In vitro shoot regeneration from organogenic callus culture and rooting of Carpathian endemic Aconitum bucovinense Zapał.

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Abstract
Aconitum bucovinense Zapał. is the European species of monkshood, endemic to the Eastern and Southern Carpathians. A protocol has been developed for the in vitro regeneration of adventitious shoots by indirect organogenesis from leaf explants. An initiation of cultures carried out on a medium (B5 macronutrients, MS micronutrients) with picloram and kinetin allowed obtaining a callus. More than a 200% FM increase of the callus and at the same time differentiation of adventitious buds were obtained on IBA and BAP supplemented medium. Excised buds were used to establish shoot cultures and multiplied after a transfer to nutrient media with an addition of BAP with IBA, IAA or NAA. Almost 70% of rooted shoots were obtained on a 1.5 mg L⁻¹ IBA and 1.0 mg L⁻¹ BAP supplemented medium with simultaneous efficient multiplication. An analysis of peroxidase activity revealed its gradual increase in shoots until the appearance of roots. For the first time, an efficient way to regenerate, multiply and root A. bucovinense shoots has been developed and can be used for ex situ conservation of this species.

Key message
The system of plant regeneration of Aconitum bucovinense from callus culture was elaborated. The analysis of the peroxidase level showed that its content increases until the first roots appear.

Keywords Micropropagation · Monkshood · Peroxidase · Ex situ conservation

Abbreviations
MS Murashige and Skoog medium
B5 Gamborg medium
BAP 6-Benzylaminopurine
IBA Indole-3-butyric acid
NAA 1-Naphthaleneacetic acid
IAA Indole-3-acetic acid
FM Fresh matter
POD Peroxidase (EC 1.11.1.7)

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Introduction

The genusaconite (monkshood) *Aconitum* L. belongs to the Ranunculaceae Juss. family and includes about 300–400 species which are characterized by a wide diversity of morphological features and occurrence in various habitats. Most of them occur in Asia and only about 10% in Central Europe (Novikoff et al. 2016; Mitka et al. 2021). *Aconitum bucovinense* Zapal. (Bucovina’s monkshood) is considered a high-mountain endemic to the Eastern and Southern Carpathians (Korzeniak 2009; Boroń et al. 2011; Novikoff and Hurdu 2015). *A. bucovinense* occurs in the subalpine and alpine zones in the Carpathians, while in the Bieszczady Mountains it grows above the upper limit of the forest on rock shelves and among hydrophilous tall herb fringe communities (Mitka 2003; Novikoff et al. 2016). Due to the limited size of the population and the few sites (currently 2 confirmed), it was under species protection and considered a critically endangered species in Poland (Mitka 2014). Research carried out in the Bieszczady Mountains on local populations confirmed their morphological distinctiveness from the other Eastern Carpathians populations (Mitka 2012). *A. bucovinense* is a diploid species 2n = 32 (Mitka 2014).

Very rare and endangered taxa, represented by small populations or related to semi-natural ecosystems, require active protection. In particular, the endemic protection strategy should include a thorough assessment of the degree and causes of the threat and the development of effective methods of both in situ and ex situ protection (Piękoś-Mirkowa and Mirek 2010). The use of monkshood in traditional folk medicine poses a risk of exploiting their natural sites. The presence of alkaloids, mainlyaconitine, causes high toxicity of plants of the genus *Aconitum*. They are one of the most toxic plants used by humans in food and medicine (Kang et al. 2012; Ali et al. 2021). With the growing understanding of detoxification methods, the medical use ofaconite is increasing (Chan et al. 2021).

Most of the reports on the in vitro propagation of the genus *Aconitum* have so far focused on Asiatic species: *A. baicalense* (Regel) Turcz. ex Rapaics (Semenov et al. 2016), *A. carmichaelii* Debeaux (Hatano et al. 1988), *A. nagarum* Stapf (Deb and Langhu 2017), *A. vilmorinianum* (Mou et al. 2022), especially from the Himalayan region: *A. ferox* Wall. ex Ser. (Singh et al. 2020), *A. violaceum* Jacquem. ex Stapf (Rawat et al. 2013b), *A. heterophyllum* Wall. ex Royle (Belay et al. 2016), *A. chasmanthum* (Rafiq et al. 2021) and *A. lethale* Griff. (Gondval et al. 2016). When it comes to European species, only for *A. napellus* L. has a micropropagation method been developed (Watad et al. 1995). The study presented here was carried out to develop an in vitro propagation protocol from shoot buds regenerated from a callus culture for *A. bucovinense* for the first time.

It has been observed in natural habitats that the factor limiting the population size of *Aconitum bucovinense* is the lack of seedlings recruitment. The germination capacity of seeds under horticultural conditions has been investigated by Boroń et al. (2011) who observed that seeds germinated two years after sowing, and their mortality exceeded 80%. Despite the conservation measures taken, the situation of high threat to this species requires the development and subsequent application of effective methods of ex situ conservation (Zemanek 2007). We have thus conducted a comprehensive study on *A. bucovinense* with the aim of elaborating the successive steps of an in vitro propagation system, involving culture initiation, callus multiplication, shoots regeneration and multiplication, and their rooting to obtain regenerated plants. The results of our experiments may contribute to effective ex situ protection.

