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

Dawid Kocot (dawid.kocot@urk.edu.pl)
University of Agriculture in Krakow: Uniwersytet Rolniczy im Hugona Kollataja w Krakowie
https://orcid.org/0000-0002-4459-642X
Barbara Nowak
University of Agriculture in Krakow: Uniwersytet Rolniczy im Hugona Kollataja w Krakowie
Ewa Sitek
University of Agriculture in Krakow: Uniwersytet Rolniczy im Hugona Kollataja w Krakowie
Anna Starzyńska-Janiszewska
University of Agriculture in Krakow: Uniwersytet Rolniczy im Hugona Kollataja w Krakowie
Józef Mitka
Jagiellonian University: Uniwersytet Jagiellonski w Krakowie

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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 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 media. 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 Massage

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.

Introduction

The genus aconite (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* Zapał. (Bucovina's monkshood) is considered to be a high-mountain endemic to the Eastern and Southern Carpathians (Korzeniak 2009, Boroń et al. 2011, Novikoff and Hurdu 2015). Endemics as a unique component of flora are the most valuable elements of biodiversity in a given area and hence a subject of special concern under any conservation measures (Piękoś-Mirkowa and Mirek 2003). *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).

Research carried out in the Bieszczady Mountains on local populations confirmed their morphological distinctiveness from the other Eastern Carpathians populations (Mitka 2012).

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, mainly aconitine, 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 of aconite 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), 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 (Belwal et al. 2016) 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 undertaken 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 the natural sites that the factor limiting the population size of *Aconitum bucovinense* is the lack of seedlings recruitment. The germination capacity of seeds in 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 undertaken conservation measures, 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.

Plant Material

The plant material for culture initiation was leaf fragments of *Aconitum bucovinense* Zapał. 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 research material was transported in polystyrene packages with an addition of ice.

Initiation of *in vitro* culture

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 ($HgCl_2$) solution and then thoroughly rinsed four times with autoclaved distilled water. The aseptic leaves were cut into squares 10 × 10 mm and 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 (Gamborg et al. 1968), micronutrients MS (Murashige & 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, supplemented with 8.0 mg L^{-1} of picloram, 5.0 mg L^{-1} of kinetin, pH 5.8.

All together 41 explants were placed in 100 mL Erlenmeyer flasks filled with 25 mL of the medium. Callus induction was carried out in a growth chamber in the darkness and a temperature of 24°C (+/-2°C). After four weeks, the callus was passaged.

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 320 mg (+/- 10 mg) pieces of the callus were placed in a single flask which was then kept in the growth chamber 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 constituted one replicate. After six 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 lenght 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 micronutrients MS with additives of vitamins, ascorbic acid and activated charcoal, similarly 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 every 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. The cultivation was carried out in a phytotron at a temperature of 24°C (+/-2°C) and in photoperiod conditions with 16/8-hr (day/night) and a photon flux density of 70 µmol $m^{-2}s^{-1}$.

Rooting and hardening

Rooting of single shoots was performed on a R0 medium, which contained macronutrients B5, micronutrients MS with vitamins, ascorbic acid and activated charcoal analogous to the K0 medium except for 0.1 mg L^{-1} thiamine and 20 g L^{-1} sucrose. The 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 in the same photoperiod conditions as those in the case of shoot multiplication. 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 2:1 mixture of horticultural soil and perlite. They were cultivated for 8 weeks in Sanyo vegetative chambers (San-Yoonoda, Japan), under 16/8-hr day/night photoperiod and a photon flux density of 70 µmol m^{-2} s^{-1}, a temperature of 24 ± 2°C, with humidity gradually lowered from 70%.

Peroxidase activity

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

Extraction: 200 mg of the plant material was homogenised in 7 mL of ice-cold 0.1M phosphate buffer (pH 6.0, containing 2 mM EDTA + 1% Poly (vinylpolypyrrolidone (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 H_20, 320 µL of 0.5% (w/v) pyrogallol, 160 µL of 0.5% (w/w) H_2O_2 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 from pyrogallol in 20 sec at pH 6.0 at 20°C.

Statistical analysis

The number of replicates was specified for each stage of the *in vitro* experiments in the methodology. The results were evaluated using the one- or two-way ANOVA module in STATISTICA ver. 13 (StatSoft Inc, Tulsa, OK, USA). A post-hoc mean separation was done 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 around the explants was observed, 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 at 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 were observed (Fig.1 A, B, 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 the FM growth of the callus was 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.1.D, E). However, it is worth noticing that in subsequent passages the rate of callus fresh matter gain increased, which resulted in increased regenerative processes (Table 1).

