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Development of the *in vitro* micropropagation protocol for the conservation of the species *Pulsatilla grandis* Wend

Lilia Brînză, Tatiana Călugăru-Spătaru, Mariana Lozinschii, Eduard Coropceanu, and Ion Botezatu

Abstract. *Pulsatilla grandis* Wend, known as the greater pasque flower, is a perennial species from the *Ranunculaceae* family, originating in Europe and Western Asia. In the Republic of Moldova, it is a species with endangered plant status. The distribution of *P. grandis* Wend in our country is limited, with some representatives found in certain protected areas and nature reserves. The plant holds pharmacological value, being utilized in folk medicine as a sedative, analgesic, diuretic, expectorant, etc. Due to its vulnerable status, conserving the natural habitats of *P. grandis* Wend is crucial. One of the conservation methods is *in vitro* micropropagation, which allows for the fast multiplication of plants and their reintroduction into nature or botanical gardens. In a recent study, *P. grandis* Wend seeds were used for micropropagation, utilizing the ½ Murashige-Skoog medium. It was found that the addition of benzyladenine (BA) to the culture medium improved the micropropagation coefficient, although a second stage was necessary for inducing rhizogenesis. Supplementation of the culture medium with NAA (1-Naphthaleneacetic acid) and IBA (Indole-3-butyric acid) (each at 0.1 mg/L) facilitates rooting and improves shoot robustness.

Keywords: *Pulsatilla grandis* Wend, *in vitro*, micropropagation, nutritive medium, conservation methods, pharmacological value.

Elaborarea protocololului de micropropagare *in vitro* pentru conservarea speciei *Pulsatilla grandis* Wend

Rezumat. *Pulsatilla grandis* Wend, cunoscută sub denumirea de *dediţelul-mare*, este o specie perenă din familia *Ranunculaceae*, originară din Europa și Asia de Vest. În Republica Moldova este o specie cu statut de plantă periclitată. Distribuția speciei *P. grandis* Wend în țara noastră este limitată, cu unele exemplare identificate în arii protejate și rezervații naturale. Planta are valoare farmacologică, fiind utilizată în medicina populară ca sedativ, analgezic, diuretic, expectorant etc. Din cauza statutului său vulnerabil, conservarea habitatelor naturale ale *P. grandis* Wend este esențială. Una dintre metodele de conservare este micropropagarea *in vitro*, care permite multiplicarea rapidă a plantelor și reintroducerea acestora în natură sau în grădini botanice. Într-un studiu recent, au fost utilizate semințele de *P. grandis* Wend pentru micropropagare, folosindu-se mediul ½ Murashige-Skoog. S-a constatat că adăugarea de benziladenină (BA) în mediul de cultură a îmbunătățit coeficientul de micropropagare, deși a fost necesară o a doua etapă pentru

inducerea rizogenezei. Mediul nutritiv suplimentat cu NAA (acid naftilacetic) – 0,1 mg/L și IBA (acid indol-3-butiric) – 0,1 mg/L contribuie atât la inițierea și dezvoltarea sistemului radicular, cât și la obținerea unei consistențe mai viguroase a lăstarului.
Cuvinte-cheie: *Pulsatilla grandis* Wend, *in vitro*, micropropagare, mediu nutritiv, metodă de conservare, valoare farmacologică.

1. Introduction

Plant biodiversity is essential to sustain human life on Earth. There are about 500 thousand plant species worldwide, and about 10% of these are used globally for various purposes such as food, fodder, fiber, medicine and horticulture. It is estimated that over 21 percent of all known vascular plants are endangered or threatened with extinction due to habitat loss, over-exploitation and fast climate change. It is important to preserve biodiversity and effectively use plant-based chemical compounds to solve problems in various fields. Recently, there has been intense activity in the development of new efficient biotechnologies, often based on the use of various chemical compounds with a stimulating effect, increasing resistance to adverse conditions, etc [2, 13].

To ensure sustainable development and ensure the continuity of the harmonious development of human society with nature, it is necessary to attract students to research activities. In order to answer current ecological challenges, it is essential to promote an education focused on inquiry and transdisciplinarity, which plays a crucial role in shaping a generation that is environmentally responsible [10].