Materials and methods

Plant material

The plant material for culture initiation was leaf fragments of *Aconitum bucovinense* Zapal. collected from plants in natural populations from Połonina Caryńska (49.14 N, 22.60 E) and Halicz (49.07 N, 22.77 E) growing in the Bieszczady Mountains (Poland) at the beginning of August 2017 with the consent of the Bieszczady National Park (license number 60/17). The approval obtained from the relevant authorities allowed to harvest a limited number of leaves) The research material was transported in polystyrene packages with an addition of ice.

Initiation of in vitro culture

The leaves were surface sterilized by immersion in 70% ethanol for 2 min, followed by immersion for 3 min. in a 0.1% (w/v) mercuric chloride (HgCl2) solution and then thoroughly rinsed four times with autoclaved distilled water. The aseptic leaves were cut into squares 10 × 10 mm (lamina without midrib) and the petioles were cut into ca. 10 mm segments and placed in such a way that the abaxial side was in contact with a K0 basal medium containing macronutrients B5 (Gamberg et al. 1968), micronutrients MS (Murashige and Skoog 1962), 2.0 mg L−1 of glycine, 1.0 mg L−1 of thiamine, 0.5 mg L−1 of pyridoxine, 0.5 mg L−1 of nicotinic acid, 100 mg L−1 of myo-inositol, 30 g L−1 of sucrose and 8.0 g L−1 of agar. K0 basal medium was supplemented with 8.0 mg L−1 of picloram, 5.0 mg L−1 of kinetin, pH was adjusted to 5.8 before autoclaving. 100 mL Erlenmeyer flasks filled with 25 mL of the medium and sealed with aluminium foil were autoclaved at 121 °C for 20 min.

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From the collected leaves, it was possible to obtain (leaf lamina and petioles) 41 explants which were placed separately in flasks. Callus induction was carried out in a growing room (phytotron) in the darkness and a temperature of 24 °C (± 2 °C). After four weeks, the callus was passaged and after obtaining a sufficient volume it was used to set up the experiment.

**Callus cultures and indirect organogenesis**

Callus cultivation and adventitious shoots regeneration were carried out on a K0 basal medium with an addition of 10 mg L⁻¹ of ascorbic acid and 0.6 g L⁻¹ of activated charcoal to prevent darkening of the medium and browning of the explants. The media were supplemented with BAP and IBA in different combinations: K1–0.5 mg L⁻¹ of BAP + 1.0 mg L⁻¹ of IBA; K2–0.5 mg L⁻¹ of BAP + 0.75 mg L⁻¹ of IBA; K3–0.5 mg L⁻¹ of BAP + 0.5 mg L⁻¹ of IBA. Three pieces of the callus (average biomass of 320 mg ± 10 mg were placed in a single flask that was then kept in the growing room in continuous darkness. Each experimental combination consisted of 10 flasks with three callus pieces per flask, and was evaluated for three consecutive passages. A single flask was one replicate. After 6 weeks of cultivation, the callus was reweighed and the number of regenerated shoots was noted. Regeneration effectiveness was expressed as the number of regenerated shoots per 1 g of callus fresh matter (FM). The percent increase in callus fresh matter was calculated according to the following formula:

\[
CFM\% = \frac{FMf - FMi}{FMi} \times 100\%
\]

where CFM%—callus fresh matter gain in %, FMi—initial fresh matter of callus (mg), FMf—final fresh matter of callus (mg).

**Shoot multiplication**

The adventitious shoots ca. 0.5 cm of length with 3–5 leaves excised from the callus were used in shoot multiplication experiments. The single shoots were cultivated on a S0 basal medium containing macro- and micro-nutrients MS with additives of vitamins, ascorbic acid, and activated charcoal, similar to the callus cultivation phase. The following combination of growth regulators was used: S1–0.5 mg L⁻¹ of BAP; S2–0.5 mg L⁻¹ of BAP + 0.75 mg L⁻¹ of IBA; S3–0.5 mg L⁻¹ of BAP + 0.75 mg L⁻¹ of NAA. The number of flasks (replicates) per combination ranged from 7 to 9, with 5 shoots in each flask. The entire experiment was repeated three times. At the end of passage, after six weeks, the number of newly formed shoots, the length of the longest leaf and the number of leaves were assessed. Culture was carried out in a growing room at a temperature of 24 °C (± 2 °C) and in photoperiod conditions with 16/8-h (day/night) and a photon flux density of 70 μmol m⁻²s⁻¹.

