Biologia i rozmnażanie rzadkich i zagrożonych gatunków roślin z rodziny Ranunculaceae

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Spis treści:

1. Wykaz skrótów

MS - pożywka Murashige i Skoog 1962

B5 - pożywka Gamborg, Miller, Ojima 1968

BAP - 6-benzyloaminopuryna

IBA - kwas indolilomasłowy

NAA - kwas 1-naftylooctowy

IAA - kwas indolilooctowy

2. Wykaz publikacji wchodzących w skład pracy doktorskiej

1. **Kocot D.**, Sitek E., Nowak B. ✉ , Kołton A., Stachurska-Swakoń A., Towpasz K. 2022. The Effectiveness of the Sexual Reproduction in Selected Clonal and Nonclonal Species of the Genus Ranunculus. Biology. 11, 85. https://doi.org/10.3390/biology11010085

IF $_{2020}$ = 5,079

MEiN $_{2022}$ = 100 pkt.

2. **Kocot D.**, Sitek E. ✉ , Nowak B., Kołton A., Towpasz K. 2022. Reproductive Biology of Dry Grassland Specialist *Ranunculus illyricus* L. and Its Implications for Conservation. Biology. 11, 873. https://doi.org/10.3390/biology11060873

IF $_{2020}$ = 5,079

MEiN $_{2022}$ = 100 pkt.

3. **Kocot D.** ✉ , Nowak B., Sitek E., Starzyńska-Janiszewska A., Mitka J. 2022. *In vitro* shoot regeneration from organogenic callus culture and rooting of Carpathian endemic *Aconitum bucovinense* Zapał. Plant Cell, Tissue and Organ Culture (PCTOC). https://doi.org/10.1007/s11240-022-02341-1

IF $_{2020}$ = 2,711

MEiN $_{2022}$ = 100 pkt.

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3. Wstęp teoretyczny

3.1. Charakterystyka rodziny jaskrowatych (Ranunculaceae)

Jaskrowate (Ranunculaceae Juss.) to rodzina botaniczna w klasie dwuliściennych. W jej obrębie klasyfikuje się około 2500 gatunków zgrupowanych w 59 rodzajach występujących w klimacie umiarkowanym i zimnym (Tamura 1993). Przeważnie są to byliny, rzadko rośliny jedno- czy dwuletnie, czasem pnącza lub krzewy (podkrzewy, szczególnie rodzaj powojnik *Clematis*). Tamura (1993) podzielił rodzinę jaskrowatych na 5 podrodzin: Helleboroideae, Ranunculoideae, Isopyroideae, Thalictroideae i Hydrastidoideae. Czasami ze względu na typ owocu i liczbę zalążków w słupku wyróżnia się jedynie dwie podrodziny: Ranunculoideae z niełupką/orzeszkiem i jednozalążkowym słupkiem oraz Helleboroideae, z wielozalążkowymi słupkami, które później przekształcają się w mieszki. Spotyka się również podział ze względu na typ chromosomów na Ranunculoideae z R(anunculus)-typem chromosomów oraz Thalictroideae z T(halictrum)-typem chromosomów (Kosuge 1994). Niektóre rodzaje klasyfikowane wcześniej do tej rodziny zostały z niej wyłączone np. *Paeonia*, *Glaucidium* czy *Circaeaster*, natomiast inne dodano np. *Hydrastis* i *Kingdonia* (Prantl 1887, Tamura 1995). Ze względu na duże zróżnicowanie gatunków oraz ich budowy w obrębie rodziny, a także prowadzone obecnie badania molekularne podziały systematyczne są wciąż aktualizowane.

W obrębie rodziny Ranunculaceae ciekawym zagadnieniem jest budowa kwiatów, ponieważ występuje duże zróżnicowanie struktury kwiatów u poszczególnych rodzajów. Kwiaty są zwykle obupłciowe, żywo zabarwione, często o wysokich walorach dekoracyjnych, posiadają liczne pręciki i od kilku do wielu słupków (rzadko jeden) (Kosuge 1994). Są zwykle o symetrii promienistej, lecz niekiedy również grzbiecistej (np. tojad *Aconitum*, ostróżka *Delphinium*). Część rodzajów wykształca kwiaty z okwiatem pojedynczym (sasanka *Pulsatilla*, zawilec *Anemone*, knieć *Caltha*), a u reszty jest on zróżnicowany na kielich i koronę. Kwiaty są pojedyncze lub zebrane w kwiatostany groniaste lub wierzchotkowe. Często występują prątniczki, funkcjonujące jako miodniki (jaskier *Ranunculus*, orlik *Aquilegia*). Większość przedstawicieli Ranunculaceae jest owadopylna, rzadko wiatropylna (rutewka *Thalictrum*) (Tamura 1993, Jasnowska i in. 2008, Xu i Deng 2017).

Najczęściej spotykanym typem owocu jest wielonasienny mieszek (knieć *Caltha*, pełnik *Trollius*, tojad *Aconitum*) oraz jednonasienna niełupka lub orzeszek (sasanka *Pulsatilla*, jaskier *Ranunculus*, przylaszczka *Hepatica*). Sporadycznie owocem może być jagoda np. w rodzaju czerniec *Actea* (Xu i Deng 2017). Liczne gatunki jaskrowatych rozmnażają się również

w sposób wegetatywny poprzez przykładowo rozłogi (nadziemne i podziemne), ukorzenianie pędów w węzłach czy tworzenie bulwek (Tutin i in. 1993).

Przedstawiciele rodziny Ranunculaceae są bogate w związki chemiczne biologicznie czynne. Wiele gatunków jest uznawanych za trujące, gdyż zawierają lakton proteoanemoninę, alkaloidy, glikozydy, flawonoidy, saponiny, kumaryny czy antocyjany, które szczególnie w stanie świeżym są toksyczne zarówno dla zwierząt, jak i ludzi. Rośliny przeważnie tracą właściwości trujące po wysuszeniu (Schrenk i in. 2013, Zuo i in. 2013, Hao 2018). Ponad 450 rodzajów alkaloidów zostało zidentyfikowanych w samym rodzaju tojad *Aconitum*. To właśnie obecność alkaloidów, szczególnie akonityny, powoduje wysoką toksyczność tojadów (Khan i in. 2017). Mimo, że uznaje się je za jedne z najbardziej trujących roślin, są używane przez człowieka zarówno jako pożywienie, jak i w medycynie, szczególnie tradycyjnej (Kang i in. 2012, Ali i in. 2021). Wraz z rozwojem nauki i technologii opisuje się nowe chemiczne i bioaktywne związki specyficzne dla Ranunculaceae, jak również zwiększa się zastosowanie w medycynie czy terapii.

Wiele gatunków jaskrowatych to cenne rośliny uprawiane w ogrodach ze względu na liczne walory dekoracyjne, a szereg z nich posiada odmiany uprawne. Szczególnie w uprawie spotyka się gatunki z rodzajów: tojad *Aconitum,* miłek *Adonis,* zawilec *Anemone,* orlik *Aquilegia,* knieć *Caltha,* pluskwica *Cimicifuga,* powojnik *Clematis,* ostróżeczka *Consolida,* ostróżka *Delphinium,* rannik *Eranthis,* ciemiernik *Helleborus,* przylaszczka *Hepatica,* czarnuszka *Nigella,* sasanka *Pulsatilla,* jaskier *Ranunculus,* rutewka *Thalictrum* czy pełnik *Trollius* (Tamura 1993). Rośliny z rodziny Ranunculaceae stanowią również ważny pożytek dla owadów, oferując głównie pyłek, ale też tworząc liczne nisze ekologiczne dla wielu dzikich zapylaczy. Czasami współzależności są bardzo silne, na przykład u tojadów jedynymi zapylaczami kwiatów są trzmiele (Antoń i in. 2014, Denisow i in. 2016).

W obrębie rodziny jaskrowatych liczne gatunki są endemitami (Dhar i Samant 1993), a w niektórych przypadkach endemiczne są całe rodzaje, na przykład: *Beesia*, *Kingdonia*, *Asteropyrum* czy *Xanthorhiza* (Ziman i Keener 1989). Nadal odkrywa się i opisuje nowe taksony dla nauki (Halamski i in. 2019, Park i in. 2022, Shchegoleva i in. 2022, Zhang i in. 2022). W Polsce w stanie dzikim występuje około 75 gatunków roślin jaskrowatych, skupionych w ponad 20 rodzajach. Wiele z nich jest objętych ochroną gatunkową. Ochroną ścisłą są objęte 22 taksony, a 9 z nich wymaga ochrony czynnej. Ochronie częściowej podlega kolejnych 12 taksonów (Rozporządzenie Ministra Środowiska z dnia 9 października 2014 r.

w sprawie ochrony gatunkowej roślin). W sumie daje to 34 taksony objęte ochroną, co stanowi około 45% krajowych taksonów/gatunków należących do tej rodziny. W Polskiej Czerwonej Księdze Roślin znalazło się 19 taksonów, w tym 6 z kategorią narażone VU, 7 zagrożone EN, 5 krytycznie zagrożone CR i 1 wymarłe w Polsce EX (Kazimierczakowa i in. 2014). Potrzeba ochrony przedstawicieli tej rodziny jest za tym wysoce uzasadniona.

3.2. Opis badanych gatunków – *Ranunculus illyricus* i *Aconitum bucovinense*

Spośród gatunków chronionych z rodziny Ranunculaceae do badań prezentowanych w niniejszej pracy wybrano dwa: *Ranunculus illyricus* i *Aconitum bucovinense*. Pierwszy z nich, jaskier iliryjski (*Ranunculus illyricus* L.), jest gatunkiem kserotermicznym i występuje w zwartym zasięgu w większości krajów Półwyspu Bałkańskiego, środkowych Włoszech, Rumunii, Ukrainie, na południu europejskiej części Rosji i w Turcji. Poza zwartym zasięgiem notowany jest w innych krajach europejskich, w populacjach rozproszonych i często izolowanych, w związku z tym najczęściej jest uwzględniany jako gatunek chroniony (Tomović i in. 2003, Kaźmierczakowa i Towpasz 2014). Natomiast w Polsce przez wiele lat był gatunkiem uznanym za wymarły, został jednak ponownie odnaleziony i obecnie podaje się dwie lokalizacje występowania jaskra iliryjskiego na terenie kraju – Miernów i rezerwat Skorocice (Zając i Zając 2001, Towpasz i Cwener 2002, Dembicz i Kozub 2015). Ze względu na ograniczone występowanie i małą liczebność populacji został objęty ochroną gatunkową i otrzymał status gatunku krytycznie zagrożonego (Kazimierczakowa i Towpasz 2014).

Siedliskiem jaskra iliryjskiego są murawy kserotermiczne, suche i nasłonecznione zbocza, stepy. Jest gatunkiem wskaźnikowym dla kwietnych muraw kserotermicznych (siedlisko 6210-3) (Erdős i in. 2014, Lachashvili 2017). Można go również znaleźć w siedliskach synantropijnych, takich jak kurhany czy stare cmentarze (Molnár i in. 2017, Bede i Csathó 2019).