Pulsatilla grandis Wend, also known as the common milkweed, is a species of perennial, hemicryptophytic plant in the family *Ranunculaceae*, native to Europe and Western Asia (Figure 1). In the Republic of Moldova, *P. grandis* Wend is an endangered plant species and is found mainly in primary steppe sectors, in the glades and steppe-like edges of downy oak forests. The distribution of this species in the Republic of Moldova is limited and can be observed in the districts of Ocnita, Dubasari, Grigoriopol, Calarasi, Straseni, Hincesti, Anenii Noi, the species is at the southern limit of its distribution. Specimens of *P. grandis* Wend can also be found in some protected areas, nature reserves of the Republic of Moldova, where they are conserved and protected against habitat degradation and anthropogenic pressure. The areas in which *P. grandis* are found are areas with moist and forested soils, less cultivated or left fallow, areas with sandy and well drained soils. It is important to note that *P. grandis* Wend is a sensitive species and may be threatened by habitat loss, harvesting, overgrazing, intensive agricultural practices and other environmental threats in the Republic of Moldova. As a result, conservation and protection of

the natural habitats where this species occurs are essential for maintaining healthy wild populations and protecting biodiversity in the Republic of Moldova [1, 5, 9, 14, 17].

Figure 1. Morphological aspect of *Pulsatilla grandis* Wend: A - early flowering stage; B - stages of flowering and fruit formation; C - fruiting stage.

Plants of *P. grandis* Wend have a long rhizome, which may be upright or oblique. The upright stems are covered with slender hairs, ranging in size from 5.0 to 30 cm, but can grow up to 40 cm at fruiting time in May. The leaves at the base of the stem are rosette-shaped, covered with hairs and deeply incised. The other leaves are elongated, finger-like and divided into three segments. *P. grandis* Wend blooms from March to April, with large, solitary flowers with numerous stamens and pistils, which appear before or with the leaves. The velvety petals are protected by a large number of peripodia. The fruit is a nut, giving the plant a decorative appearance during fruiting.

In folk medicine, *P. grandis* Wend is used as a sedative, analgesic, diuretic and expectorant. It is also used in homeopathy. *P. grandis* Wend is poisonous because it contains a significant amount of essential oils, which includes anemonol. But in the process of drying the plant anemonol is converted to anemonin, which determines its pharmacological value: bactericidal, antitumor, antispasmodic activity with a broad spectrum of action and sedative properties. In modern Western medicine (USA and Western Europe), lumbago is known as a sedative, analgesic, antispasmodic, diuretic and expectorant; it is included in the French and English Pharmacopoeias. This plant provides bees with a large amount of nectar. It is also used in veterinary medicine. Some species are grown as ornamental plants.

P. grandis Wend is a protected plant with endangered status, which is increasingly rare due to over-harvesting. Seed germination capacity in open ground is very low. Conservation of endangered plant species through *ex situ* conservation, including *in vitro* culture, prevents biodiversity loss and allows the transfer of propagated plants to natural

habitats or botanical gardens. In the field of biotechnology, various plant conservation methods and techniques are available. Among these, *in vitro* culture is one of the most important and effective techniques for ex situ conservation of various endangered plant species, including representatives of the species *P. grandis* Wend. Modern *in vitro* culture techniques, compared to traditional propagation methods, allow much faster plant reproduction. Micropropagation is an advanced technique used for vegetative propagation, which results in the generation of clone plants with similar characteristics of the mother plants, being healthy and of high quality. Using this method, a whole plant can be produced from a section of leaf, shoot, stem, meristematic tissue, etc. Plants generated by *in vitro* culture have a well-developed root system, vigorous stems and show increased resistance to diseases and pests.

The aim of the current research was to develop successive stages of micropropagation involving initiation of culture, multiplication of micro-rootstocks and stimulation of their rhizogenesis to generate new plants of *P. grandis* Wend and to protect the germplasm of this endangered species.

2. Materials and Methods

The research presented in the article was carried out in the ”Plant Genetics, Physiology and Biochemistry” laboratory of the ”Plant Biology” Department of ”Ion Creanga” State Pedagogical University of Chisinau.

Seeds of *P. grandis* Wend (Figure 2) were used as research material. To ensure sterilization, the achenes were sterilized with the chlorine-based preparation for 15 min and then washed three times with sterile water. These light, bristly fruits spread efficiently over long distances, helping the plant to colonize new habitats. The dried achenes (fruits) were collected. The beads that facilitate anemocorous dispersal can be seen (Figure 2).