**Rooting and hardening**

Rooting of single shoots was performed on a R0 medium, which contained macronutrients B5, micronutrients MS with vitamins analogous to the K0 basal medium except for 0.1 mg L⁻¹ thiamine and 20 g L⁻¹ sucrose and supplemented with of 10 mg L⁻¹ of ascorbic acid and 0.6 g L⁻¹ of activated charcoal. The rooting media differed in the combination of growth regulators used: R1–0.5 mg L⁻¹ of BAP + 0.75 mg L⁻¹ of IBA; R2–1.0 mg L⁻¹ of BAP + 1.5 mg L⁻¹ of IBA; R3–1.0 mg L⁻¹ of BAP + 1.5 mg L⁻¹ of IAA; R4–1.0 mg L⁻¹ of BAP + 1.5 mg L⁻¹ of NAA; R5–2.0 mg L⁻¹ of BAP + 3 mg L⁻¹ of IBA; R6–2.0 mg L⁻¹ of BAP + 3.0 mg L⁻¹ of IAA; R7–2.0 mg L⁻¹ of BAP + 3.0 mg L⁻¹ of NAA. Each combination consisted of 7–16 250 mL Erlenmeyer flasks with five shoots each; one flask being replication. The cultivation was carried out under the same photoperiod conditions as those in the case of shoot multiplication in the growing room. The evaluation was carried out at the end of passage after eight weeks and concerned the number of rooted shoots, the number of roots and their length, but also the number of shoots formed.

Eighty-six rooted shoots were used for acclimatization and were planted into pots filled with a 1:2 mixture of perlite and commercially available potting soil AURA® from Agaris Poland. They were cultivated for 8 weeks in Sanyo vegetative chambers (San-Yoonoda, Japan), under 16/8-h day/night photoperiod and a photon flux density of 70 μmol m⁻²s⁻¹, a temperature of 24 ± 2 °C, with humidity gradually lowered from 70%.

**Ploidy assessment**

Plant ploidy analysis based on flow cytometry was performed in the Cytogenetics Laboratory of the Sugar Beet Breeding Station in Kutno (Poland). The ploidy level was determined for samples of young leaves collected separately from each plant and prepared according to the Galbraith method (1989) modified by Thiem and Śliwińska (2003). Chopped plant material in 2 mL of lysis buffer with the addition of fluorochrome dye DAPI was filtered and analysed using a Partec CyFlow Ploidy Analyser (Sysmex). A random selection of ten in vitro regenerated plants was evaluated, the seed-origin plant served as a reference.

**Peroxidase activity**

Peroxidase (POD) levels were determined for selected media (R1, R2, R3, R4—with the lower PGR content) at the beginning of rooting and after two and four weeks (0, 2, and 4
weeks). On the day of the analysis, from each medium, the aboveground parts of the rooted explants were obtained for measurements in six laboratory repetitions.

Extraction: 200 mg of FM plant material was homogenised in 7 mL of ice-cold 0.1 M phosphate buffer (pH 6.0, containing 2 mM EDTA + 1% Poly (vinylpolyvinylpyrrolidone (PVPP)) and
underwent centrifugation (4 °C for 15 min. at 4800 ×g) after which the supernatant was immediately analysed.

Peroxidase (POD) activity assay was performed according to the Sigma-Aldrich Enzymatic Assay of Peroxidase (EC 1.11.1.7) protocol.

Pyrogallol was used as a substrate, which is oxidised by POD to purpurogallin. All the reagents were prepared in ultrapure water. The reaction mixture consisted of 2100 µL of H₂O₂, 320 µL of 0.5% (w/v) pyrogallol, 160 µL of 0.5% (w/w) H₂O₂ and 420 µL of enzyme extract. The absorbance of the coloured reaction product was measured at 420 nm. The enzymatic activity was calculated considering the linear part of the curve. One unit of peroxidase was defined as the amount of the enzyme that forms 1.0 milligram of purpurogallin in 20 s at pH 6.0 at 20 °C.

Statistical analysis

Completely randomized experimental design was performed in the rooting experiment while factorial design was applied for callus cultures and shoot multiplication (factors: media and subsequent passages) and peroxide activity (factors: media and subsequent weeks of rooting). The number of replicates was specified for each stage of the in vitro experiments in the methodology. The results were evaluated using the one-way (shoot multiplication) or two-way (callus culture, shoot multiplication and peroxide activity) ANOVA module in STATISTICA ver. 13 (StatSoft Inc, Tulsa, OK, USA). A post-hoc mean separation was performed using the Tukey’s test at P ≤ 0.05.

Results

Initiation of in vitro culture

The decontamination parameters used resulted in 39.1% decontamination of the explants but none of the disinfected explants died and the callus began to differentiate on all of them, both leaf and petiole fragments, originating from Halicz as well as Połonina Caryńska. However, darkening of the medium was observed around the explants, which may be a result of potentially harmful oxidation processes taking place in the explants; therefore, activated carbon and ascorbic acid were introduced to the medium in all subsequent stages of cultivation. Earlier, a callus appeared on petioles, but on leaf explants the callus grew more vigorously and for further experiments the callus on leaf explants from Połonina Caryńska was chosen. The single adventitious buds developing asynchronously were observed after 4–6 weeks of cultivation (Fig. 1a–c).