Rodzaj *Ranunculus*, jako jeden z najbogatszych w gatunki w rodzinie jaskrowatych, został podzielony na podrodzaje i sekcje. Jaskier iliryjski należy do podrodzaju Ranunculus, sekcji Ranunculastrum, dla której jest gatunkiem typowym. Sekcja ta liczy około 70 taksonów (Hörandl i Emadzade 2012).

Charakterystyczny wygląd jaskra iliryjskiego sprawia, że jest łatwy do rozpoznania dzięki gęsto owłosionym częściom nadziemnym. Wyróżniają się również wąskie, klapowane,

trójsegmentowe liście. Jest to wieloletni geofit o krótkim okresie wegetacji trwającym od kwietnia do czerwca i stosunkowo długim okresie spoczynku. Kwiaty ma stosunkowo duże, o żółtych płatkach korony, a kwitnienie przypada na przełom maja i czerwca (Fot. 1 A). Zaraz po zawiązaniu owoców cała część nadziemna rośliny zasycha. Kwiaty zapylane są przez owady, a rozmnaża się przez nasiona (jednonasienne niełupki) lub wegetatywnie poprzez rozłogi (Kaźmierczakowa i Towpasz 2014).

W literaturze nie ma zbyt wielu doniesień badań dotyczących jaskra iliryjskiego. Dostępne dane dotyczą morfologii nasion i niełupki oraz anatomii owocni, która według autorów składa się z jednej warstwy grubościennych komórek (Mourad i in. 2000). Gherghişan (2013) przedstawiła morfologię siewek, a także budowę nasion, które miały średnicę 2-5 mm, barwę żółto-brązową, jajowaty kształt oraz biały zarodek, zlokalizowany w centrum nasiona. Badania potwierdziły w zielu jaskra iliryjskiego obecność kumaryn i flawonoidów o właściwościach antybakteryjnych, dzięki czemu może być ono stosowane w kąpieli ziołowej w przypadku niektórych schorzeń (Akbari 2017).

Drugim gatunkiem jest tojad bukowiński (*Aconitum bucovinense* Zapał.), uznawany za gatunek endemiczny dla wschodnich i południowych Karpat. Występuje w piętrze subalpejskim i alpejskim. W Polsce obecnie jest notowany jedynie w Bieszczadach na dwóch stanowiskach powyżej górnej granicy lasu - Połoninie Caryńskiej i Haliczu (Zając i Zając 2001, Mitka 2003, Mitka 2014). Tojad bukowiński zasiedla półki skalne wśród ziołorośli. W 2006 roku utworzono populację zachowawczą tojadu w eksperymentalnym ogrodzie stacji edukacyjno-przyrodniczej Bieszczadzkiego Parku Narodowego w Suchych Rzekach (Boroń i in. 2011).

Ze względu na ograniczone występowanie w Polsce został objęty ochroną gatunkową (w Rozporządzeniu Ministra Środowiska z dnia 9 października 2014 r. w sprawie ochrony gatunkowej roślin podawany jako *Aconitum tauricum*). Posiada status krytycznie zagrożonego wyginięciem (Mitka 2014). Według Zapałowicza (1908) tojad bukowiński ma pochodzenie hybrydowe i jest utrwalonym mieszańcem między *A. napellus* a *A. paniculatum* (*A. degeni* subsp. *degenii*) (Mitka 2008). Dotychczasowe obserwacje wskazują, że dominującym systemem zapylenia u tego gatunku jest geitonogamia, a występująca izolacja przestrzenna ogranicza przepływ genów. Analizy molekularne ISSR potwierdziły genetyczną odrębność sąsiednich populacji. Obie populacje różniły się nie tylko od siebie nawzajem, ale również wykazano wyraźne różnice w zmienności wewnątrzgatunkowej (Boroń i in. 2011).

Tojad bukowiński jest byliną, hemikryptofitem o wysokości około 30-60 cm. Kwitnienie przypada na okres od lipca do sierpnia. Kwiaty mają charakterystyczną budowę: górna działka kielicha tworzy hełm, w kolorze ciemnoniebieskim lub fioletowym. Są zebrane w groniasty kwiatostan, rozgałęziony u dołu (Fot. 1B). W przypadku tojadu bukowińskiego hełm jest owłosiony, co odróżnia go od mieszańca *A.* × *nanum*. Słupki są trzy, nagie, natomiast pręciki owłosione. Ma liście łodygowe dłoniasto klapowane, 5(7)-dzielne, o lancetowatych odcinkach. Korzenie są wrzecionowato wydłużone. Owocem tojadu jest mieszek (Rottensteiner i Mitka 2001, Mitka 2008, Mitka 2014).

Fotografia 1. A – kwiat jaskra iliryjskiego w optimum kwitnienia, B – kwiatostan tojadu bukowińskiego na stanowisku naturalnym na Połoninie Caryńskiej (Źródło: D. Kocot)

Nasiona tojadu bukowińskiego są przechowywane w Centrum Ochrony Przyrody Polskiej Akademii Nauk w Warszawie (Niemczyk i in. 2015). W celu zachowania unikalnego genotypu populacji krajowych, rośliny uzyskane z nasion powinny być utrzymywane w ogrodach botanicznych w formie kolekcji zachowawczych, żeby w perspektywie stanowić rezerwuar osobników dla ewentualnych zabiegów ochrony czynnej np. reintrodukcji lub tworzenia populacji zastępczych (Boroń i in. 2011). Obserwacje prowadzone w ramach planu

ochrony gatunków rzadkich w Bieszczadzkim Parku Narodowym wskazują, że istotnym czynnikiem ograniczającym liczebność krajowych populacji jest brak efektywnej rekrutacji siewek. W warunkach ogrodowych nasiona kiełkowały dwa lata po wysiewie, lecz śmiertelność siewek przekraczała 80% (Boroń i in. 2011).

3.3. Ochrona gatunków w warunkach *ex situ*

Szacuje się, że od 20 do 39% różnorodności roślin jest w jakimś stopniu zagrożona wyginięciem. Oszacowanie stanu zagrożenia jest kluczowe w celu opracowania planu ochrony. Globalnej ocenie ryzyka wyginięcia zostało poddanych około 10% roślin. Przyczyny wymierania gatunków są różne, lecz w ostatnich latach za główną przyczynę wymierania gatunków uznaje się zmiany klimatyczne (Holz i in. 2022).

Najbardziej właściwa wydaje się ochrona gatunków roślin w ich naturalnych siedliskach, czyli *in situ* . Przykładami takiej ochrony jest tworzenie parków narodowych, rezerwatów czy innych obszarów ochrony. Niestety taka forma ochrony nie zawsze jest wystarczająca, a w niektórych przypadkach wydaje się wręcz niemożliwa. W takich sytuacjach konieczne jest wprowadzenie ochrony *ex situ*, czyli poza naturalnym miejscem występowania gatunku, na wypadek jego wyginięcia. Tworzone kolekcje *ex situ* stanowią źródło materiału roślinnego do celów badawczych, jak i ewentualnej reintrodukcji gatunku. Ochrona *ex situ* staje się jedyną formą zachowania gatunku w przypadku, gdy naturalne stanowiska danej rośliny przestają istnieć. Lista takich gatunków jest naprawdę długa, a przykładami mogą być *Franklinia alatamaha*, *Cochlearia polonica* czy *Sophora toromiro* (Oldfield 2007, Abeli i in. 2019).

Badania naukowe są niezbędne do opracowania skutecznych programów ochrony populacji zagrożonych gatunków. Placówkami, które najlepiej są przystosowane do prowadzenia ochrony *ex situ*, są ogrody botaniczne, które oprócz prowadzenia kolekcji roślin coraz częściej dysponują laboratoriami, szklarniami, poletkami doświadczalnymi czy bankami nasion. Przykładem może być utworzenie Milenijnego Banku Nasion przy Królewskich Ogrodach Botanicznych w Kew (Sarasan i in. 2006, Mounce i in. 2017) oraz banku nasion przy Górskim Ogrodzie botanicznym w Zakopanem czy też banku nasion flory rodzimej Ogrodu Botanicznego PAN – CZRB w Powsinie (Kapler i in. 2014).

Ochrona *ex situ* jest szczególnie istotna w przypadku endemitów, gdyż występują one na bardzo ograniczonym terenie. Taksony bardzo rzadkie i zagrożone, reprezentowane przez małe populacje często wymagają ochrony czynnej. Szczególnie strategia ochrony endemitów powinna obejmować wnikliwą ocenę stopnia i przyczyn zagrożenia oraz opracowanie skutecznych metod ochrony zarówno *in situ* , jak i *ex situ* (Piękoś-Mirkowa i Mirek 2010). Małe populacje często są narażone na dryf genetyczny i chów wsobny, które prowadzą do erozji genetycznej oraz zwiększają ryzyko wyginięcia z powodu losowych fluktuacji środowiska. Niezwykle istotne są również badania związane z biologią kwitnienia i zapylenia gatunków rzadkich, w celu zidentyfikowania ewentualnych zaburzeń, które mogą wpływać ograniczająco na utrzymanie populacji (Denisow i in. 2014).

Coraz częściej w ochronie *ex situ* obok klasycznych technik ogrodniczych korzysta się z różnych metod biotechnologicznych, np. mikrorozmnażania w warunkach *in vitro*. Mikrorozmnażanie jest szczególnie przydane w przypadku, gdy naturalne populacje zostały mocno ograniczone, czasem do pojedynczych osobników, oraz gdy dany gatunek z trudnościami rozmnaża się generatywnie (Marszał-Jagacka i in. 2005, Chandana i in. 2018, Grigoriadou i in. 2019, Sitek i in. 2020). Kolejna metodą jest krioprezerwacja, polegająca na przechowywaniu tkanek roślinnych w bardzo niskich temperaturach w ciekłym azocie (Mikuła i in. 2013). Stosunkowo nową metodą jest technologia uzyskiwania sztucznych nasion, która może być niezwykle pomocna w ochronie bioróżnorodności gatunków mających zaburzenia w tworzeniu żywotnych i zdolnych do kiełkowania nasion (Kamińska i in. 2018, Faisal i Alatar 2019).

4. Główne cele i zadania badawcze pracy doktorskiej

Jaskrowate to ważna rodzina botaniczna we florze Polski ze względu na liczne gatunki, z których wiele jest objętych ochroną gatunkową. Poznanie szczegółów związanych z biologią rozmnażania rzadkich gatunków jest kluczowe do opracowania efektywnych metod ich ochrony w warunkach zarówno *in situ* , jak i *ex situ*. Rozpoznanie ewentualnych zaburzeń związanych z rozmnażaniem zagrożonych taksonów umożliwia odpowiedni dobór metod uzyskania materiału roślinnego do ewentualnej reintrodukcji. Najlepsze dla zabiegów restytucji są metody wykorzystujące naturalne strategie reprodukcji roślin, a w przypadkach jeśli to niemożliwe dopiero metody biotechnologiczne.