Figure 2. Morphological appearance of achenes (A, B, C) of *P. grandis* Wend

Inoculation of plant explants was carried out under aseptic conditions in a sterile airflow laminar. Aseptic achenes of *P. grandis* Wend were placed for germination on half-strength Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) [11] devoid of phytohormones but supplemented with 100 mg/L myo-inositol, 0.2 mg/L thiamine, 0.5 mg/L pyridoxine, 0.5 mg/L nicotinic acid, 2.0 mg/L glycine, 30 g/L sucrose and 6.0 g/L agar.

Culture media were adjusted to pH 5.8 before autoclaving at 121°C for 21 min. Germination was carried out in the culture chamber at 25 ± 2°C with 16/8 h photoperiod (light/dark cycle) provided by cool white fluorescent bulbs (1500-3000 Lx).

3. Results and Discussions

In the literature, information on micropropagation of *Pulsatilla* specimens, especially *P. grandis* Wend, is very limited and incomplete. The protocol developed by Zabicka [15] on the micropropagation of *P. grandis* Wend plants was not reproducible, although the same culture conditions, explants, plant growth regulators, culture media were used. Only a few important contributions that have been published present in vitro culture protocols of European [12, 15, 16] and Asian [7, 8] species of *Pulsatilla*.

The first stage of micropropagation of *P. grandis* Wend seedlings consisted of germination of sterile achenes on half-strength MS medium under in vitro conditions. Thus, despite drastic treatment to decontaminate the surface of the achenes, it amounted to about 90%. Research by some investigators [6, 16] also demonstrated a high germination percentage, given in dependence on the culture substrate. Inoculated *P. grandis* Wend achenes germinated after 25 days of cultivation. Seedling growth and development was normal. The appearance of the developed mini-sprouts is shown in Figure 3. During the propagation process, some of the regenerated rosettes were in poor condition with anthocyanin staining, and others dried over time.

During the second stage of micropropagation, two variants of the MS 100% medium were tested: 1 – without phytohormones (Figure 3 D; E); 2 – supplemented with BA+ charcoal – 0.1; 0.3; 0.5 mg/L (Figure 3 A; B; C); and MS 100%: 3 – without phytohormones but supplemented with activated charcoal. Mini-rootstocks multiplied on both media tested, but the highest multiplication coefficient was determined in the second variant with the presence of BA.

On culture media supplemented with BA, increased induction of lateral shoots on the axis of the seedling was observed, which favors a shoot, which later serves as a source of biological material for micropropagation and increased clone number. The

