passage was 3-9 pieces. Thus, the media were alternated at each passage. Moreover, after the use of medium # 5 the large microshoots specimens were planted into the rooting medium, and the small ones were planted into medium # 4 for further propagation.



Figure 3. The rooting stage of Potentilla alba

The resulting shoots with a height of not less than 20 mm were transferred to the rooting medium. The growing medium contained 50% of the mineral salts concentration by the MS prescription with the addition of 3 μ M NAA, 30 g/l sucrose, and 7–9 g/l agar at pH 5.6-5.8. On the 20–24th day of rooting, the *P. alba* shoots had roots and reached the standard size (Figure 3).

Acclimatization of in vitro plants

The staged system of the adaptation of *P. alba* regenerated plants to *ex vitro* conditions was developed. For the rhizogenesis stimulation and easier transfer to the soil conditions, the adaptation to the non-sterile conditions was carried out at the hydroponic installation using ¹/₄ of the MS medium's mineral composition (Figure 4).



Figure 4. The regenerated *Potentilla alba* plant after its adaptation to the hydroponic installation

After 25–30 days of adaptation, the seedlings were replanted into pots with soil. The final growth period lasted for 30 days in winter greenhouses. 90% from the total number of regenerated plants successfully completed the adaptation and final growth. As a result of the growth, we had standard seedlings of *P. alba* with closed root systems, ready to be replanted into the open ground (Figure 5).



Figure 5. Potentilla alba closed root seedlings

On the basis of the theoretical calculation using the formula $Bn = B1 \times G^{(n-1)}$ (where *n* is the number of culture months, *B1* is the number of microshoots, *G* is the propagation factor) and using the proposed culture technique, it is possible to get 177147 *P. alba* regenerated plants from one plant over 12 months, if the propagation factor is 3.0. The proposed method of producing the *P. alba* planting material is industrially applicable and allows for the possibility to create high-quality seedlings while simplifying the steps in the process (Russian Federation Patent # 2525676). The tissue culture application helps to reduce the material and labour costs and production areas.

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REFERENCES

Arkhipova, E.V. (2012). The influence of Potentilla alba L. extract and the

complex agent «Tireoton» on the experimental hypothyroidism. Thesis

of Doctoral Dissertation. Ulan-Ude, Russia. (In Russian).

Bashylau, A.V. (2012). To a question on a pharmacolo-biachemical substantiation of practical of *Potentilla alba* L. *Proceedings of the National*

Academy of Sciences of Belarus 1, 3-7.

- Butenko, R.G. (1999). Biology of higher plant cells *in vitro* as the basis for biotechnology. FBK-PRESS, Moscow. (In Russian).
- Damien Dorman, H.J., Shikov, A.N., Pozharitskaya, O.N. & Hiltunen, R. (2011). Antioxidant and pro-oxidant evaluation of a *Potentilla alba* L. rhizome extract. *Chemistry & Biodiversity* 8(7), 1344-1356. doi: 10.1002/cbdv.201100043.
- Gritsenko, O.M. & Smyk, G.K. (1977). The phytochemical study of *Potentilla alba*. *Pharmaceutical journal* 32(1), 88-92. (In Ukrainian).



- Kalinin, F.A., Sarnatskaya, V.V. & Polischuk, V.E. (1980). Tissue culture methods in plants physiology and biochemistry. Kiev. (In Russian).
- Kitaeva, M.V., Kot, A.A. & Spiridovich, E.V. (2014). The comparative analysis of species *Potentilla* L. – *Potentilla alba* L., *Potentilla recta* L., *Potentilla rupestris* L. as the producers of biologically active substances by secondary metabolities in the Central region agroclimatic conditions of the Republic of Belarus. *Bulletin of Bryansk dpt. of RBS* 1(3), 67-70. (In Russian).
- Kosman, V.M., Faustova, N.M., Pozharitskaya, O.N., Shikov, A.N. & Makarov,
 V.G. (2013). The accumulation of biologically active substances in the underground parts of *Potentilla alba* L. depending on the culture period. *Chemistry of Plant materials* 2, 139-146. (In Russian). doi: 10.14258/jcprm.1302139.
- Lavrenov, V.K. & Lavrenova, G.K. (1999). The Complete Encyclopedia of Medicinal Plants, Vol. 1. St.-Petersburg. (In Russian).

Biological Bulletin of Bogdan Chmelnitskiy Melitopol State Pedagogical University 443 Murashige, T. & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bioassay with Tobacco Tissue cultures. *Physiologia Plantarum* 15(3), 473-497. doi: 10.1111/j.1399-3054.1962.tb08052.x.

- Prikhod'ko, E.I. (1976). Treatment of patients with thyrotoxicosis by the grass of *Potentilla alba*. *Vrachebnoye Delo* 6, 66-71. (In Russian).
- Semyonova, E.F. & Presnyakova, E.V. (2001). The chemical composition of Potentilla alba and its use for therapeutic purposes. Chemistry and Computational Simulation. Butlerov Communications 2(5), 32-36. (In Russian).
- Shikov, A.N., Lazukina, M.A., Pozharitskaya, O.N., Makarova, M.N., Golubeva, O.V., Makarov, V.G., & Djachuk, G.I. (2011). Pharmacological evaluation of *Potentilla alba* L. in mice: adaptogenic and central nervous system effects. *Pharmaceutical Biology* 49(10), 1023-1028. doi: 10.3109/13880209.2011.560162.
- Smyk, G.K. & Krivenko, V.V. (1975). Potentilla alba as an effective way of thyroid diseases treatment. Pharmaceutical Journal 2, 58-62. (In Ukrainian).
- Smyk, G.K. (1976). The use of *Potentilla alba* as a new medical plant, restoration of its natural reserves and culture possibilities. In: Smyk G.K., New cultures in the economy and Medicine. Medicine, Moscow. (In Russian).



Shimko, O.M. & Khishova, O.M. (2010). The assessment of the *Potentilla alba* grass. *Journal of Pharmacy* 1(47), 17-24. (In Russian).

- Vechernina, N.A. (2004). Biotechnology methods in plant breeding, propagation and gene pool preservation. ASU, Barnaul. (In Russian).
- Zaiats, O. & Mitrofanova, I. (2013). Features of induction of morphogenesis *in vitro* in some species of genus *Potentilla* L. *Biologija* 59(3), 309-318.

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