Callus cultures and indirect organogenesis

The use of ascorbic acid and activated carbon in the medium prevented the darkening of the explants that was observed at the initiation stage at the same time as the FM growth of the callus increased. Regardless of the applied growth regulators and the duration of the culture, the efficiency of the differentiation processes was constant and from 1 g of a callus one adventitious bud was formed (Fig. 1d, e). However, it is worth noting that in subsequent passages the rate of callus fresh matter gain increased, resulting in increased regenerative processes (Table 1).

Shoot multiplication

Evaluation of the shoot multiplication process showed that application of BAP cytokinin alone is sufficient for development of new adventitious shoots at the basal part of explants. On the medium supplemented with BAP alone (S1), the highest number of shoots (3.5) of the best quality (with the highest number of leaves and the longest leaves) was observed, see Table 2.

The medium S1 with BAP alone produced the highest number of shoots and the differences became significant in the third passage. The number of the regenerated shoots on the media S2 and S3 remained constant in the subsequent passages, so the multiplication capacity of the shoots did not change. On the other hand, the size of the new shoots gradually increased on the medium with cytokinin BAP alone: the shoots had more and longer leaves and finally, after the third passage, were significantly bigger. During the multiplication in a few explants, single roots appeared, but it was rather accidental.

Rooting and hardening

The rooting process was slow—the first roots were observed in the 4th week of the culture. The highest number of roots was obtained on the medium with the addition
of 1.0 mg L\(^{-1}\) of BAP + 1.5 mg L\(^{-1}\) of IBA (Table 3). The resulting roots were characterized by slow growth, reaching an average length of less than 1 cm (Fig. 1i–k). It is noticeable that the shoots multiplied simultaneously with rooting, moreover the number of shoots recorded was higher than on the so-called multiplication media (S1, S2, S3) and was the same on all the media regardless of the growth regulator used (Fig. 1f–h) (within the range of the tested concentrations and types of growth regulators).

Table 1 The increase in callus fresh matter (CFM\%\) and the number of regenerated shoots (pcs g\(^{-1}\) of callus) of *Aconitum bucovinense* evaluated after six weeks of cultivation on media supplemented with various combinations of BAP and IBA during three consecutive passages

| Growth regulators (mg L\(^{-1}\)) | Evaluated characteristic | Passage 1 | Passage 2 | Passage 3 | Mean |
|----------------------------------|--------------------------|-----------|-----------|-----------|------|
| K1: 0.5 BAP + 1.0 IBA            | CFM\%                   | 183.8\(^a\) | 249.2\(^abc\) | 290.6\(^bc\) | 241.2\(^A\) |
|                                  | Shoots                   | 0.66\(^a\)  | 0.76\(^a\)  | 1.43\(^a\)  | 0.95\(^A\)  |
| K2: 0.5 BAP + 0.75 IBA           | CFM\%                   | 230.5\(^ab\) | 284.6\(^bc\) | 310.3\(^c\)  | 275.1\(^B\)  |
|                                  | Shoots                   | 0.94\(^a\)  | 1.34\(^a\)  | 1.25\(^a\)  | 1.18\(^A\)  |
| K3: 0.5 BAP + 0.5 IBA            | CFM\%                   | 219.4\(^ab\) | 255.5\(^abc\) | 282.4\(^bc\) | 252.4\(^Ab\) |
|                                  | Shoots                   | 0.94\(^a\)  | 0.79\(^a\)  | 0.69\(^a\)  | 0.81\(^A\)  |
| Mean                             | CFM\%                   | 211.3\(^a\) | 263.1\(^B\)  | 294.4\(^C\)  | 263.1\(^A\)  |
|                                  | Shoots                   | 0.84\(^a\)  | 0.97\(^a\)  | 1.11\(^A\)  | 0.84\(^A\)  |

*The mean values followed by the same letter for the evaluated characteristic in rows and columns were not significantly different at \(P \leq 0.05\). Two-way ANOVA was performed: one factor: medium (capital letter italics), second factor: subsequent passages (capital letter), interaction: medium × subsequent passage – lower case

Table 2 The influence of the various combinations of growth regulators and the duration of the culture on the number and quality (the number of leaves and the length of the longest leaf) of the produced shoots of *Aconitum bucovinense* on MS based media