Table 1 The increase of callus fresh matter (CFM%) and the number of regenerated shoots (pcs 1 g^{-1} of callus) of *Aconitum bucovinense* assessed 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 feature	Passage 1	Passage 2	Passage 3	Mean
K1:	CFM%	183.8 a*	249.2 abc	290.6 bc	241.2 A’
0.5 BAP + 1.0 IBA	Shoots	0.66 a	0.76 a	1.43 a	0.95 A’
K2:	CFM%	230.5 ab	284.6 bc	310.3 c	275.1 B’
0.5 BAP + 0.75 IBA	Shoots	0.94 a	1.34 a	1.25 a	1.18 A’
K3:	CFM%	219.4 ab	255.5 abc	282.4 bc	252.4 AB’
0.5 BAP + 0.5 IBA	Shoots	0.94 a	0.79 a	0.69 a	0.81 A’
Mean	CFM%	211.3 A	263.1 B	294.4 C	
	Shoots	0.84 A	0.97 A	1.11 A	

*Means followed by the same letter for evaluated feature in rows and columns were not significantly different at $P = 0.05$

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.

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

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

***** Explanation: see Table 1

Both added auxins (IBA and NAA) had a negative effect on the number and quality of the regenerated shoots. The number of the regenerated shoots 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 4^{th} week of the culture. The greatest 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.1 I, J, 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 (Fig1. F, G, H) (within the range of the

tested concentrations and types of growth regulators). The higher number of emerging shoots may be due to the cultivation time that was two weeks longer or a result of the different mineral compositions of the media.

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 a	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 = 0.05

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.

Out of the acclimatized shoots, 81% survived the process of hardening and were destined for outdoor planting in open field conditions (Fig.1 L, M).

Peroxidase activity

Table 4 Peroxidase activity in rooted shoot cultures of *A. bucovinense* (u mg^{-1} FM) at the beginning of subculture (day 0 – control) and after two and four 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 f	1.62 B’
R4: 1.0 BAP + 1.5 NAA	1.00 a	1.48 de	2.40 h	1.63 B’
Mean	1.25 A	1.48 B	2.17 C	

*Explanation: see Table 1

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, a higher peroxidase activity was observed. The highest activity was recorded on the medium stimulating the formation of the highest number of roots.

Discussion

Among the endangered species, numerous are endemic, forming island populations at the margin of their range. Their protection is particularly important in the context of biodiversity conservation and they require implementation of special conservation programs. Two such populations of *A. bucovinense*, whose distinctiveness has been confirmed, occur in the western Bieszczady Mountains (Boroń et al. 2011, Mitka 2012). Propagation of species from the family Ranunculaceae is often difficult because of poor seed germination and low survival of seedlings, which also proves to be the case with *A. bucovinense* seeds (Boroń et al. 2011). In such situations, for species that do not reproduce vegetatively, tissue culture techniques are used for propagation purposes in conservation programs.

The results presented here show the propagation possibility of *A. bucovinense* from leaf explants by indirect organogenesis through an intermediate callus stage. A callus 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).

In view of the difficulty of germinating the few seeds *A. bucovinense* produced, cultures were initiated from leaf fragments. For many *Aconitum* species, for the same reasons, cultures were initiated in a similar manner and next shoots of *A. balfourii*, *A. violaceum*, *A. hetrophyllum*, *A. ferrox* (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 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 reported also 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. On the other hand, one can ask whether it would be beneficial in the restitution of a small population by providing it with some variability (Fay 1994, Żabicki et al. 2019).

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. Their extraction efficiency can be increased in hairy root cultures obtained after *Agrobacterium rhizogenes* transformation. Such roots grow much faster and may have a higher content of secondary metabolites. The first such root modification was performed for *A. heterophyllum* (Giri et al. 1997). Today, the hairy root cultures are becoming increasingly important for the production of secondary metabolites.

During our experiments on the selected media, peroxidase levels in the cultivated shoots were found. 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).

The physiological importance of peroxidase is great, because it is involved in processes of broadly understood stress response, including wound healing, but also in growth and lignification of cell walls. 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 unfavorable growth conditions (Dąbrowska et al. 2007). The observed gradual increase in the peroxidase activity during the rooting of *A. bucovinense* is not likely to be a reaction to stress related to injury, as such occur 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 for 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).

Aconitum bucovinense is a rare species that requires 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 processes of root differentiation of *A. bucovinense* and can be recognised as an important indicator of tissue differentiation.

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)

Declarations

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Author contributions:

JM, BN, DK conceived and designed the experiments. AS, DK performed the experiments and collected the data. DK, BN, AS analysed the data. ES, DK, BN wrote the manuscript and contributed to manuscript revisions.

