# *In vitro* cultivation and root initiation of the endangered plant *Pulsatilla patens*

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**Abstract**

*Pulsatilla patens* (L.) Mill. is endangered due to habitat loss and degradation by various factors including heavy overgrowth of vegetation and a dense moss layer. In the present study, a suitable rooting medium for the difficult-to-root species *P. patens* was developed on the basis of multistage rooting experiments. The best rooting was obtained in darkness for five days on a half-diluted Murashige-Skoog medium supplemented with 5 mg $L^{-1}$ α-naphthaleneacetic acid and riboflavin followed by auxin photooxidation. It was concluded that, for *in vitro* formation of good quality roots on *P. patens* explants, it is important to provide exogenous auxins during root induction and to reduce auxin content during the expression stage. In vitro rooted plantlets of *P. patens* successfully acclimatized *ex vitro* in a peat substrate mixed with sand, and they developed well-branched roots and healthy leaf rosettes. After transplantation to semi-natural conditions, plants started to flower after a year.

**Key words:** auxins, *ex situ* conservation, *in vitro* propagation, plantlet establishment, *Pulsatilla patens*, rooting.
**Abbreviations:** IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige-Skoog; NAA, α-naphthaleneacetic acid.

## Introduction

*Pulsatilla patens* (L.) Mill. is one of the species under investigation in a wide study on conservation of the endangered flora of Latvia. This species has the largest distribution area of all of the highly polymorphic species of the genus *Pulsatilla* (Zāmels 1926). The species occurs in the submeridional and temperate zones of the north-eastern part of Central and Eastern Europe, western Siberia and North America. In Latvia, *P. patens* is distributed mainly in the eastern part of the country while it is quite rare in the western part. It mainly inhabits dry pine forests on fairly poor mineral soil, mesotrophic forests with pine and spruce, rich forests with a pine overstorey, slightly mixed with deciduous trees, forest margins and heath (Andrušaitis 2003).

P. patens has declined in the last decades and is included in the national Red Lists of many European countries and in Annex II and IV of the EC Habitats Directive as well as Annex I of the Bern Convention (Council of Europe 1979; Commission of the European Communities 1992; Ingelög et al. 1993). *P. patens* is included in the Red Data Book of Latvia in the category IV as a data-deficient species (Andrušaitis 2003). There are 100 to 200 known locations of *P. patens* in Latvia and it grows in 25 especially protected areas (V. Baroniņa, personal communication) but a comprehensive monitoring of these locations has not been performed during recent years.

Changes in habitat management, especially intensive forestry, are suggested as the main reasons for a decrease in the number of *P. patens* individuals (V. Baroniņa, personal communication). Picking of flowers takes place mainly in areas close to the largest cities. In addition to habitat reduction and degradation, heavy overgrowth by vegetation, including formation of a dense moss layer, are suggested as possible threats. Growth and regeneration of the species can be promoted by fire as this decreases the density of overgrowth vegetation and the thick moss and litter layers (Kalamees et al. 2005; Kalliovirta et al. 2006). Thinning of forest stands also benefits the growth of *P. patens*. In the seedling stage, *P. patens* requires well-illuminated habitats without competition from individuals of other plant species (Uotila 1969).

Identification of growth requirements and an *in vitro* propagation protocol were determined for *P. patens* were set as the first step in establishing a tissue bank for this species (Kļaviņa et al. 2004). However, difficulties in rooting of *P. patens* explants was experienced, which did not allow for *ex vitro* cultivation. The aim of the present study was to establish experimentally conditions for successful rooting of *P. patens in vitro*. In addition, *ex vitro* acclimatization and further development of micropropagated plants were monitored.

## Materials and methods

Ripe seeds of *Pulsatilla patens* (L.) Mill. were collected from a natural population in Dārziņi, Riga (56°52' N, 24°16' E). The

seeds were surface sterilized with an undiluted commercial bleach ACE containing 4.85% sodium hypochlorite for 10 min followed by three rinses with sterile distilled water. The sterilized seeds were individually aseptically placed into 19 × 110 mm test tubes containing 10 mL hormone-free half-diluted Murashige-Skoog (MS) medium supplemented with 0.1 g $L^{-1}$ myo-inositol, 0.5 mg $L^{-1}$ tiamine HCl, 0.5 mg $L^{-1}$ pyridoxine HCl, 0.5 mg $L^{-1}$ nicotinic acid, 20 g $L^{-1}$ sucrose and 5 g $L^{-1}$ plant agar (basal medium). The medium was adjusted to pH 5.8 with NaOH before autoclaving for 20 min at 121 °C. To promote germination the seeds maintained in a growth chamber at 22 to 25 °C under a 16-h photoperiod (40 μmol $m^{-2}$ $s^{-1}$ of photosynthetically active radiation). Effect of duration of seed storage was evaluated by initiation of cultures at different periods after seed collection. Fifteen seeds in three to five replications were used for germination studies.