| Growth regulators (mg L\(^{-1}\)) | Number of shoots (pcs) | Length of the longest leaf (cm) | Number of leaves (pcs) |
|----------------------------------|------------------------|-------------------------------|------------------------|
|                                  | Passage 1 | Passage 2 | Passage 3 | Mean | Passage 1 | Passage 2 | Passage 3 | Mean | Passage 1 | Passage 2 | Passage 3 | Mean |
| S1: 0.5 BAP                      | 3.3\(^ab\) | 3.2\(^ab\) | 3.7\(^b\)  | 3.5\(^g\) | 1.5\(^a\)  | 1.9\(^a\)  | 2.8\(^b\)  | 2.3\(^g\) | 9.4\(^a\)  | 10.8\(^ab\) | 16.6\(^b\) | 12.7\(^g\) |
| S2: 0.5 BAP + 0.75 IBA           | 2.9\(^ab\) | 2.4\(^ab\) | 2.0\(^a\)  | 2.4\(^A\)  | 1.5\(^a\)  | 1.6\(^a\)  | 2.1\(^a\)  | 1.8\(^A\)  | 9.7\(^a\)  | 8.9\(^a\)  | 7.6\(^a\)  | 8.7\(^A\)  |
| S3: 0.5 BAP + 0.75 NAA           | 2.3\(^ab\) | 2.6\(^ab\) | 2.7\(^b\)  | 2.5\(^A\)  | 1.5\(^A\)  | 1.7\(^a\)  | 2.1\(^a\)  | 1.8\(^A\)  | 7.0\(^a\)  | 8.1\(^a\)  | 11.0\(^ab\)| 8.9\(^A\)  |
| Mean                             | 2.8\(^A\)  | 2.8\(^A\)  | 2.9\(^A\)  | 2.9\(^A\)  | 1.5\(^A\)  | 1.8\(^A\)  | 2.4\(^B\)  | 2.4\(^B\)  | 8.7\(^A\)  | 9.3\(^A\)  | 12.1\(^B\) | 12.1\(^B\) |

*The mean values followed by the same letter for the evaluated characteristic in rows and columns were not significantly different at \(P \leq 0.05\). Two-way ANOVA was performed: one factor: medium (capital letter italics), second factor: subsequent passages (capital letter), interaction: medium × subsequent passage – lower case

Table 3 The influence of the various combinations of growth regulators on the rooting process and the number of the produced shoots of *Aconitum bucovinense* on media with B5 macronutrients and MS micronutrients

| Growth regulators (mg L\(^{-1}\)) | Rooting (%) | Number of roots (pcs) | Length of roots (cm) | Number of shoots (pcs) |
|----------------------------------|-------------|----------------------|----------------------|-----------------------|
|                                  | R1: 0.5 BAP + 0.75 IBA | 69.33\(^b\) | 1.26\(^ab\) | 0.65\(^a\) | 5.19\(^a\) |
|                                  | R2: 1.0 BAP + 1.5 IBA   | 53.33\(^ab\) | 2.51\(^b\)  | 0.58\(^a\) | 6.19\(^a\) |
|                                  | R3: 1.0 BAP + 1.5 IAA    | 46.67\(^ab\) | 1.43\(^ab\) | 0.53\(^a\) | 5.51\(^A\) |
|                                  | R4: 1.0 BAP + 1.5 NAA     | 54.00\(^ab\) | 1.16\(^ab\) | 0.42\(^a\) | 4.55\(^A\) |
|                                  | R5: 2.0 BAP + 3.0 IBA    | 47.50\(^ab\) | 1.62\(^ab\) | 0.31\(^a\) | 4.17\(^A\) |
|                                  | R6: 2.0 BAP + 3.0 IAA     | 54.29\(^ab\) | 1.53\(^ab\) | 0.35\(^a\) | 5.93\(^A\) |
|                                  | R7: 2.0 BAP + 3.0 NAA     | 42.67\(^a\)  | 2.04\(^ab\) | 0.52\(^a\) | 4.24\(^a\) |

*Means followed by the same letter in columns were not significantly different at \(P \leq 0.05\); one-way ANOVA was performed.

The higher number of emerging shoots may be due to the cultivation time that was 2-week longer or a result of the different mineral compositions of the media.

Summarizing all the stages of the regeneration of *A. bucovinense*, it can be concluded that the whole process of differentiation, multiplication, and rooting of this species was slow, but it was possible to identify a group of media on which shoots multiplied and rooted simultaneously and with good efficiency. The highest number of rooted shoots was observed on medium R1 supplemented with 0.5 mg L\(^{-1}\) BAP and 0.75 mg L\(^{-1}\) IBA but the highest number of roots on shoots was noted on medium R2 supplemented with 1.0 mg L\(^{-1}\) BAP and 1.5 mg L\(^{-1}\) IBA.
Out of the acclimatized shoots, 81% survived the hardening process and were destined for outdoor planting under open field conditions (Fig. 1L, m).

**Ploidy assessment**

Results of flow cytometric analysis presented on DNA histograms (Fig. 2) showed a distribution of relative DNA content with dominant peaks corresponding to the 2 C level in the G1 phase of the cell cycle of the seed-origin plant of *A. bucovinense* (control plant, Fig. 2a). The DNA content analysed for all plants after indirect organogenesis indicated that they did not differ from seed-origin plants and they were diploids (Fig. 2b).