Do badań wybrano dwa gatunki należące do Ranunculaceae, które są krytycznie zagrożone wyginięciem w Polsce – jaskra iliryjskiego *Ranunculus illyricus* i tojada bukowińskiego *Aconitum bucovinense*. Głównym celem pracy doktorskiej było poznanie szczegółów związanych z biologią rozmnażania jaskra iliryjskiego dla stworzonej populacji *ex situ* oraz określenie potencjału tworzenia potomstwa na drodze wegetatywnej i generatywnej. W przypadku tojadu bukowińskiego celem było opracowanie efektywnego protokołu mikrorozmnażania w warunkach *in vitro*, które umożliwi uzyskanie dużej liczby roślin, z przeznaczeniem do wykorzystania w badaniach nad biologią tego gatunku, ale też do tworzenia kolekcji *ex situ* czy potencjalnej rehabilitacji gatunku na stanowiskach naturalnych.

Aby zrealizować powyższe cele wyznaczono następujące zadania badawcze:

1. Zbadanie kilku wybranych czynników kształtujących potencjał (ilość słupków w kwiecie, żywotność pyłku) i efektywność (zawiązanie nasion, ich żywotność i zdolność do kiełkowania) rozmnażania generatywnego jaskra iliryjskiego oraz porównanie ich z innymi gatunkami z rodzaju *Ranunculus* rozmnażającymi się w sposób klonalny i aklonalny.

2. Opisanie rocznego cyklu życiowego jaskra iliryjskiego dla populacji *ex situ* wraz z obserwacją wzrostu i rozwoju roślin uzyskanych na drodze generatywnej.

3. Określenie potencjału jaskra iliryjskiego do rozmnażania na drodze wegetatywnej przy pomocy skupień bulwek tworzących się na rozłogach podziemnych i porównanie efektywności rozmnażania wegetatywnego z efektywnością rozmnażania generatywnego.

4. Uzyskanie kultur kalusa tojadu bukowińskiego na pożywkach stałych.

5. Indukcja organogenezy pośredniej w celu uzyskania kultur pędowych tojadu bukowińskiego oraz przeprowadzenie skutecznego ukorzeniania pędów i ich aklimatyzacji do warunków *ex vitro*.

5. Omówienie szczegółowych celów i wyników poszczególnych publikacji wchodzących w skład pracy doktorskiej

Pierwsza praca wchodząca w skład rozprawy doktorskiej, która stanowi część połączonych ze sobą tematycznie publikacji: **„The effectiveness of the sexual reproduction in selected clonal and nonclonal species of the genus *Ranunculus*"** opisuje wybrane parametry związane z biologią kwitnienia kilku krajowych gatunków jaskrów oraz ziarnopłonu wiosennego. Weryfikowaną hipotezą było założenie, że gatunki jaskrów, które mogą rozmnażać się w sposób klonalny, rozmnażają się mniej efektywnie w sposób generatywny. Rośliny dużą część swojej energii i składników odżywczych przeznaczają na rozmnażanie. Zgodnie z objętą strategią życiową gatunki klonalne dzielą swoje zasoby na zarówno tworzenie nasion, jak i organów wegetatywnych służących rozmnażaniu. Natomiast gatunki nieklonalne energię przeznaczoną na rozmnażanie kierują jedynie na tworzenie nasion, dlatego proces ten potencjalnie może być bardziej efektywny.

Podczas przeprowadzania obserwacji szczególną uwagę zwrócono na jaskra iliryjskiego *Ranunculus illyricus*, który jest gatunkiem rzadkim w niektórych krajach europejskich, w związku z czym zebrane szczegółowe dane na temat jego biologii są cenne w kontekście jego ochrony. Do badań wybrano następujące gatunki: *Ficaria verna*, *Ranunculus auricomus*, *R. bulbosus*, *R. cassubicus*, *R. lanuginosus* oraz *R. illyricus*. Ziarnopłon wiosenny *F. verna*, który we wcześniejszych ujęciach systematycznych był klasyfikowany w obrębie rodzaju *Ranunculus*, znalazł się wśród gatunków objętych obserwacjami ze względu na podobną biologię rozmnażania do rozmnażającego się klonalnie jaskra iliryjskiego. Badane gatunki jaskrów podzielono na dwie grupy: gatunki rozmnażające się wyłącznie generatywnie za pomocą nasion (nieklonalne) oraz gatunki - nazwane klonalnymi – które mogą się rozmnażać zarówno w sposób generatywny, jak i wegetatywny – przy pomocy różnych organów, takich jak bulwki, rozłogi. Badania porównawcze wybranych gatunków dotyczyły kilku parametrów kształtujących potencjał i efektywność rozmnażania generatywnego, czyli żywotność pyłku, liczba słupków w kwiecie, efektywność zawiązania owoców, żywotność nasion oraz ich zdolność do kiełkowania i wrażliwość na czynniki przerywające spoczynek. Obserwacje mikroskopowe pozwoliły również na porównanie rozmiarów ziaren pyłku, ocenę skuteczności zapylenia i rozwoju łagiewki pyłkowej na znamieniu słupka.

Do oceny żywotności pyłku wykorzystano metodę barwienia Aleksandra (Alexander 1969). Żywotność pyłku dla badanych gatunków była zróżnicowana, od 38% dla

R. auricomus do 91% dla *R. bulbosus*. Dalsze obserwacje mikroskopowe w świetle fluorescencyjnym (Martin 1959) przeprowadzane na utrwalonych pod koniec kwitnienia słupkach pozwoliły na potwierdzenie efektywnego zapylenia oraz kiełkowania i wzrostu łagiewki pyłkowej w szyjce słupka. W przypadku jaskra iliryjskiego blisko połowa ziaren obecnych na znamieniu nie kiełkowała oraz nie zaobserwowano łagiewek wrastających do zalążni, co może być efektem mechanizmów samoniezgodności.

Jednym z czynników kształtujących potencjał do wiązania owoców jest liczba wykształconych w kwiecie słupków. Natomiast miarą efektywności zawiązania owoców jest odsetek słupków, z których powstały owoce. Średnia liczba słupków w kwiatach dla poszczególnych gatunków była zróżnicowana (od 12 dla *F. verna* do 146 dla *R. illirycus*). Gatunki różniły się również pod względem efektywności zawiązania owoców. Jaskier iliryjski, posiadający największą liczbę słupków w kwiecie, miał jednocześnie najniższy procent zawiązania owoców (11%). Największą efektywność zawiązania owoców odnotowano dla *R. bulbosus* – 64%. Gatunki klonalne (*F. verna* i *R. illirycus*), w porównaniu do gatunków nieklonalnych, miały wyraźnie mniejszą efektywność zawiązania owoców.

Efektywność rozmnażania generatywnego jest skutkiem nie tylko liczby wykształconych nasion (w przypadku jaskrów równej liczbie jednonasiennych owoców), ale też ich żywotności i zdolności do kiełkowania. Do oceny żywotności zawartych w owocach nasion wykorzystano metodę barwienia tetrazoliną (Peters 2000). Podobnie jak w przypadku liczby słupków w kwiecie, skrajne wartości odnotowano dla jaskra iliryjskiego (najwyższa żywotność – 100%) i ziarnopłonu wiosennego (najniższa – 7%). Oceniana z wykorzystaniem testów bibułowych zdolność zebranych nasion do kiełkowania była największa dla gatunków rozmnażających się wyłącznie generatywnie: *R. lanuginosus* (67%) i *R. bulbosus* (68%); zdolność do kiełkowania nasion *R. illyricus*, pomimo ich wysokiej żywotności, była niska (16%). Wobec powyższego oceniono również efekt zastosowania dwóch czynników najczęściej stosowanych do przerywania spoczynku nasion: niskiej temperatury i gibereliny.

Zastosowano czterotygodniową stratyfikację w niskiej temperaturze (4°C), moczenie w roztworze kwasu giberelinowego GA_3 przed siewem przez 24 godziny oraz kombinacje obu czynników. Zabiegi przerywania spoczynku nasion nie zwiększyły zdolności do kiełkowania nasion dla *F. verna* i *R. illyricus*. Natomiast dla *R. auricomus*, *R. cassubicus* i *R. lanuginosus* przedsiewne traktowanie GA_3 zwiększyło liczbę kiełkujących nasion. Co ciekawe, w przypadku *R. bulbosus* odnotowano zmniejszenie zdolności do kiełkowania pod wpływem

stratyfikacji w niskiej temperaturze, co może wynikać z ekologicznych powiązań z siedliskiem występowania (miejsca suche, często kserotermiczne). Jednoczesne stosowanie obu czynników przerywających spoczynek nie poprawiło parametrów kiełkowania w porównaniu do efektu, jaki otrzymano przy stosowaniu ich oddzielnie.

Dla zweryfikowania założonej hipotezy badawczej, w celu wyodrębnienia grup o podobnych parametrach rozmnażania przeprowadzono analizę skupień w oparciu o średnicę i żywotność pyłku, liczbę słupków w kwiecie, zawiązanie owoców, żywotność nasion i ich zdolność do kiełkowania. Analiza skupień wykazała, że zależności były bardziej złożone niż w przyjętej w hipotezie. W efekcie wyodrębniono 4 grupy różniące się efektywnością rozmnażania generatywnego. Do klastra 1 zostały zaliczone dwa gatunki nieklonalne *R. bulbosus* i *R. lanuginosus*, które miały największą żywotność pyłku, najwyższy procent zawiązanych owoców oraz charakteryzowały się wysoką żywotnością nasion zdolnych do kiełkowania. Klaster 2 obejmował pozostałe dwa gatunki uznane za nieklonalne, *R. auricomus* i *R. cassubicus*, które wyróżniały się najmniejszą żywotnością pyłku, średnią liczbą słupków w kwiecie oraz nasionami o małej zdolności do kiełkowania. Gatunki klonalne przydzielono do oddzielnych klastrów. Klaster 3 zawierał *R. illyricus*, który miał największą liczbę słupków w kwiecie, ale charakteryzował się najmniejszą efektywnością wiązania nasion o bardzo dużej żywotności. W ostatnim, czwartym klastrze znalazł się *F. verna* charakteryzujący się wysoką żywotnością pyłku, najmniejszą liczbą słupków w kwiecie oraz nasionami o niskiej żywotności, które nie kiełkowały. Dodatkowo wyodrębniony klaster 2 z *R. auricomus* i *R. cassubicus* zgrupował gatunki apomiktyczne. Co ciekawe, rozdział klastrów odpowiada przyporządkowaniu badanych gatunków do sekcji w obrębie rodzaju *Ranunculus* opisanych na podstawie cech morfologicznych we Flora Europaea (Tutin i in. 1993). Uzyskane wyniki potwierdziły też fakt, że gatunki znacznie efektywniej rozmnażają się generatywnie, jeśli jest to ich jedyny sposób rozmnażania. Efektywność rozmnażania generatywnego u gatunków rozmnażających się dodatkowo w sposób wegetatywny jest mniejsza z uwagi na niższy potencjał (mała liczba słupków, jak u ziarnopłonu) lub różne czynniki ograniczające ten potencjał (mała efektywność wiązania nasion oraz mała ich zdolność do kiełkowania, jak u jaskra iliryjskiego).

Drugą pracą wchodząca w skład rozprawy doktorskiej był artykuł o tytule: **„Reproductive biology of dry grassland specialist *Ranunculus illyricus* L. and its implications for conservation”**, który dotyczył rozmnażania jaskra iliryjskiego, zarówno w sposób generatywny, jak i wegetatywny. Celem pracy było opisanie szczegółów biologii

gatunku w postaci cyklu rocznego. W artykule weryfikowano hipotezę, że efektywność rozmnażania u jaskra iliryjskiego wyrażona liczbą wykształconych roślin w kolejnych sezonach na drodze wegetatywnej jest równa efektywności rozmnażania w sposób generatywny w warunkach *ex situ*.