To optimize basal medium composition for shoot multiplication, *in vitro* cultivation with different cytokinins (0.2 to 0.75 mg $L^{-1}$ 6-benzyladenine; 0.1 to 0.5 mg $L^{-1}$ topolin and 0.1 to 5 mg $L^{-1}$ kinetin) was performed. The cultures were placed in a growth chamber at 25 °C and illuminated with white fluorescent lamps with a 16-h photoperiod. Shoots obtained during the multiplication stage were used as explants in rooting experiments.

For rooting of *P. patens* shoots, basal medium with auxins indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA) was tested. Shoots were cultivated for rooting in test tubes (2.5 × 15 cm) with plastic caps, one explant per tube.

In the first series for root induction, explants were incubated for one week with one of the three auxins at concentrations of 2.5, 5 and 10 mg $L^{-1}$, after which they were transferred to a hormone-free medium. In the second experiment series, the basal medium with auxins IBA and NAA (1 and 5 mg $L^{-1}$) and two types of iron compounds (Fe-EDTA and Fe-EDDHA, 0.2 mM) (Molassiotis et al. 2004) were used. After a three-week induction period explants were placed on a fresh root expression medium without auxins supplemented with activated charcoal (2 and 20 g $L^{-1}$) plus the same Fe forms as in the induction stage. In the third series of experiments, basal medium with NAA (1 and 5 mg $L^{-1}$) and riboflavin (3.76 and 7.52 mg $L^{-1}$) and dark conditions for two, five or seven days were used. After this period, test tubes with shoots were transferred to light (16-h photoperiod) for auxin photooxidation. The number of roots was counted after four and six weeks.

In all rooting experiments, control explants were cultivated on an auxin-free medium. The rooting of 10 to 15 explants was evaluated in each treatment and expressed both as rooting percentage and root number per plantlet. The 95% confidence intervals of rooting and number of roots per plantlet in different media were used to determine statistical differences. Each series of experiments were repeated two to three times. Replicates showed similar results. Only the data from the representative experiment are reported.

Rooted plantlets were removed from culture tubes and planted into commercial neutralized peat or peat mixed with sand, cultivated in a growth chamber and illuminated with white fluorescent lamps using a 16-h photoperiod. In spring, after acclimatization to *ex vitro* conditions for six weeks, the plants were planted in single pots and placed outdoors. During the summer these plants were kept in a sunny place and sprayed with water when needed. In

**Table 1.** *Pulsatilla patens* shoot rooting induced with 1 mg $L^{-1}$ indole-3-butyric acid (IBA 1) and 1 or 5 mg $L^{-1}$ α-naphthaleneacetic acid (NAA 1, NAA 5) for three weeks and subsequent root emergence on hormone-free media with 2 or 20 g $L^{-1}$ activated charcoal (C 2, C20). *, Fe-EDTA; **, Fe-EDDHA 0.2 mM. Means followed by different letters within columns indicate significant differences (P = 0.05; n = 8 to 10).

| Root induction medium | Root expression medium | Rootings (%) | Number of roots |
|---|---|---|---|
| NAA 1* | C 2 | 20.0 b | 0.1 b |
| NAA 1* | C 20 | 22.2 b | 0.6 b |
| NAA 1** | C 2 | 33.3 b | 0.6 b |
| NAA 1** | C 20 | 77.8 a | 1.3 b |
| NAA 5* | C 2 | 50.0 b | 1.3 b |
| NAA 5* | C 20 | 77.8 a | 1.1 b |
| NAA 5** | C 2 | 50.0 b | 1.3 b |
| NAA 5** | C 20 | 60.0 b | 2.0 a |
| IBA 1* | C 2 | 0.0 b | 0.0 b |
| IBA 1* | C 20 | 0.0 b | 0.0 b |
| IBA 1** | C 2 | 10.0 b | 0.2 b |
| IBA 1** | C 20 | 0.0 b | 0.0 b |