**Peroxidase activity**

There was a gradual increase in peroxidase activity from the time the shoots were transferred onto the media until the fourth week of cultivation when the first growing rootlets were observed. POD activity was also determined by the type of the growth regulators used in the culture medium. In the explants cultivated on the media with higher doses of the growth regulators, increased peroxidase activity was

Fig. 2 Histograms of relative DNA content in the nuclei of *Aconitum bucovinense* leaf cells: a control seed-origin plant; b plant originating from in vitro (indirect organogenesis); G1, G2—phases of cell life
of picloram with 9.5 µM kinetin for *Phoenix canariensis* (Huong et al. 1999). So far, in genus *Aconitum* for callus induction other combinations of PGRs: 2,4-D alone (*A. bai-calense*—Semenov et al. 2016; *A. ferox*—Singh et al. 2020) or in combination with kinetin (*A. violaceum*—Rawat et al. 2013b; *A. vilmorinianum*—Mou et al. 2022) and NAA or BAP (*A. heterophyllum*—Jabeen et al. 2006) or TDZ and/or NAA for *A. balfourii* (Gondval et al. 2016) have been applied.

Using high concentrations of growth regulators can increase the likelihood of somaclonal variation. A preliminary step in its assessment can be the evaluation of the ploidy level. Such evaluation carried out for *A. bucovicense* showed that all estimated plants were diploid, just like the reference plant (Fig. 2). In the genus *Aconitum*, variability after propagation in tissue culture was evaluated only for plants regenerated from callus of *A. balfourii* were no changes in ploidy level reported (Pandey et al. 2004). The genetic fidelity of micropropagated shoots of *A. heterophyllum* (Belwal et al. 2016) was confirmed using ISSR technique. Molecular methods provide definitive confirmation of genetic fidelity and used in future for *A. bucovicense*, together with the evaluation of the biology of the obtained plants after acclimatization, may bring new interesting data.

In efforts aimed at augmentation of existing natural populations, it is recommended that micropropagated plants from seeds should be used to provide as much genetic diversity as possible. In cases where this is not possible, callus cultures proved to be the only multiplication technique available. However, it is important to be aware that this mode of cultivation can be particularly susceptible to the occurrence of somaclonal variation, which can arise spontaneously among micropropagated plants (Krishna et al. 2016) but was also reported even if the callus stage was omitted (Prado et al. 2005; Farahani et al. 2011; Sivanesan and Jeong 2012). Such variability is considered undesirable in the conservation of naturally occurring plant resources. In contrast, any emerging variability may be important in the selection of new breeding lines used for different purpose. Callus cultures can be used not only in plant regeneration but also as a potential source of biologically active substances. The genus *Aconitum* is rich in diterpene alkaloids and flavonoids (Rawat et al. 2013a; Wani et al. 2021) which can easily turn into less toxic alkaloids by heating or alkaline treatment. The callus and shoots cultivated on culture media can be used to extract these compounds and use after detoxification in medicine. After determining the content of active substances in cultivated tissues, *A. bucovicense* could become a source of biologically active substances.

In most available source data, MS mineral medium was used for callus and shoot cultivation, and rooting of the genus *Aconitum* (Giri et al. 1993; Watad et al. 1995; Padney et al. 2004; Jabeen et al. 2006; Rawat et al. 2013a; Belwal

### Table 4 Peroxidase activity in rooted shoot cultures of *A. bucovicense* (µg mg⁻¹ FM) at the beginning of subculture (day 0—control) and after 2 and 4 weeks of cultivation

| Growth regulators (mg L⁻¹) | Control | 2nd week | 4th week | Mean |
|---------------------------|---------|----------|----------|------|
| R1: 0.5 BAP + 0.75 IBA    | 1.21 b  | 1.20 b   | 1.83 g   | 1.42 a|
| R2: 1.0 BAP + 1.5 IBA     | 1.34 c  | 1.55 e   | 2.33 h   | 1.74 c|
| R3: 1.0 BAP + 1.5 IAA     | 1.45 d  | 1.70 f   | 1.71 i   | 1.62 b|
| R4: 1.0 BAP + 1.5 NAA     | 1.00 a  | 1.48 g   | 2.40 h   | 1.63 b|
| Mean                      | 1.25 A  | 1.48 B   | 2.17 C   |      |

*The mean values followed by the same letter for the evaluated characteristic in rows and columns were not significantly different at *P* ≤ 0.05. Two-way ANOVA was performed: one factor: medium (capital letter italics), second factor: subsequent weeks of culture (capital letter), interaction: medium × subsequent weeks of culture—lower case observed. The highest activity was recorded on the medium that stimulated the formation of the highest number of roots (Table 4).

### Discussion

One of the reasons that have contributed to the declining population of *A. bucovicense* is its limitations of generative reproduction: a small number of flowering individuals setting a small number of seeds that germinate with difficulty. Propagation for *ex situ* conservation in such cases is often only possible using tissue culture technique. The results presented here show the possibility of propagation of *A. bucovicense* from leaf explants by indirect organogenesis through an intermediate callus stage.