Doświadczenia i obserwacje prowadzono w latach 2016-2019 na populacji *ex situ* stworzonej przy Wydziale Biotechnologii i Ogrodnictwa Uniwersytetu Rolniczego w Krakowie. W pierwszym sezonie posadzono 39 roślin (macierzystych skupień bulwek) pojedynczo w doniczkach o średnicy 7 cm wypełnionych substratem torfowym i odkwaszonym torfem w stosunku 1:1. Na podstawie obserwacji wzrostu i rozwoju części nadziemnych i podziemnych w kilku sezonach opisano roczny cykl życiowy jaskra iliryjskiego. Pod ziemią wykształcany był system korzeni wiązkowych, z których część zaczynała gromadzić substancje zapasowe i przekształcać się w korzenie bulwiaste. Korzenie bulwiaste były wieloletnie i tworzyły skupienia z pąkiem w centralnej części. U podstawy pąka, między korzeniami bulwiastymi, jesienią mogły się tworzyć rozłogi, które były organem rozmnażania wegetatywnego tego gatunku, bo na nich powstawały potomne skupienia bulwek. Pod koniec czerwca cała cześć nadziemna rośliny, korzenie niezmodyfikowane oraz rozłogi zamierały, a cała roślina przyjmowała postać skupienia bulwek z pąkiem, które wchodziły w stan trzymiesięcznego, letniego spoczynku. Dalszy rozwój rozpoczynał się pod koniec września, gdy pojawiały się nowe cienkie korzenie, rozłogi, a pąk pędowy rozpoczynał wzrost – w takiej postaci roślina zimuje. Wiosną następował dalszy wzrost części nadziemnych jednocześnie z rozwojem części podziemnych i wytwarzaniem potomnych skupień bulwek na rozłogach. Rozwój pędów kwiatostanowych przypadał na maj, a kwitnienie trwało około 3 tygodni na przełomie maja i czerwca. Po dojrzeniu owoców cała cześć nadziemna zamierała, a roślina wchodziła w spoczynek letni.

W celu określenia efektywności rozmnażania wegetatywnego rośliny corocznie wyciągano z doniczek w czasie spoczynku, dokonując pomiarów: ilości skupień bulwek potomnych, masy (zarówno skupienia matecznego, jak i potomnych), liczby bulwek tworzących skupienie. Dzięki oznakowaniu skupień bulwek i corocznym monitoringom udało się prześledzić historię życia poszczególnych klonów oraz ocenić ich zdolność do rozmnażania wegetatywnego. W każdym sezonie pojedyncze skupienie bulwek wytwarzało średnio od 3-4 skupienia potomne, maksymalnie 5. Każde skupienie mogło produkować potomne skupienia przez co najmniej dwa lata. Podsumowując, wśród roślin, które rozmnażały się wegetatywnie, potomstwo po 2 latach to średnio 8 skupień (3-19, w zależności od klonu), a po 3 latach – od

13 do 51 skupień. Zaobserwowano również, że potencjał do rozmnażania wegetatywnego zmieniał się z wiekiem skupienia bulwek. Bulwki potomne, wytworzone w danym sezonie, na końcach rozłogów podejmowały wzrost i rozwijały część nadziemną i zwykle kwitły po okresie spoczynku już w pierwszym sezonie. Najwięcej skupień potomnych (2,2), o największej masie (682 mg) i z największą liczbą bulwek w skupieniu (22) wykształcały skupienia jednoroczne, w kolejnym roku wielkość tych parametrów malała, co świadczy o obniżaniu potencjału wraz z wiekiem.

Charakterystyka rozmnażania generatywnego uzupełniała obserwacje ujęte w poprzedniej pracy o kolejne lata i nowe parametry. Oceniono odsetek kwitnących osobników w populacji i wykazano, że w 2018 roku kwitło 57,6 % osobników, a w 2019 85%. Rośliny kwitnące na pędzie wykształcały od 1 do 4 kwiatów z 66 pręcikami i 147 słupkami. Wysoki potencjał do rozmnażania generatywnego tego gatunku wynika nie tylko z dużej liczby słupków w kwiecie, ale również bardzo dużej produkcji ziaren pyłku, których liczba oceniona została przy pomocy obserwacji mikroskopowych metodą hemocytometryczną (Godini 1981) i wynosiła prawie 140 tysięcy ziaren pyłku na kwiat; żywotność ziaren pyłku wynosiła 53,6–68,5%. Mimo dużego potencjału do rozmnażania generatywnego, ilość zawiązanych nasion była niewielka (najwięcej 12,8%) i kształtowała się na niskim poziomie niezależnie od sezonu wskazując na potencjalne ograniczenia związane ze słabym zapyleniem lub obcopylnym charakterem kwiatów. Ten drugi przypadek może być konsekwencją efektywnego rozmnażania wegetatywnego, co skutkuje dużym udziałem osobników o tym samym genotypie w populacji. Fakt taki ogranicza skutecznie wzrost łagiewki pyłkowej u gatunków obcopylnych.

Ponieważ żywotne nasiona *R. illyricus* mają trudności z kiełkowaniem, oceniono skuteczność innych czynników przerywających spoczynek w porównaniu do stosowanej już wcześniej gibereliny i stratyfikacji w niskiej temperaturze. W efekcie oceniono wpływ zimnej (4°C) lub gorącej stratyfikacji (przedsiewne moczenie w wodzie o temp. 50°C), temperatury kiełkowania (10 lub 20°C) oraz traktowania kwasem giberelinowym na skuteczność przerywania spoczynku nasion. Okazało się, że kluczowym czynnikiem wpływającym na efektywność kiełkowania jest niska temperatura, szczególnie jeśli jest to temperatura kiełkowania. Niezależnie od zastosowanych czynników nasiona kiełkowały między 10 a 18 dniem od wysiewu. Obserwacje przeprowadzone dla siewek przeniesionych do ziemi w doniczkach pozwoliły na śledzenie ich rozwoju i przeżywalności w kolejnych 3 latach. Okazało się, że w warunkach *ex situ* przeżywalność siewek wynosi 50% w pierwszym roku,

pierwsze bulwki zapasowe powstają w tym samym roku u części osobników. Rozłogi służące do rozmnażania wegetatywnego wykształcane są u połowy osobników w drugim roku. Pierwszy kwitnący osobnik został zaobserwowany po trzech latach od wysiewu.

Zakładając najbardziej optymistyczne parametry, które mają wpływ na efektywność rozmnażania generatywnego uzyskane w trakcie badań *ex situ*, czyli liczbę nasion na kwiat - 19 i kiełkowanie nasion na poziomie 40%, do uzyskania jednego osobnika potrzebne są nasiona z więcej niż trzech kwiatów, a taka roślina zakwita dopiero po 3 latach. Z kolei efektywność rozmnażania wegetatywnego opisana dla przykładowego klonu 11, pokazuje, że po trzech latach można uzyskać 57 skupień potomnych z jednej rośliny matecznej. Dodatkowo potomstwo uzyskane na drodze wegetatywnej od razu w pierwszym sezonie podejmuje wzrost i kwitnie, rozmnażając się zarówno wegetatywnie, jak i generatywnie.

Wyniki badań nie potwierdziły założonej hipotezy, ponieważ wykazano, że rozmnażanie wegetatywne było efektywniejsze niż rozmnażanie generatywne, gdyż w ciągu jednego sezonu można było uzyskać więcej osobników potomnych (skupienia bulwek) i były one w stanie w następnym sezonie rozmnażać się wykorzystując oba typy reprodukcji. Uzyskane w warunkach *ex situ* osobniki mogą być stosowane do wzmocnienia istniejących populacji lub do zakładania nowych populacji zastępczych. Mimo że rozmnażanie generatywne jest ograniczone przez niską wydajność zawiązywania owoców oraz słabą zdolność do kiełkowania nasion, jest ono niezbędne do zwiększenia różnorodności genetycznej w obrębie populacji, jak również ułatwienia zapylenia krzyżowego między osobnikami o różnych genotypach.

Artykuł trzeci, który wchodzi w skład powiązanego ze sobą tematycznie cyklu, dotyczy drugiego badanego gatunku, czyli tojadu bukowińskiego i nosi tytuł: **„*In vitro* shoot regeneration from organogenic callus culture and rooting of Carpathian endemic *Aconitum bucovinense* Zapał.”**. Jest to pierwsze doniesienie naukowe dotyczące kultur tkankowych dla tego gatunku. Po raz pierwszy opisano protokół mikrorozmnażania, obejmujący inicjację kultur, kultury kalusa i indukcję organogenezy, namnażanie pędów, ich ukorzenianie i aklimatyzację. W pierwszej hipotezie badawczej założono, że z powodu braku zdolnych do kiełkowania nasion, wykorzystanie eksplantatów liściowych umożliwi indukcję kalusa oraz uzyskanie jego kultur zdolnych do organogenezy. Druga hipoteza badawcza zakładała, że dodane do pożywki MS (Murashige i Skoog 1962) regulatory wzrostu w postaci cytokininy BAP oraz w połączeniu z auksynami IBA lub NAA spowodują namnożenie pędów

przybyszowych. Trzecia postawiona hipoteza zakładała, że dodatek do pożywki zawierającej makroskładniki B5 (Gamborg i in. 1968), mikroskładniki MS i różnej zawartości cytokininy BAP w połączeniu z auksynami IBA, IAA, NAA spowoduje ukorzenianie pędów oraz że proces indukcji ryzogenezy połączony jest ze zwiększoną aktywnością peroksydazy.

Odkażone powierzchniowo przy pomocy alkoholu i chlorku rtęci $HgCl_2$ eksplantaty zostały wyłożone na pożywkę zawierającą makroskładniki B5 oraz mikroskładniki MS z dodatkiem 8,0 mg L^{-1} pikloramu i 5,0 mg L^{-1} kinetyny, co zaindukowało tworzenie się tkanki kalusowej. W celu optymalizacji warunków wzrostu kalusa i różnicowania się pąków przybyszowych zastosowano w pożywce kombinacje BAP z IBA w różnych proporcjach. Zastosowanie regulatorów wzrostu w postaci 0,5 mg L^{-1} BAP z 0,5-1,0 mg L^{-1} IBA spowodowało 2,4-2,8 krotny przyrost masy kalusa w ciągu 6 tygodni trwania pasażu z równoczesnym tworzeniem się pąków przybyszowych.

W kolejnym etapie przeprowadzonych doświadczeń oceniono wpływ auksyn IBA oraz NAA w kombinacji z BAP na proces namnażania pędów przybyszowych na pożywce MS. Okazało się, że zastosowane auksyny nie różniły się pod względem skuteczności namnażania pędów. Jednak najlepsze parametry namnażania, tzn. liczbę nowo powstałych pędów oraz ich jakość (największa liczba liści i najdłuższe liście) uzyskano na pożywce zawierającej jedynie cytokininę BAP w dawce 0,5 mg L^{-1}, a efekt taki utrzymywał się w 3 kolejnych pasażach. Uzyskane wyniki potwierdziły założoną hipotezę, ale najlepsze efekty uzyskano, stosując samą cytokininę.