**Table 2.** Rooting of shoot explants of *Pulsatilla patens* on medium with α-naphthaleneacetic acid (NAA) and riboflavin. Means followed by different letters within columns indicate significant differences (P = 0.05; n = 6 to 15)

| Medium | | Rooting (%) | | Number of roots | |
|---|---|---|---|---|---|
| NAA (mg $L^{-1}$) | Riboflavin (mg $L^{-1}$) | Darkness 5 days | Darkness 7 days | Darkness 5 days | Darkness 7 days |
| 0 | 0 | 0.0 b | 0.0 b | 0.0 b | 0.0 b |
| 0 | 3.76 | 0.0 b | 0.0 b | 0.0 b | 0.0 b |
| 0 | 7.52 | 0.0 b | 0.0 b | 0.0 b | 0.0 b |
| 2 | 3.76 | 28.8 b | 0.0 b | 0.4 b | 0.0 b |
| 3 | 3.76 | 33.3 b | 23.1 b | 0.7 b | 0.8 b |
| 4 | 3.76 | 73.3 a | 36.4 b | 1.4 a | 0.9 b |
| 5 | 3.76 | 91.8 a | 28.6 b | 2.7 a | 0.1 b |
| 2 | 7.52 | 23.1 b | 14.3 b | 0.2 b | 0.1 b |
| 3 | 7.52 | 0.0 b | 53.3 a | 0.0 b | 1.3 a |
| 4 | 7.52 | 0.0 b | 40.0 b | 0.0 b | 1.1 b |
| 5 | 7.52 | 15.4 b | 50.0 b | 0.3 b | 0.1 b |

autumn they were transplanted near pine trees in a semi-natural habitat. Further growth and flowering of acclimated plants were monitored.

## Results and discussion

Seeds of *P. patens* germinated *in vitro* on half-diluted MS medium in light. The germination percentage of recently collected ripe seeds was 80 to 100%, and after a few months of storage – only 60%. These results are similar to the germination ability of *P. pratensis* reported previously (Šediva 2002; Naumovski et al. 2009).

For multiplication, either benzyladenine or topolin at concentrations of 0.25 and 0.5 mg $L^{-1}$ was sufficient (multiplication rate three), but kinetin at such concentrations only strengthened plantlets. A multiplication rate of about two was obtained with a relatively high kinetin concentration (5 mg $L^{-1}$). For further micropropagation, the basal medium was supplemented with topolin 0.5 mg $L^{-1}$. The use of benzyladenine, kinetin and zeatin for shoot multiplication of various *Pulastilla* species has been described (Danova et al. 2009; Naumovski et al. 2009; Šauliene, Brynkyte 2009; Lin et al. 2010).

In the previous rooting experiments, shoots were exposed to auxins IAA, IBA and NAA at concentrations of 0.5 to 1.5 mg $L^{-1}$ during the entire experiment (6 weeks), as it is commonly done for rooting of various cultures (Kļaviņa, unpublished data). *P. patens* shoots *in vitro* did not form roots when incubated in media with the respective concentrations of auxins for prolonged periods of time. Further, concentrations (2.5, 5 and 10 mg $L^{-1}$ of IAA, IBA and NAA) used for a 6-week period also showed no apparent stimulation of rooting. Failure of root formation in *P. patens* is not surprising as it is difficult to transplant *Pulsatilla* sp. individuals from the wild due to an easily damaged root system that regenerates poorly. After various manipulations the process of root formation of *in vitro* shoots of *Pulsatilla vulgaris* has also been unsuccessful (Šauliene, Brinkyte 2009). Hence, the rooting of shoots is a critical stage in the propagation of *P. patens* and rooting is not possible in the usual way through cultivation of shoot explants with continuous presence of exogenous auxins.

As a theoretical basis for explaining why some species are difficult-to-root, it should be considered that the rooting process is a sequence of three physiologically distinct stages characterized by different metabolism and dominant endogenous regulators, e.g., induction, initiation and expression of root formation. Auxin is necessary for root induction, but during the initiation stage it must be enzymatically catabolized as it prevents further rooting (Gaspar et al. 1992). To solve the problem of initially unsuccessful rooting and taking into account the above considerations, three additional series of rooting experiments were performed. In the first experiment shoots were exposed to auxins IAA, IBA, NAA at concentrations of 2.5, 5 and 10 mg $L^{-1}$ for root induction only for a week. Thereafter, explants were transferred to a hormone-free basal medium for root expression. However, this approach also did not result in root appearance.