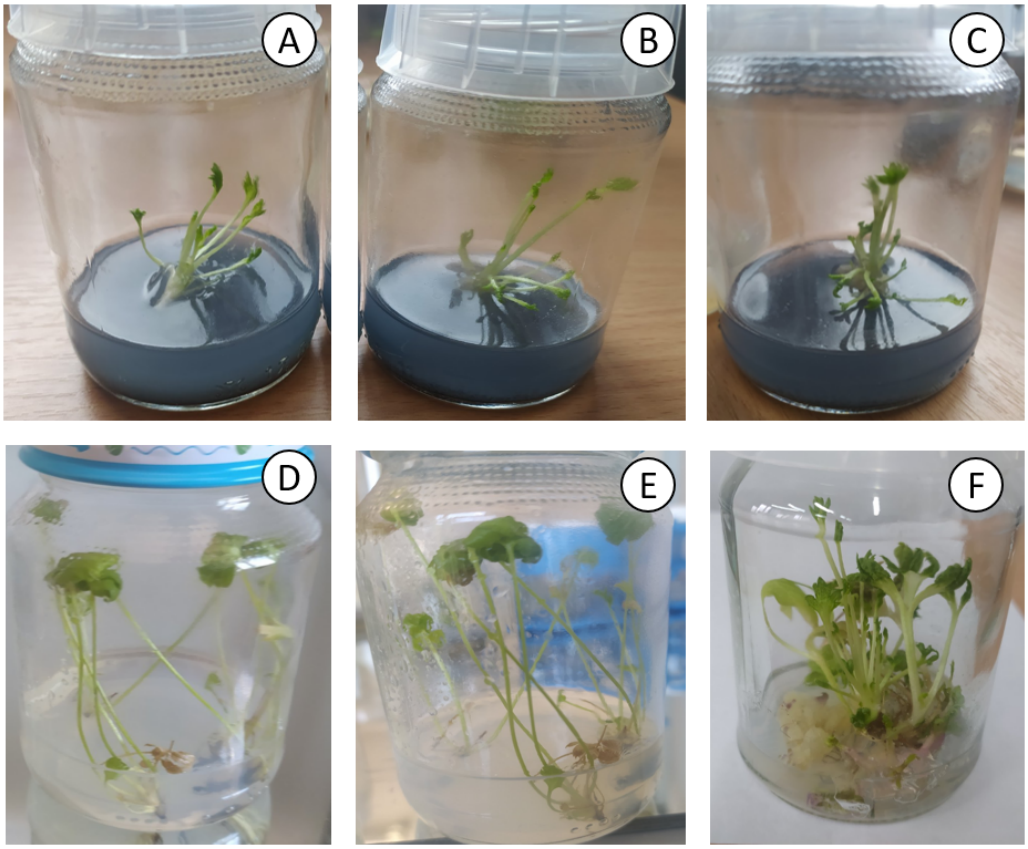

Figure 3. Seedlings of *P. grandis* Wend grown under *in vitro* conditions on ½ MS medium: A – MS + 0.1 mg/L BA + coal; B – MS + 0.3 mg/L BA + coal; C – MS + 0.5 mg/L BA + coal; D – MS 50%; E – MS 100%; F – MS + BA 0.5 mg/L + NAA 0.1 mg/L.

micropropagation coefficient was 5-7 mini-rootstocks per explant on medium No. 5 (Figure 3 B; C; F).

Increasing the concentration of BA in the nutrient medium from 0.1 to 0.3 mg/L improves the vigor of the rosette (from moderate to vigorous), and contributes to the formation of lateral shoots in number 1.50-3.00 but the shoot height remains constant (6-7 cm). Nutrient medium No. 5 supplemented with cytokinin BA 0.5 mg/L only, without auxin, produces moderate callus, marking the beginning of organogenesis but a poorly developed or even absent root system, which is expected, as cytokinins stimulate cell division and multiplication without supporting rooting. On nutrient medium No. 6,

Table 1. Evaluation of the effect of phytohormones on in vitro development in *P. grandis* Wend

No.	Nutrient medium	Number of explants per inoculum	Shoot height. cm	Presence of callus	Rosette vigor	Root system
1.	**MS ½**	1.00 ± 0.02	6.04 ± 0.12	-	+	Poorly developed
2.	**MS 100%**	1.50 ± 0.01	6.11 ± 0.07	-	++	Poorly developed
3.	**MS BA 0.3 mg/L**	3.00 ± 0.05	7.23 ± 0.09	-	+++	Poorly developed
4.	**MS BA 0.1 mg/L**	1.50 ± 0.02	6.19 ± 0.18	-	+++	Poorly developed
5.	**MS BA 0.5 mg/L**	7.00 ± 0.08	5.41 ± 0.07	+	+++	Poorly developed
6.	**MS BA 0.5 mg/L + NAA 0.1 mg/L**	6.00 ± 0.06	7.38 ± 0.10	++	++++	Developed
7.	**MS NAA 0.1 mg/L**	1.00 ± 0.02	6.98 ± 0.10	-	+++	Moderately developed
8.	**MS IBA 0.1 mg/L**	1.00 ± 0.01	6.79 ± 0.18	-	+++	Moderately developed

Note: '**MS**' - Murashighe and Scoog, '**½ MS**' half salt concentration, '**100% MS**' - full salt concentration; '**-**' no root; '**+**' root present.

this combination produced more abundant callus and excellent vigor along with the development of a consistent root system, confirming the effectiveness of the auxin:cytokinin relationship in concomitant use.

Nutrient medium No. 6 gives the best result: shoot length 7 cm, robust rosette, induction of lateral shoots, presence of callus and a well-developed root system. Nutrient media No. 7 and No. 8 (Figure 4 C; D) did not induce axial shoot establishment but contributed to root system initiation and development and more vigorous shoot consistency compared to growth on No. 1 and No. 2.

The rest of the nutrient media do not generate callus, forming a poorly developed root system, although the rosette is well formed, emphasizing the importance of hormone supplementation.

The study by Priede and Klavina [12] demonstrated that these plants are difficult to root and it is usually necessary to develop a multi-step protocol for the induction of rhizogenesis. Rooting difficulties may be related to fluctuations and deteriorations in the content and activity of some biochemical parameters, which could be considered as markers of rhizogenesis. These may include the content of phenolic compounds, carbohydrates and the activity of antioxidant enzymes, which would change during successive rooting stages [3, 4, 6]. During 4 weeks of cultivation, the mini-rhizomes of the phytohormone-free variant (variant no. 1) regenerated the root system more efficiently compared to the rhizomes grown on the medium containing cytokinin BA. This may be due to activated charcoal, which was included in the culture medium to reduce tissue oxidation due to phenolic compounds removed during explant growth and development. In parallel with the formation of mini-rootstocks, callus formation was also observed, which had a crumbly, whitish, non-morphogenic consistency (Figure 4 B). The explants from the BA-containing variant were subsequently transferred to medium devoid of phytohormones but supplemented with activated charcoal to induce root system formation.