Given the difficulty of germinating the few seeds *A. bucovicense* produced, cultures were initiated from leaf fragments. For many species of *Aconitum*, for the same reasons, cultures were initiated in a similar manner and next shoots of *A. balfourii*, *A. violaceum*, *A. heterophyllum*, *A. ferox* (Pandey et al. 2004; Jabeen et al. 2006; Rawat et al. 2013b; Gondval et al. 2016; Singh et al. 2020) or embryos of *A. heterophyllum* (Giri et al. 1997) were regenerated via a callus. In many of these cases, rare species were propagated and the goal was to protect their natural resources (Rafiq et al. 2021). In presented experiments picloram and kinetin, at relatively high concentrations, were used for the first time in the genus *Aconitum* for callus induction on leaf explants. There are reports about picloram alone (Gantait and Nahanta 2021) or in combination with kinetin (Farjaminezhad and Garoosi 2019) used for callus induction. Some authors have reported also the effectiveness of high concentrations of picloram for dedifferentiation processes: up to 5 mg L⁻¹ for *Paspalum scrobiculatum* (Kaur and Kothari 2004), 50 µM for *Leucojum aestivum* (Ptak et al. 2013) or 100 µM of picloram with 9.5 µM kinetin for *Phoenix canariensis* (Huong et al. 1999). So far, in genus *Aconitum* for callus induction other combinations of PGRs: 2,4-D alone (*A. bai-calense*—Semenov et al. 2016; *A. ferox*—Singh et al. 2020) or in combination with kinetin (*A. violaceum*—Rawat et al. 2013b; *A. vilmorinianum*—Mou et al. 2022) and NAA or BAP (*A. heterophyllum*—Jabeen et al. 2006) or TDZ and/or NAA for *A. balfourii* (Gondval et al. 2016) have been applied.

Using high concentrations of growth regulators can increase the likelihood of somaclonal variation. A preliminary step in its assessment can be the evaluation of the ploidy level. Such evaluation carried out for *A. bucovicense* showed that all estimated plants were diploid, just like the reference plant (Fig. 2). In the genus *Aconitum*, variability after propagation in tissue culture was evaluated only for plants regenerated from callus of *A. balfourii* were no changes in ploidy level reported (Pandey et al. 2004). The genetic fidelity of micropropagated shoots of *A. heterophyllum* (Belwal et al. 2016) was confirmed using ISSR technique. Molecular methods provide definitive confirmation of genetic fidelity and used in future for *A. bucovicense*, together with the evaluation of the biology of the obtained plants after acclimatization, may bring new interesting data.

In efforts aimed at augmentation of existing natural populations, it is recommended that micropropagated plants from seeds should be used to provide as much genetic diversity as possible. In cases where this is not possible, callus cultures proved to be the only multiplication technique available. However, it is important to be aware that this mode of cultivation can be particularly susceptible to the occurrence of somaclonal variation, which can arise spontaneously among micropropagated plants (Krishna et al. 2016) but was also reported even if the callus stage was omitted (Prado et al. 2005; Farahani et al. 2011; Sivanesan and Jeong 2012). Such variability is considered undesirable in the conservation of naturally occurring plant resources. In contrast, any emerging variability may be important in the selection of new breeding lines used for different purpose. Callus cultures can be used not only in plant regeneration but also as a potential source of biologically active substances. The genus *Aconitum* is rich in diterpene alkaloids and flavonoids (Rawat et al. 2013a; Wani et al. 2021) which can easily turn into less toxic alkaloids by heating or alkaline treatment. The callus and shoots cultivated on culture media can be used to extract these compounds and use after detoxification in medicine. After determining the content of active substances in cultivated tissues, *A. bucovicense* could become a source of biologically active substances.

In most available source data, MS mineral medium was used for callus and shoot cultivation, and rooting of the genus *Aconitum* (Giri et al. 1993; Watad et al. 1995; Padney et al. 2004; Jabeen et al. 2006; Rawat et al. 2013a; Belwal
Only *A. baicalense* callus (Semenov et al. 2016) was obtained from etiolated seedlings on B5 medium. In the results presented here, the authors used macronutrients from B5 medium with MS micronutrients at most stages. Only for the shoot multiplication the full MS was used. However, analysis of results from the rooting stage performed on B5 showed that the application of full MS was not beneficial for shoot multiplication. The maximum number of shoots obtained then was 3.7 (Table 2), while on rooting medium with macroelements B5—more than 6 (Table 3). MS nutrient solution is more abundant in macroelements, except for potassium, in comparison to B5. Nitrogen itself is there almost twice as much; there are also changed proportions between its ionic forms. In the B5 medium the proportions are significantly shifted in favour of the nitrate form (Murashige and Skoog 1962; Gamborg et al. 1968). The results obtained for *A. bucovinense* may suggest that this mountain species has lower requirements for the richness of the medium (or soil) and the use of media poorer in macronutrients (as B5 in comparison with MS) will positively influence the effects of in vitro cultivation.