Na etapie ukorzeniania oceniono skuteczność auksyn IAA, IBA lub NAA w kombinacji z BAP w różnych wzajemnych proporcjach oraz dawkach. Okazało się, że wszystkie zastosowane kombinacje stymulują proces ryzogenezy, lecz największa liczba korzeni powstawała na pożywce z dodatkiem 1,0 mg L^{-1} BAP i 1,5 mg L^{-1} IBA. Co ciekawe, na wszystkich pożywkach z tego etapu odnotowano również większą liczbę formowanych pędów przybyszowych w porównaniu do etapu wcześniejszego. Wpłynął na to zapewne fakt wydłużenia czasu kultywacji o 2 tygodnie oraz zastosowanie innych – uboższych w składniki mineralne – pożywek. Badana aktywność peroksydazy wzrastała od momentu przeniesienia pędów na pożywkę ukorzeniającą do czasu formowania pierwszych korzeni (4 tydzień trwania kultury). W eksplantach kultywowanych na pożywkach z wyższymi dawkami regulatorów wzrostu zaobserwowano zwiększoną aktywność peroksydazy. Pożywka, na której powstawało najwięcej korzeni, charakteryzowała się również największą aktywnością peroksydazy, co

może sugerować, że aktywność peroksydazy jest związana nie tylko z indukcja ryzogenezy, ale też z liczbą tworzonych korzeni. Przeprowadzona ocena ploidalności metodą Galbraith (1989) zmodyfikowaną przez Thiem i Śliwińską (2003) nie wykazała zmian, pomimo zastosowanych wysokich stężeń regulatorów wzrostu na etapie indukcji kalusa na eksplantatach liściowych, dlatego uzyskany materiał roślinny mógłby być wykorzystany do działań konserwatorskich.

6. Wnioski – weryfikacja postawionych hipotez badawczych

1. Gatunki klonalne (*Ranunculus illyricus* i *Ficaria verna*) rozmnażają się mniej efektywnie w sposób generatywny w porównaniu do ocenianych nieklonalnych gatunków z rodzaju *Ranunculus*.

2. Wydzielony w obrębie gatunków nieklonalnych dodatkowy klaster skupia gatunki apomiktyczne o cechach pośrednich - najniższa żywotność pyłku przy małej zdolności kiełkowania nasion i średniej liczbie słupków na kwiat.

3. Jaskier iliryjski posiada duży potencjał rozmnażania generatywnego (duża liczba słupków, duża produkcja pyłku), lecz proces ten jest mało efektywny w związku z ograniczeniami wynikającymi z małego udziału zawiązanych nasion i słabego ich kiełkowania.

4. Rozmnażanie wegetatywne jaskra iliryjskiego pozwala na szybsze uzyskanie większej liczby osobników potomnych, które w kolejnym sezonie są zdolne do rozmnażania wegetatywnego i generatywnego. W związku z tym rozmnażanie wegetatywne jest bardziej efektywne dla badanej populacji *ex situ* w porównaniu do rozmnażania generatywnego.

5. Odkażone powierzchniowo eksplantaty pochodzące z fragmentów liści wyłożone na pożywkę zawierającą makroskładniki B5 oraz mikroskładniki MS z dodatkiem 8,0 mg L^{-1} pikloramu i 5,0 mg L^{-1} kinetyny umożliwiły indukcje kalusa, a BAP w kombinacji z IBA jego dalszy wzrost i różnicowanie.

6. Zastosowanie pożywki MS z dodatkiem samej cytokininy BAP w ilości 0,5 mg L^{-1} jest wystarczające do tworzenia się nowych pędów przybyszowych. Ten wariant pożywki zapewniał powstawanie największej liczby pędów i o najlepszej jakości (z największą liczbą liści i najdłuższymi liśćmi).

7. Największa liczba korzeni powstawała na pożywce zawierającej makroskładniki B5 oraz mikroskładniki MS z dodatkiem 1,0 mg L^{-1} BAP i 1,5 mg L^{-1} IBA. Aktywność peroksydazy wzrastała od momentu przeniesienia pędów na pożywkę do czasu formowania pierwszych korzeni.

7. Podsumowanie

Wyniki badań uzyskane w ramach przedstawionej rozprawy doktorskiej przyczyniły się do uzupełnienia szczegółów dotyczących biologii krytycznie zagrożonych gatunków z rodziny Ranunculaceae. Szczególną uwagę poświęcono aspektom związanym z rozmnażaniem. Metodą pierwszego wyboru pozyskiwania roślin do celów konserwatorskich powinno być rozmnażanie generatywne. Oceniono efektywność rozmnażania generatywnego jaskra iliryjskiego oraz podjęto próbę optymalizacji kiełkowania nasion poprzez testowanie rożnych sposobów przełamywania spoczynku nasion. Niestety udało się zwiększyć kiełkowanie jedynie do 40%. Ze względu na mało efektywne rozmnażanie generatywne gatunku warto w programach ochrony wykorzystać również możliwość rozmnażania jaskra iliryjskiego na drodze wegetatywnej. Zastosowanie połączenia obu metod w przypadku jaskra wydaje się być najbardziej korzystne, gdyż umożliwi zwiększenie liczby roślin oraz zachowanie odpowiedniej różnorodności genetycznej populacji.

W przypadku, gdy dany gatunek zawiązuje niewiele nasion, które słabo kiełkują oraz gdy rozmnażanie wegetatywne jest niemożliwe lub obarczone dużym ryzykiem warto wykorzystać metody biotechnologiczne w celu pozyskania nowych osobników. Przykładem takiego gatunku jest tojad bukowiński. Użycie technik biotechnologicznych, w tym przypadku kultur *in vitro*, umożliwiło opracowanie alternatywnej metody rozmnażania tojadu bukowińskiego.

Prezentowana praca doktorska pokazała, że lepsze poznanie możliwości reprodukcyjnych gatunków chronionych stanowi podstawę do dopasowania odpowiedniej metody pozyskiwania nowych osobników oraz opracowania skutecznych planów ochrony.

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9. Streszczenie pracy doktorskiej w języku polskim

Licznie przedstawiciele rodziny jaskrowatych Ranunculaceae występują w różnorodnych siedliskach głównie na półkuli północnej. Również w Polsce spotykamy szereg gatunków, z czego duża ich część to taksony rzadkie i zagrożone, dlatego zostały objęte ochroną. Rodzina ta charakteryzuje się również dużym zróżnicowaniem budowy kwiatów. Często są to rośliny o właściwościach trujących, ponieważ zawierają liczne związki biologicznie czynne.

Jaskier iliryjski (*Ranunculus illyricus* L.) i tojad bukowiński (*Aconitum bucovinense* Zapał.) to gatunki reprezentujące tę rodzinę, które są krytycznie zagrożone w Polsce. Ze względu na ograniczoną liczbę stanowisk i niewielką liczebność populacji zostały objęte ochroną ścisłą. Brak jest jednak literaturowych wiadomości na temat możliwości reprodukcyjnych tych gatunków.

Prezentowana praca doktorska składa się z cyklu trzech powiązanych tematycznie, oryginalnych publikacji naukowych. Tematem dwóch pierwszych artykułów była biologia rozmnażania jaskra iliryjskiego oraz ocena efektywności rozmnażania generatywnego i wegetatywnego. Przedmiotem badań w trzeciej publikacji był tojad bukowiński, dla którego opracowano protokół mikrorozmnażania w warunkach *in vitro*.

Głównymi celami pracy było: (1) określenie potencjału i efektywności rozmnażania jaskra iliryjskiego na podstawie kilku wybranych parametrów oraz porównanie z innymi gatunkami z rodzaju *Ranunculus*, (2) określenie potencjału do rozmnażania w sposób wegetatywny jaskra iliryjskiego i porównanie efektywności wegetatywnego rozmnażania z generatywnym, (3) opisanie rocznego cyklu życiowego jaskra iliryjskiego dla populacji *ex situ*, (4) uzyskanie kultur kalusa tojadu bukowińskiego oraz indukcja organogenezy pośredniej pędów w celu opracowania protokołu mikrorozmnażania dla gatunku jako alternatywnej metody rozmnażania.

Aby zrealizować postawione cele badawcze, zbadano wybrane czynniki kształtujące efektywność rozmnażania generatywnego jaskra iliryjskiego na tle innych gatunków jaskrów, które podzielono na gatunki klonalne i nieklonalne. Oceniono również efektywność rozmnażania wegetatywnego jaskra iliryjskiego na podstawie corocznych obserwacji liczby skupień bulwek potomnych, ich masy (zarówno skupienia matecznego, jak

i potomnych) oraz liczby bulwek tworzących skupienie. Uzyskane wyniki umożliwiły porównanie efektywności rozmnażania wegetatywnego z generatywnym.

Do inicjacji kultur tojadu bukowińskiego wykorzystano eksplantaty pochodzące z fragmentów liści, które odkażono powierzchniowo i wyłożono na pożywkę zawierającą makroskładniki B5 oraz mikroskładniki MS z dodatkiem 8,0 mg L^{-1} pikloramu i 5,0 mg L^{-1} kinetyny uzyskując kalus, o zdolnościach do organogenezy. Podjęto próbę ukorzeniania pędów na kilku pożywkach z dodatkiem cytokininy BAP w połączeniu z IBA, IAA lub NAA.

Uzyskane wyniki pozwoliły na wyciągnięcie następujących wniosków: (1) jaskier iliryjski ma duży potencjał rozmnażania generatywnego, lecz rozmnażanie to jest mało efektywne, (2) rozmnażanie na drodze wegetatywnej pozwala na szybsze uzyskanie większej liczby osobników potomnych, które w kolejnym sezonie są zdolne do rozmnażania obydwoma sposobami, dlatego rozmnażanie wegetatywne należy uznać za bardziej skuteczne dla badanej populacji *ex situ*, (3) wykorzystanie eksplantatów pochodzących z fragmentów liści umożliwiło uzyskanie kalusa na pożywce zawierającej makroskładniki B5 oraz mikroskładniki MS z dodatkiem 8,0 mg L^{-1} pikloramu i 5,0 mg L^{-1} kinetyny, (4) zastosowanie w pożywce MS samej cytokininy BAP w ilości 0,5 mg L^{-1} pobudza tworzenie się nowych pędów przybyszowych, (5) zastosowanie 1,0 mg L^{-1} BAP i 1,5 mg L^{-1} IBA w pożywce zawierającej makroskładniki B5 oraz mikroskładniki MS powoduje powstawanie największej liczby korzeni.

10. Streszczenie pracy doktorskiej w języku angielskim

Numerous representatives of the Ranunculaceae family occur in a variety of habitats, mainly in the northern hemisphere. Also in Poland we meet a number of species, many of which are rare and endangered taxa, which is why they have been placed under protection. This family is also characterized by a wide diversity of flower structure. They are often poisonous plants because they contain numerous biologically active compounds.

The Illyrian buttercup (*Ranunculus illyricus* L.) and the Bukovina's monkshood (*Aconitum bucovinense* Zapał.) are critically endangered species in Poland belonging to Ranunculaceae. Due to the limited number of sites and the small size of the population, they have been placed under strict protection. Relatively little information is available about the reproductive potential of these species.