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

Data availability:

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

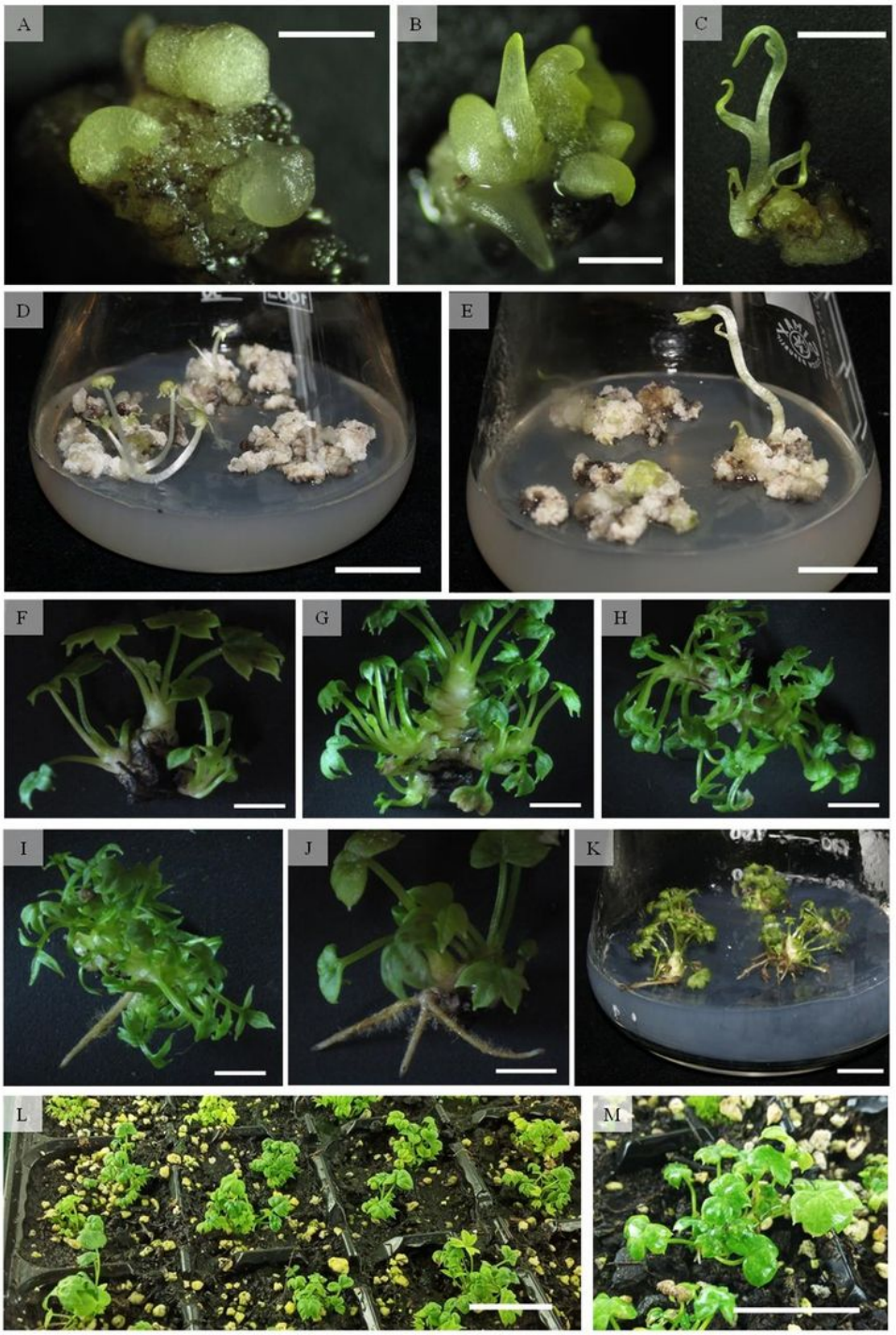

Figure 1

Callus induction, differentiation of adventitious buds and rooting of shoots of *Aconitum bucovinense*. Developing callus (A) differentiating buds (B) developing shoots (C) on medium K0 with 8.0 mg L^{-1} of picloram and 5.0 mg L^{-1} of kinetin; D - callus cultures and differentiating shoots after six weeks of cultivation on medium K1 with 0.5 mg L^{-1} of BAP and 1.0 mg L^{-1} of IBA; E - callus cultures and

differentiating shoots after six weeks of cultivation on medium K3 with 0.5 mg L^{-1} of BAP and 0.5 mg L^{-1} of IBA; F – adventitious shoots developing at the base of cultivated shoots after eight weeks of cultivation on medium R4 with 1.0 mg L^{-1} of BAP and 1.5 mg L^{-1} of NAA; G and H – numerous adventitious shoots developing after eight weeks of cultivation on medium R1 with 0.5 mg L^{-1} of BAP and 0.75 mg L^{-1} of IBA; rooted shoots after eight weeks of cultivation on: I - medium R3, J - medium R1, and K - medium R4. L and M plants planted for hardening: on the first day and after eight weeks. A - C, F – J: bar = 5 mm, D, E, L and M: bar = 2 cm, K: bar = 1.5 cm.