Two media can be identified that stand out in terms of rooting effects: R1 (0.5 mg L\(^{-1}\) BAP + 0.75 mg L\(^{-1}\) IBA), on which a high percentage of rooted shoots was obtained, and R2: (1.0 mg L\(^{-1}\) BAP + 1.5 mg L\(^{-1}\) IBA), where the rooted shoots formed the most roots. These media differ in the level of growth regulators used, but in the proportions of BAP : IBA in both cases are the same − 1:1.5. Applied at a lower dose, they are more favourable for the number of rooted shoots, while at a higher dose for the number of developed roots. It is also worth noting that in both cases a high multiplication factor was observed simultaneously - higher than in the multiplication phase on MS medium.

During the rooting of the shoots, the formation of the first roots was observed in the 4th week of cultivation. This time is similar to that observed for *A. heterophyllum* during rooting (25–45 days) (Jabeen et al. 2006). On the other hand, the initiation of rooting of *A. balfourii* shoots was faster (15–18 days) (Pandey et al. 2004). Interestingly, studies on *A. chasmamnthus* failed to induce root formation at all tested media (Rafiq et al. 2021). The roots obtained in our research were short, not even 1 cm, and relatively sparse. However, they allowed for the effective acclimatization of plants, taking up further growth under ex vitro conditions. A greater number of roots was obtained in studies conducted on *A. nagarum* on media with the addition of NAA, obtaining even 5.3 roots per shoot in the best variant, but the percent of acclimatized plants was lower (65%) (Deb and Langhu 2017).

During our experiments on the selected media, peroxidase levels in the cultivated shoots were estimated. There are several biochemical markers whose levels in plant tissue are related to the differentiation process. These include phenolic compounds, soluble sugars, and peroxidases (Goel et al. 2018; Wang et al. 2018; Hanus-Fajerska et al. 2021; Oulbi et al. 2021). Numerous reports have shown that peroxidase levels are the highest just before root emergence (Gaspar et al. 1992; Rout 2006; Goel et al. 2018). During rooting of *A. bucovinense*, irrespective of the rooting medium used, an increase in peroxidase activity was observed from the moment of shoot planting to the 4th week of rooting, when the first differentiating roots appeared. The highest peroxidase activity was recorded for the medium that yielded the highest number of roots rather than the highest percentage of rooted shoots. This implies that there may be a relationship between the number of roots produced and the level of peroxidase in the rooting shoots. This is difficult to demonstrate directly in an experiment: shoots scheduled for analysis are not further observed for rooting and the number of roots is impossible to predict. We can only rely on the averages of the combinations.

The physiological importance of peroxidase is great, because it is involved in processes of broadly understood stress response, including wound healing, but also in cell wall growth and lignification. In all plants, they play an important role in regulating growth and development processes (for review, see: Barcelo and Pomar 2002; González-Rábade et al. 2012; Pandey et al. 2017). In vitro cultures are particularly stressful conditions for plants, as there are continuous changes in the concentration and direction of the transport of trophic substances, the concentration of growth regulators, oxygen conditions, and the concentration of ethylene. The peroxidase activity increases, i.a., when plants are under unfavourable growth conditions (Dąbrowska et al. 2007). The observed gradual increase in peroxidase activity during rooting of *A. bucovinense* is not likely to be a reaction to stress related to injury, as it occurs in a relatively short period of time. Instead, it may be a result of root formation with well-developed vascular tissue, i.e. a result of enhanced lignification processes especially as the peroxidase levels continued to rise until the roots emerged. It has been shown in previous studies that peroxidase can be considered a marker enzyme in the somatic embryogenesis of pumpkin (Krsnik-Rasol 1991) and in the induction and beginning of root initiation phase of *Bacopa monnieri* and *Camellia sinensis* (Rout 2006; Goel et al. 2018).

A callus of *A. bucovinense* with durable regenerative capacity was obtained and media on which shoots simultaneously multiplied and rooted effectively were identified. Only minor differences in regeneration potential were noted for the growth regulators used, while a dependence on the mineral composition of the nutrient solutions was apparent. On the medium with macroelements B5 (Table 3) used for rooting, more than twice as many shoots were obtained as in the so-called shoot multiplication phase, which was carried
out using the MS mineral medium (Table 2). The medium on which the highest number of shoots produced and at the same time the high percentage of rooted shoots was observed was R1 medium with macronutrients B5 and micronutrients MS with the addition of 0.5 mg L\(^{-1}\) BAP with 0.75 mg L\(^{-1}\) IBA.

\textit{Aconitum bucovinense} is a rare species that requires the implementation of different active conservation programs. Our results demonstrate that it can be efficiently propagated using tissue culture techniques from leaves, which is valuable when propagation from seeds is limited. From a small amount of a mother material, taken without harming the mother plant, numerous rooted plants can be obtained and used in conservation programs. Moreover, such cultures can be used as a potential source of biologically active compounds. Furthermore, we confirm that an increase in peroxidase activity precedes the root differentiation processes of \textit{A. bucovinense} and can be recognised as an important indicator of tissue differentiation.

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**Data availability** The data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors declare that they have no relevant conflict of interest.

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