The doctoral dissertation presented consists of a series of three original scientific publications that are thematically related. The subject of the first two articles was the reproductive biology of Illyrian buttercup and the assessment of the effectiveness of generative and vegetative reproduction. The subject of research in the third publication was the Bukovina's monkshood for which a protocol of micropropagation for *in vitro* conditions was developed.

The main goals of the study were: (1) determining the potential and efficiency reproduction of Illyrian buttercup on the basis of several selected parameters and comparison to other species of the genus *Ranunculus* (2) determination of the potential for vegetative reproduction of Illyrian buttercup and comparison of the efficiency of vegetative reproduction to generative (3) describing the annual life cycle of Illyrian buttercup for the *ex situ* population (4) obtaining Bucovina's monkshood callus cultures and induction of indirect shoot organogenesis in order to develop a micropropagation protocol for the species as an alternative method of reproduction.

To achieve the set research goals, selected factors affecting the efficiency of generative reproduction of Illyrian buttercup were examined and compared to other buttercups species, which were divided into two groups - clonal and non-clonal species. The efficiency of the vegetative reproduction of the Illyrian buttercup was assessed on the basis of annual measurements of the number of progeny tuber clusters, their weight (both mother and progeny

cluster) and the number of tubers in the cluster. The obtained results made it possible to compare the efficiency of vegetative and generative reproduction.

For the initiation of the Bukovina's monkshood culture, the decontaminated explants from leaf fragments were used, which were placed on a medium containing B5 macronutrients and MS micronutrients with the addition of 8.0 mg L^{-1} picloram and 5.0 mg L^{-1} kinetin to obtain organogenic callus. An attempt was made to root the shoots on several media supplemented with cytokinin BAP in combination with IBA, IAA or NAA.

The results obtained allowed us to draw the following conclusions: (1) the Illyrian buttercup has a high potential for generative reproduction, but this reproduction is ineffective, (2) vegetative reproduction allows faster obtaining of more progeny plants, which are capable of reproducing in the next season both methods, therefore vegetative reproduction should be considered more effective for the studied population *ex situ*, (3) the use of explants derived from leaf fragments made it possible to obtain callus on a medium containing B5 macronutrients and MS micronutrients with the addition of 8.0 mg L^{-1} picloram and 5, 0 mg L^{-1} kinetin, (4) application of BAP cytokinin alone in the MS medium in the amount of 0.5 mg L^{-1} stimulates the formation of new adventitious shoots, (5) application of 1.0 mg L^{-1} BAP and 1.5 mg L^{-1} IBA in the medium containing B5 macronutrients and MS micronutrients causes the greatest number of roots.

11. Wykaz publikacji i doniesień konferencyjnych niewchodzących w zakres pracy doktorskiej

11.1. Wykaz opublikowanych rozdziałów w monografiach naukowych

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11.2. Wykaz opublikowanych artykułów w czasopismach naukowych

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IF $_{2020}$ = 2,711

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2. Koźmińska A., Hanus-Fajerska E., **Kocot D.** 2016. Wpływ jonów kadmu na *Alyssum montanum* w warunkach kultur *in vitro*. Prace Naukowe Uniwersytetu Ekonomicznego we Wrocławiu. Wybrane zagadnienia z bioekonomii. Wydawnictwo Uniwersytetu Ekonomicznego we Wrocławiu. 461, s. 105-112.

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11.3. Wykaz doniesień naukowych na konferencjach polskich i międzynarodowych

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3. Koźmińska A., **Kocot D.**, Hanus-Fajerska E. 2016. Oczyszczalnie hydrofitowe – alternatywna technologia poprawy jakości środowiska wodnego. I Interdyscyplinarna Akademicka Konferencja Ochrony Środowiska. Gdańsk, 18-20.03.2016.

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10. **Kocot D.** 2017. Wstępne badania nad mikrorozmnażaniem goździka Knappa. III Toruńskie Sympozjum Doktorantów Nauk Przyrodniczych. Toruń, 01-02.04.2017.

11. **Kocot D.**, Koźmińska A., Hanus-Fajerska E. 2017. *In vitro* cultivation of *Pulsatilla turczaninovii*. 6 th International Conference For Young Researchers Multidirectional Research In Agriculture, Forestry and Technology. Kraków, 24-25.04.2017.

12. **Kocot D.** 2017. Indukcja kallusa i wstępne obserwacje organogenezy pośredniej u *Dianthus knappii*. Ogólnopolska Konferencja Młodych Biologów BiologUS – „Innowacje w naukach biologicznych". Szczecin, 27-28.04.2017.

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14. **Kocot D.** 2017. Żywotność pyłku ziarnopłonu wiosennego dla wybranych populacji Krakowa. IV Ogólnopolska Konferencja Młodych Naukowców Nauk Przyrodniczych „Wkraczając w Świat Nauki". Wrocław, 07-08.09.2017.

15. **Kocot D.** 2017. Wpływ czynników przerywających spoczynek na zdolność do kiełkowania nasion *Ranunculus lanuginosus* L. i *Ranunculus bulbosus* L. Ogólnopolska Konferencja Naukowa „ Zrozumieć Naukę". Łódź, 30.09.2017.

16. **Kocot D.** 2018. Inicjacja kultur tkankowych sasanki łąkowej *Pulsatilla pratensis* (L.) Mill. III Interdyscyplinarna Akademicka Konferencja Ochrony Środowiska. Gdańsk, 13-15.04.2018.

17. **Kocot D.** 2018. Indukcja i namnażania kalusa *Ranunculus illyricus* w kulturach *in vitro*. IV Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen. Łódź, 24-25.05.2018.

18. **Kocot D.** 2018. Prospects and limitations of *in vitro* propagation of rare, native plant species from Ranunculaceae family. XV Ogólnopolska Konferencja Kultur *In vitro* I Biotechnologii Roślin. Rogów, 17-20.09.2018.

19. **Kocot D.**, Nowak B., Sitek E. 2019. Różnicowanie paków przybyszowych w kulturach kalusowych *Aconitum bucovinense.* 58. Zjazd Polskiego Towarzystwa Botanicznego „Botanika bez granic". Kraków, 1-7.07.2019.

20. Stachurska-Swakoń A., Towpasz K., Binkiewicz B., Mitka J., Sitek E., **Kocot D.**, Słomka A. 2019. Stan populacji *Ranunculus illyricus* w Polsce. 58. Zjazd Polskiego Towarzystwa Botanicznego „Botanika bez granic". Kraków, 1-7.07.2019.

21. **Kocot D.**, Nowak B., Sitek E. 2019. The use of *in vitro* cultures in the protection of rare species of the family Ranunculaceae on the example of *Aconitum bucovinense* Zapał. and *Ranunculus illyricus* L. XI Konferencja „Kultury *in vitro* w biotechnologii i fizjologii roślin". Kraków, 4-6.12.2019.

22. **Kocot D.**, Sitek E., Nowak B., Mitka J. 2022. Wybrane zagadnienia związane z biologią kwitnienia tojadu bukowińskiego (*Aconitum bucovinense*). LIX Zjazd Polskiego Towarzystwa Botanicznego. Warszawa, 26.06.- 03.07.2022.

11.4. Informacje o projektach naukowych

Kierownik projektu w ramach dotacji celowej na prowadzenie badań naukowych lub prac rozwojowych oraz zadań z nimi związanych, służących rozwojowi młodych naukowców oraz uczestników studiów doktoranckich w 2017 roku - BM-4508.

Tytuł projektu: „Wybrane aspekty rozmnażania generatywnego u rodzimych ratunków z rodzaju *Ranunculus*"

11.5. Informacje o odbytych stażach naukowych

07.08.- 18.08.2017- Staż w Centrum Badań i Ochrony Roślin Górskich Polskiej Akademii Nauk w Zakopanem

12. Cykl publikacji stanowiący pracę doktorską
Publikacja 1

biology

MDPI

Article

The Effectiveness of the Sexual Reproduction in Selected Clonal and Nonclonal Species of the Genus *Ranunculus*

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Simple Summary: The genus *Ranunculus* (buttercup) includes over 600 species, some of which are endangered, e.g., Illyrian Buttercup. Knowledge of the reproductive biology of such species may be crucial for conservation action. For this purpose, six species with different reproduction modes (nonclonal reproducing sexually by seeds only, clonal propagating by seeds and additionally vegetatively and apomictic) were observed. Selected features related to the efficiency of sexual reproduction were described: pollen viability, number of fruit set, seed viability and germination. It has been shown that in clonal species, which include the Illyrian Buttercup, the efficiency of sexual reproduction is lower compared to nonclonal species. The results will support conservation action taken for this species.

Abstract: Generative processes have been evaluated in six European buttercup species in order to verify the hypothesis that the reproduction efficiency of clonal species is lower than that of nonclonal ones. The study covered common species (*Ficaria verna*, *Ranunculus auricomus*, *R. bulbosus*, *R. cassubicus*, *R. lanuginosus*) and the endangered *R. illyricus*. The following properties have been assessed: pollen viability (staining method), pollen grain germination and the pollen-tube elongation in pistil tissues (fluorescence microscopy), seed formation efficiency, seed viability (tetrazolium test) and germination ability by introducing factors interrupting dormancy (low temperature and gibberellin application). Additionally, the pistil morphology was documented for *R. bulbosus*, *R. illyricus* and *R. cassubicus* using SEM techniques. It was demonstrated that the reproductive efficiency, expressed as the production of viable seeds able to germinate, was significantly higher in the species reproducing sexually (especially in *R. lanuginosus*) compared to the clonal ones. However, the complexity observed leads to separation of an additional group (cluster) of apomictic species: *R. auricomus* and *R. cassubicus*, distinguished by the lowest pollen viability and a low ability of the seeds to germinate. In the vegetatively reproducing *R. illyricus*, the seed formation efficiency was just 13.2% despite the having highest number of pistils in its flowers. The developed seeds of this species observed in our experiment were viable, but in general effective methods to stimulate their germination have not been proposed yet. Here, the first comparative study concerning the biology of sexual reproduction of *R. illyricus* is presented in the context of its decreasing distribution in natural habitats.

Keywords: apomictic species; fruit set; pollen viability; *Ranunculus illyricus*; reproductive biology; seed dormancy

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1. Introduction

There are two modes of reproduction in plants: generative using seeds and vegetative (clonal) using bulbs, stolons or tubers. Numerous plant species are able to produce offspring

both in vegetative and sexual ways, and the balance between the two reproductive modes may vary widely across and within the species. The problem of the trade-off between the resources allocated to vegetative versus generative reproduction within a plant has been investigated over recent decades [1–3]. The shares of sexual and clonal progeny may vary and depend on ecological or genetic factors that limit one or the other reproductive mode [4]. A question worth answering is to what extent the efficiency of sexual reproduction depends on the genetically determined reproduction mode.

An interesting choice for such investigations is the *Ranunculus* (buttercups) genus, which comprises about 600 species prevalent in both hemispheres, mainly in the subtropics and temperate zones [5]. Taking into account its taxonomic diversity, this genus is divided into 20 sections [6]. These are annual or perennial herbaceous plants growing in a variety of habitats, from damp meadows through forests up to xerothermic grasslands and mountain areas, as well as in streams and water reservoirs [5,7]. In the previous systematics, the genus *Ranunculus* also included representatives of the contemporary genus *Ficaria* [8,9].