In the second series shoots were exposed with auxins for a relatively longer period in medium with two forms of Fe. Cultivation of shoots during three weeks with 1 mg $L^{-1}$ NAA and Fe-EDDHA as well as with 5 mg $L^{-1}$ NAA and both forms of Fe was suitable for root induction. Further, roots appeared after transfer of explants to a hormone-free root expression medium containing activated charcoal (Table 1). This corresponds to findings by other authors that rooting enhancement is gained with the addition of Fe-EDDHA (Molassiotis et al. 2004) or activated charcoal (Dumas, Monteuuis 1995). Fe-EDDHA promotes root formation by changes in soluble fractions and ionically bound cell wall peroxidase activity and catalase activity as well as by changes in isoenzyme activity. Similar behavior was not observed in plantlets on medium with Fe-EDTA (Molassiotis et al. 2004), which might be related to the lignification of root primordia cells and/or synthesis of a specific protein needed for rooting. Activated charcoal may interfere with polyphenolic compounds, which affects organogenic potential including root development. Nevertheless, in spite of the relatively high percentage of rooted shoots (50 to 78%) on medium with NAA, this can not be considered as a complete success, because plantlets formed large basal calli with futher negative effect on *ex vitro* growth.

In the third series of experiments rooting was improved by adding of riboflavin to the medium and exposure to NAA 1 and 5 mg $L^{-1}$ for two, five or seven days in

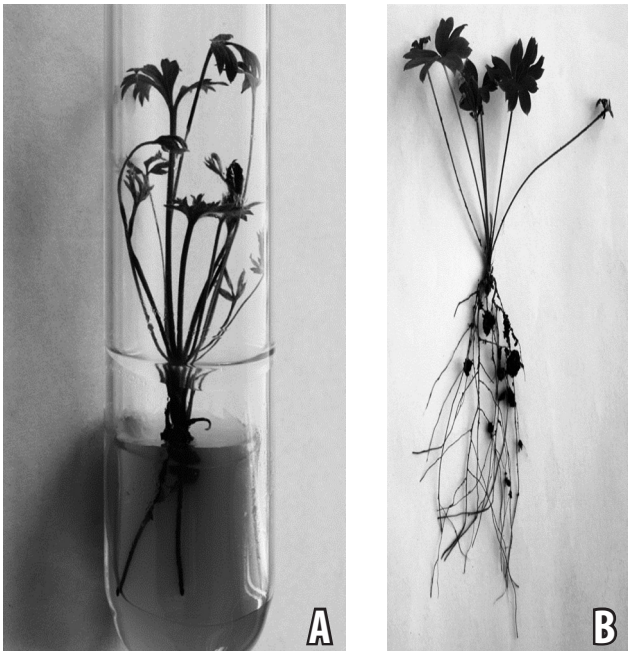


**Fig. 1.** *In vitro* rooted plantlet of *Pulsatilla patens* (A), after onemonth of *ex vitro* acclimatization (B).

darkness with subsequent transfer to light. The ability of riboflavin to break down auxin in the medium in light but not in the dark has been used as a method to control the period of exposure to exogenous auxin (Drew 1991; Kaity et al. 2009). Exposure of plantlets for five days to NAA at a concentration of 4 or 5 mg $L^{-1}$ was optimal in terms of both rooting percentage and mean root number per shoot after photooxidation of exogenous auxin by 3.76 mg $L^{-1}$ riboflavin. However, seven days of exposure to NAA appeared to be too long and satisfactory rooting was obtained only with a doubled concentration of riboflavin (Table 2). Root induction with NAA at concentrations of 4 and 5 mg $L^{-1}$ resulted in 73 and 91% rooting respectively. For a related species, *P. pratensis* ssp. *nigricans*, 83% rooting was achieved with 1.5 mg $L^{-1}$ IAA (Naumovski et al. 2009). Differences in rooting ability between these two species might be partly related to differences in auxin uptake and metabolism (Baraldi et al. 1993).

*In vitro* rooted plantlets of *P. patens* were successfully acclimatized (average survival rate of 60%) in a peat substrate mixed with sand. The plantlets placed in *ex vitro* conditions grew fast, and developed well-branched roots and healthy leaf rosettes in a period of one month (Fig. 1). After transplantation to semi-natural conditions, plants started to flower after a year and appeared to be true to type.

In the present study, a suitable rooting medium for the difficult-to-root species *P. patens* was developed in multistage rooting experiments. The best rooting was obtained in darkness for five days on a half-diluted MS medium supplemented with 5 mg $L^{-1}$ NAA and riboflavin followed by auxin photooxidation. For the *in vitro* formation of good quality roots on *P. patens* explants, it is important to provide exogenous auxins during root induction and to reduce auxin content during the expression stage. The production of *in vitro* plantlets of *P. patens* appears to represent an effective means for future *ex situ* conservation programmes.

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