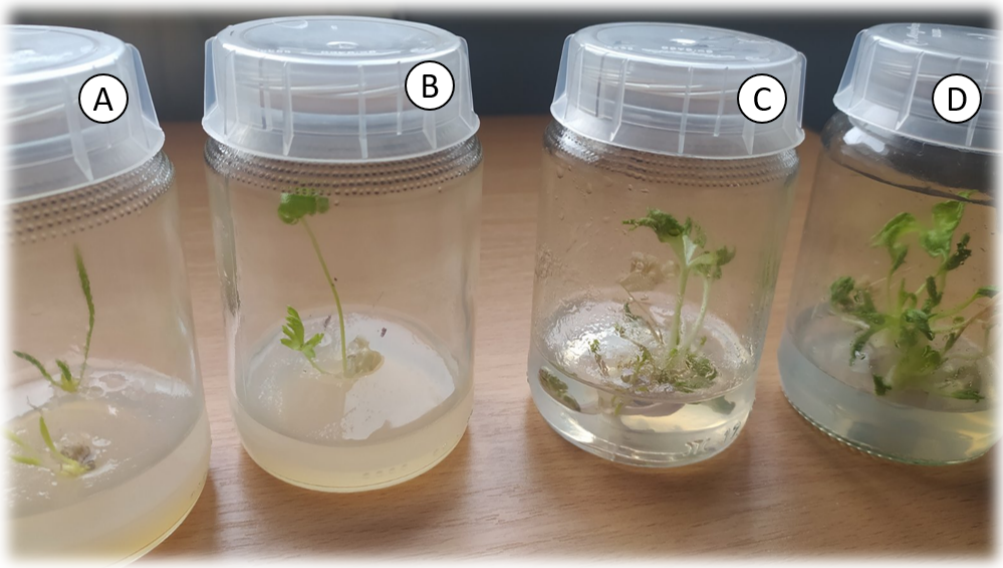

Figure 4. Seedlings of *P. grandis* Wend grown under *in vitro* conditions on MS medium: A, B - without phytohormones; C, D - in the presence of IBA.

Another option would be to develop nutrient media combined with cytokinin/auxin hormone balances, as in nutrient media No. 6. Nutrient media No. 7 and No. 8 supplemented with NAA - 0.1 mg/L and IBA - 0.1 mg/L contribute to the initiation and development of the root system and more vigorous shoot consistency.

Therefore, the micropropagation of *P. grandis* Wend plants grown on half-strength MS medium lacking phytohormone but supplemented with activated charcoal can be achieved in a single step with mini-rootstock formation and parallel root formation, compared to plants grown in the presence of BA, which have to go through two steps (mini-rootstock formation and subsequent induction of rhizogenesis). We note that the micropropagation coefficient in the BA variant is higher than in the phytohormone-free variant.

4. Conclusions

(1) The use of ½ Murashige-Skoog nutrient medium induced seed germination and subsequent seedling growth and development of *Pulsatilla grandis* Wend.

(2) The micropropagation coefficient of *Pulsatilla grandis* Wend seedlings in the 100% Murashige-Skoog variant supplemented with BA was higher than that in the phytohormone-free variant.

(3) The combination of BAP + NAA in the nutrient media gives the best practical results: stimulation of shoot growth, initiation of callus mass, development of lateral shoots, formation of root system. The nutrient medium supplemented with NAA and IBA in minimal amounts contributes to root system initiation and development and more vigorous shoot consistency.

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(Lilia Brînză, Eduard Coropceanu, Ion Botezatu) "Ion Creangă" State Pedagogical University of Chisinau
E-mail address: brinza.lilia@upsc.md, coropceanu.eduard@upsc.md, botezatu.ion@upsc.md

(Tatiana Călugăru-Spătaru) Institute of Genetics, Physiology and Plant Protection, Moldova State University
E-mail address: tcalugaru@yahoo.com

(Mariana Lozinschii) Centre of Excellence in Medicine and Pharmacy "Raisa Pacalo", Chisinau
E-mail address: marianalozinschii@gmail.com