Buttercups are characterized by different reproduction modes. They comprise species reproducing only generatively—self-fertilizing species or self-incompatible species—and those that have developed effective ways of vegetative reproduction [10–12]. There are species developing tubers in the axils of lower leaves (*Ficaria verna* Huds.), aboveground and underground stolons (*R. asiaticus* L., *R. cymbalaria* Pursch, *R. repens* L.), shoots rooting in the nodes (*R. flammula* L., *R. hederaceus* L., *R. reptans* L.), and other permanent underground storage organs (*R. asiaticus, R. illyricus* L., *R. nigrescens* Freyn, *F. verna*) [6]. Some species are optionally apomictic, asexually reproducing via seeds, for example, *R. auricomus* L. or *R. kuepferi* Greuter & Burdet [13–17]. Different strategies preventing self-pollination have been described in cross-pollinated species, such as protogyneous or protandrous flowers [11,18,19]. Both forms of temporal separation of gender phases have been described in *Ranunculus*, especially among the alpine species. Protogyny appeared to be more common and was described for alpine species of New Zealand, USA, lowland populations of *R. acris* L. and *R. repens* in the United Kingdom [18] and cultivated *R. asiaticus* [20]. Protandry of different degrees was reported for *Ficaria verna* [18] and New Zealand populations of *R. acris* and *R. flammula* [21].

The effectiveness of the sexual reproduction noted for *Ranunculus* species varies significantly and results from a proper course of successive stages: production of pollen, pollination, fertilization, seeds' development, their viability and their ability to germinate. The course of these processes is affected by abiotic environmental factors, such as temperature [22–24], light [25] or water [26], as well as the presence of pollinators, which is the case for the self-compatible species *Ranunculus adoneus* A. Gray [22].

The regular course of generative processes depends not only on the availability of pollen, but also on its quality; for self-incompatible species, a precondition for seed formation is pollen of a different genotype [11] high viability. In the case of buttercups, studies have also looked at the size and viability of pollen grains in apomictic species [17,20,27–29]. Izmaiłow [16] conducted research on the apomictic complex of *R. auricomus,* and showed that a change in the plants ploidy level clearly reduced pollen viability and ability to germinate by ca. 30% and 75% in triploids and diploids, respectively.

For the reproductive success of a species, the formation of seeds that are alive and able to germinate is crucial. In many Ranunculaceae species at the time of diaspore dispersal, seed dormancy is determined by an undeveloped embryo or the structure of the seed coat [30–32]. Tiwari et al. [33] identified the dormancy of seeds in buttercups as an endogenous type resulting from underdevelopment of the embryo or physiological state. The natural ability of buttercup seeds to germinate varies across species, from 30% in *R. testiculatus* Crantz [34], to 50% in the case of *R. cortusifolius* Willd. [35], to even 96% for *R. peltatus* subsp. *baudotii* (Godron) Meikle ex C.D.K. Cook [30]. There are many ways in which the dormancy of seeds can be interrupted, and this effect has been studied for several buttercup species. Most commonly, seeds are exposed to low or high temperatures and growth regulators [30,34–37].

The aim of our research was to verify the hypothesis that the clonal species of *Ranunculus* exhibit limited effectiveness of generative reproduction (assessed based on pollen viability, number of pistils per flower, efficiency of fruit set, viability of seeds and ability of seeds to germinate) compared to nonclonal ones. Special attention was paid to *Ranunculus illyricus*—a rare species protected in some central European countries, the biology of which has not yet been studied and reported in detail.

2. Materials and Methods

2.1. Plant Material

Six species from the Ranunculaceae family, including five buttercups and one lesser celandine, were studied. The species come from a temperate climate, but from a range of habitats, and they differ in terms of their reproduction modes (Tables 1 and 2). They are common in Central and Eastern Europe, with the exception of xerothermic *R. illyricus*, which is endangered in some countries in Europe including Poland [38–40].

Table 1. Systematics affiliation, ploidy level, reproduction mode and breeding system of investigated *Ficaria verna* and *Ranunculus* species.

Species	Section [a]	Ploidy Level and Chromosome Number [b]	Reproduction Type [c]	Breeding System [d]
Ficaria verna Huds.	Ficaria	4x = 32	G, Cl	FAl
Ranunculus illyricus L.	Ranunculastrum	4x = 32	G, Cl	Al, SI
Ranunculus bulbosus L.	Ranunculus	2x = 16	G	Al, SI
Ranunculus lanuginosus L.	Ranunculus	4x = 28	G	FAl
Ranunculus auricomus L.	Auricomus	4(2,5,6)x = 16–48	G	Al, SI, FAp
Ranunculus cassubicus L.	Auricomus	4x = 32	G	FAp

[a] according to Tutin et al. [6]; [b] according to PLADIAS [19]; [c] according to Erikson [41], Sarukhán and Harper [12], Troll [42], and Tutin et al. [6]: G—generative, Cl—clonal; [d] according to PLADIAS: FAl—facultative allogamy, Al—allogamy, SI—self-incompatibility, FAp—facultative apomixis.

Table 2. Locality and date of plant material collection of *Ficaria verna* and *Ranunculus* species.

Species	Collection Locality	GPS Coordinates	Collection Date	
			Flowers	Fruits
F. verna	oak hornbeam forest, Bielany, Kraków (Kraków Gate mezoregion)	50°02′54.6″ N 019°50′15.0″ E	21 April 2017 10 April 2019	15 May 2017 1 May 2019
R. illyricus	pot cultivation, collection of University of Agriculture in Kraków (Kraków-Częstochowa Upland)	50°05′03.6″ N 019°57′01.3″ E	7 June 2017 10 June 2019	12 July 2017 10 July 2019
R. bulbosus	grasslands from *Festuco-Brometea*, Mydlniki, Kraków (Kraków-Częstochowa Upland)	50°05′03.8″ N 019°51′34.7″ E	13 May 2017 20 May 2019	20 June 2017 26 June 2019
R. lanuginosus	oak hornbeam forest, Bielany, Kraków (Kraków Gate mezoregion)	50°02′54.6″ N 019°50′15.0″ E	8 May 2017 1 May 2019	22 May 2017 29 May 2019
R. auricomus	moist meadow from Molinietalia coeruleae, Wola Radziszowska, Wieliczka Foothills (Western Carpathians)	49°53′51.1″ N 019°46′16.8″ E	19 May 2017 12 May 2019	16 June 2017 6 June 2019
R. cassubicus	oak hornbeam forest, Uniejów-Rędziny, Miechów Upland (Małopolska Upland)	50°26′39.5″ N 019°58′43.7″ E	19 May 2017 22 May 2019	10 June 2017 12 June 2019

Species reproducing only by seeds are regarded as nonclonal, while those that produces vegetative offspring using bulbs, tubers, rhizomes or stolons are called clonal [43]. Based on this criterion, our own observations (Table 2) and the literature, *R. lanuginosus* [6],

R. bulbosus [12], *R. auricomus* [14,16] and *R. cassubicus* [44–46] were classified as non-clonal species.

The second group—clonal—was represented by *Ranunculus illyricus* and *Ficaria verna*, which are perennial geophytes producing underground clusters of tuberous roots. *Ficaria verna* was previously included in genus *Ranunculus* as *R. ficaria* [9]. In Central Europe, one can also come across the tetraploid *Ficaria verna* subsp. *bulbifera* Á. Löve & D. Löve, which additionally produces descendant tubers in leaf axils [9,47].

The test material comprised flowers and achenes collected from randomly selected individuals in their natural habitats (Table 2), except for *R. illyricus*, which was gathered from a collection at the Faculty of Biotechnology and Horticulture of the University of Agriculture in Kraków. Cultivated plants represented natural resources from one of the two known localities in Poland [39,48,49] and were grown in thermal, light, moisture and edaphic conditions in line with Ellenberg indicator values [50]. The research material was obtained at the optimum times for flowering and fruiting, which were different for each species (Table 2). Specimens in populations did not bloom synchronously, hence it was possible to collect at the same time flowers with open anthers, to assess the viability of pollen, and overblown flowers (flowers with a wilting corolla), to examine pollination effectiveness and pollen-tube elongation.

For every species, 30 and at least 50 (50–132) flowers or multiple fruits were collected in 2017 and 2019, respectively, each from a separate individual. The taxa chosen for the research form an apocarpous gynoecium, from which a spherical cluster of single seed fruits (achenes) develops. The effectiveness of pollination and fruit set were observed in the conditions of open—pollination of plants in natural stands (in situ)— or in the case of *R. illyricus*, an outdoor collection (ex situ).

Table 1 presents the systematic division of the genus into sections following Tutin et al. [6], the chromosome number and breeding system in accordance with PLADIAS [19], and the reproduction type according to Erikson [41], Sarukhán and Harper [12], Troll [42], and Tutin et al. [6].

2.2. Pollen Quality and Pistil Morphology

Anthers and pistils were excised from the flowers to assess pollen quality, germination and pistil morphology. Pollen viability was evaluated using the indirect staining method [51] on the collection day. The assessment was conducted in three repetitions. A single repetition involved a mixture of pollen collected from 10 flowers, each coming from a separate plant. In total, 300 grains of pollen were assessed in each repetition using a Zeiss Axio Imager M2 microscope (Carl Zeiss, Jena, Germany). Photographs were taken using an EOS 450D digital camera (CANON, Tokyo, Japan). At the same time, the diameters of pollen grains were measured using computer graphics program AxioVision 4.8 in three repetitions with 30 grains of pollen each.

Pistils from overblown flowers from the middle part of the receptacle were fixed in FAA (formalin, glacial acetic acid, ethyl alcohol 1:1:8 *v*/*v*/*v*) for 10–12 h, and then macerated in a 30% NaOH solution for two to three hours and cleared with a 6% H_2O_2 solution. Next, pistil tissues were stained with aniline blue for three hours [52] and squeezed between microscopic slides. The observations of pollination, pollen germination and the pollen-tube growth from the stigma to the ovary were conducted in fluorescent ultraviolet light with a wavelength of about 356 nm, using a Zeiss Axio Imager M2 microscope (Carl Zeiss, Jena, Germany). in the fluorescence mode. This allowed for an assessment of the percentage of pollinated pistils, and among them, the share of pistils with germinating and nongerminating pollen. There were at least 50 pistils per species analyzed.

For *R. bulbosus*, *R. illyricus* and *R. cassubicus*, additional documentation was prepared for pistil pollination and morphology using the SEM technique. The material was fixed in FAA, dehydrated in ethyl alcohol and dried in vacuum. The dried tissues were sputter-coated with gold and viewed under a Phenom™ ProX Desktop SEM electron microscope (ThermoFisher Scientific™, Waltham, MA, USA).

2.3. Efficiency of Seed Formation, Their Viability and Ability to Germinate

The efficiency of seed (single seed fruit) formation expressed as percentage was calculated as the number of ripe achenes per the number of pistils.

Seed viability was assessed using the tetrazolium method [53] in the year 2019. The achenes were soaked in water for 24 h, and then, after removing the pericarp, in a tetrazolium solution for the same period of time. The viability was evaluated for 15 to 58 seeds per species.

Seed material was also evaluated in terms of its ability to germinate using the blotter test in Petri dishes under a 16/8 h photoperiod and a temperature of 20 ± 2 °C [54]. The influence of factors interrupting seed dormancy was examined for a few variants: 4-week low-temperature stratification, pre-sowing conditioning with gibberellic acid (GA_3), and a combination of both factors. Each treatment (and control) involved four Petri dishes (repetitions) with 25 seeds from each species.

In the stratification treatment, the seeds were kept at a low temperature (4 °C) for a period of four weeks. Application of gibberellin consisted of soaking seeds in a GA_3 solution at a concentration of 1.0×10^{-3} mM for 24 h before sowing. Seeds that did not undergo this treatment were soaked in water for the same time and were used as control. The number of germinated seeds was evaluated after 4 weeks.

2.4. Statistical Analysis

All statistical analyses were performed with STATISTICA v. 13.3. Quantitative variables (efficiency of fruit set, germination of seeds per plate, number of pistils per flower, pollen diameter, pollen viability per sample) were tested for the normality of distribution and the homogeneity of variance. The normality of data in groups (species or clusters) was tested by a means of the Shapiro–Wilk test. The homogeneity of variance in groups (species or clusters) was tested by employing the Levene test. When comparing the groups with anormal distribution and homogeneity of variance, parametric tests (ANOVA) were used. However, when comparing the groups characterized by the lack of a normal distribution or the lack of homogeneity of variance, nonparametric tests (Kruskal–Wallis test) were applied. The null hypothesis tested stated that there were no differences between the groups (species or clusters), and the rejection of the null hypothesis allowed accepting the alternative hypothesis implying the existence of differences between the groups. Details of the tests used are provided in the captions of tables or figures. To assess the relationship between qualitative data, multiway contingency tables and the chi-squared test were used. The null hypothesis stated that there was no association between the group (species or cluster) and the parameter (viability of seeds). After rejection of the null hypothesis, it was possible to accept the alternative hypothesis that there was a relationship between the group and the parameter (viability of seeds). Additionally, cluster analyses were carried out to divide the species into groups of similar characteristics (Euclidean distance, Ward's method). The significance level was $\alpha = 0.05$.

3. Results

3.1. Pollen Quality and Pistil Morphology

The pollen of the species studied in our work differed in its viability, from 38% for *R. auricomus* to 91% for *R. bulbosus* (Table 3, Figure 1a–c). As far as size is concerned, pollen grains were similar to each other across the species (Table 3).

The SEM-aided observation revealed differences in the morphology of pistils across the species studied in our research (Figure 2). The differences concerned the pistil shape and the presence of ovary hairs: in *R. illyricus*, the ovary was glabrous (Figure 2a,d), single long hairs were present at the base of the *R. bulbosus* ovary (Figure 2b,e), whereas in *R. cassubicus*, a hairy ovary was observed (Figure 2c,f). Distinct differences were also noticed on the surface covered with papillae (stigma). A straight pistil of *R. illyricus* was covered with papillae up to about one-third of its height (Figure 2d). In the case of *R. bulbosus*, papillae

concentrated only around the top part of the pistil (Figure 2e), while *R. cassubicus* had papillae unilaterally covering the bent upper part of the pistil up to the ovary (Figure 2f).

Table 3. Traits of generative reproduction of investigated *Ficaria verna* and *Ranunculus* species. Values represent means of parameter ±SE; letters indicate differences between species according to statistical analysis for each parameter, separately. Based on chi-squared analysis, it can be only stated that clusters differ from each other.

Species	Pollen Viability Per Sample (%/Sample)	Pollen Diameter (µm)	Number of Pistils Per Flower (pcs/Flower)	Efficiency of Fruit Set (%)	Viability of Seeds (%)	Germination of Seeds Per Plate (%/Plate)
F. verna	84 ± 3.1 bc	37 ± 0.2	12 ± 0.3 a	22 ± 1 b	7	0 ± 0 a
R. illyricus	59 ± 2.1 abc	36 ± 0.4	146 ± 3.2 e	11 ± 1.2 a	100	16 ± 2.1 a
R. bulbosus	91 ± 2.2 c	36 ± 1.7	32 ± 0.8 c	64 ± 3.2 d	97	68 ± 2.8 b
R. lanuginosus	85 ± 2.7 bc	41 ± 0.2	22 ± 0.6 b	51 ± 3.2 cd	67	67 ± 4.7 b
R. auricomus	38 ± 1.9 a	35 ± 0.7	49 ± 2.6 cd	35 ± 1.8 c	35	24 ± 6.4 a
R. cassubicus	54 ± 2 ab	36 ± 0.5	84 ± 2.8 d	33 ± 1.6 c	48	22 ± 5.8 a
Test	Kruskal–Wallis test	Kruskal–Wallis test	Kruskal–Wallis test	Kruskal–Wallis test	Chi-square	Kruskal–Wallis test
p-value	0.0000	0.1036	0.000	0.000	0.00000	0.0000

Despite the different dates of flowering across the species, both in the case of the natural sites and in the collection, the pollination (in the open-field conditions) was effective because the majority of the observed stigmas were covered with pollen (Table 4, Figure 1d–i). There were numerous germinating pollen grains observed on stigma of all the species. For the majority of the species (not in *R. illyricus*), a single pollen tube entering the ovary was also seen.

Table 4. Pollination effectiveness and pollen-tube elongation in pistil tissues of investigated *Ficaria verna* and *Ranunculus* species.

Species	Pollinated Pistils(%)	Pistils with Nongerminating Pollen	Pistils with Germinating Pollen Not Entering Ovary	Pistils with Pollen Tubes Entering Ovary
		% of Pollinated Pistils		
F. verna	100.0	6.5	45.1	48.4
R. illyricus	91.0	55.5	54.5	0
R. bulbosus	86.2	4	68	28
R. lanuginosus	80.0	0	93.7	6.3
R. auricomus	78.6	31.8	59.1	9.1
R. cassubicus	95.2	15	80	5

3.2. Seeds' Formation Efficiency, Their Viability and Ability to Germinate

The number of pistils per single flower varied across the species studied here (Table 3); extreme values were noted for two clonal species and ranged from 12 per flower for *F. verna* to 146 for *R. illyricus*. The species differed in terms of the effectiveness of fruit setting. The fruit set per flower estimated for *R. bulbosus* was 64%, while for *R. illyricus* it was only 11%. In this case, the clonal species were less effective than the other species, but also differed from each other. The highest viability of seeds was noted for *R. illyricus* and the lowest for *F. verna*. The species differed significantly in terms of the ability of seeds to germinate, with the best result observed for *R. lanuginosus* and *R. bulbosus* (Table 3, Figure 3). The species also responded in different ways to dormancy-breaking factors.

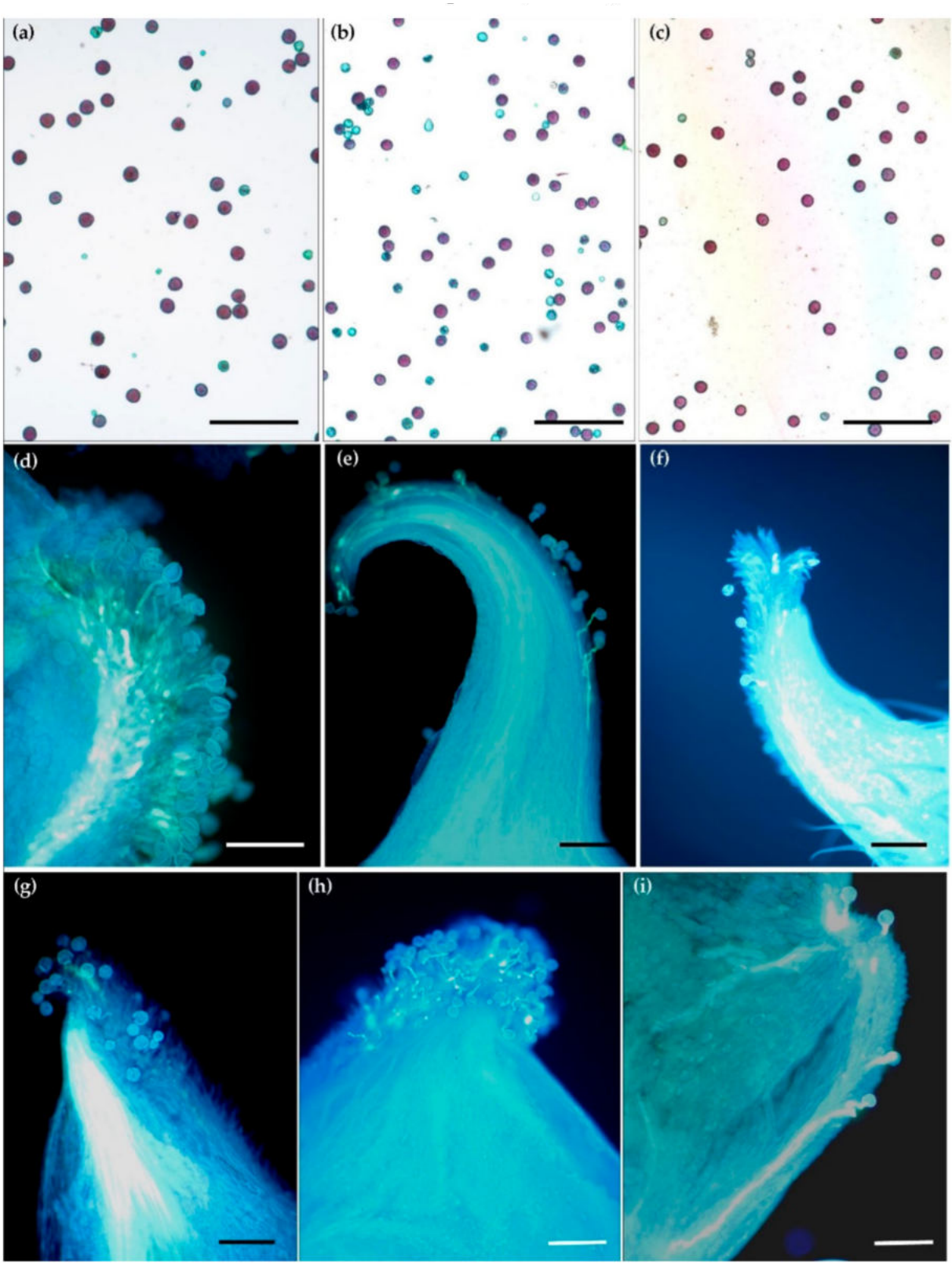

Figure 1. Pollen grains' viability and germination for *Ranunculus* species. Pollen grains' viability for (**a**) *R. illyricus*, (**b**) *Ficaria verna*, (**c**) *R. bulbosus* after Alexander method staining: red—alive, green—dead. (**d**–**i**) Pollen grains' and pollen tubes' growth in fluorescent ultraviolet light after aniline blue staining; (**d**)—germinating pollen grains on stigma of *F. verna*, (**e**) *R. lanuginosus*, (**f**) *R. auricomus*, (**g**) *R. illyricus*, (**h**,**i**) *R. bulbosus*, bar = 200 µm.

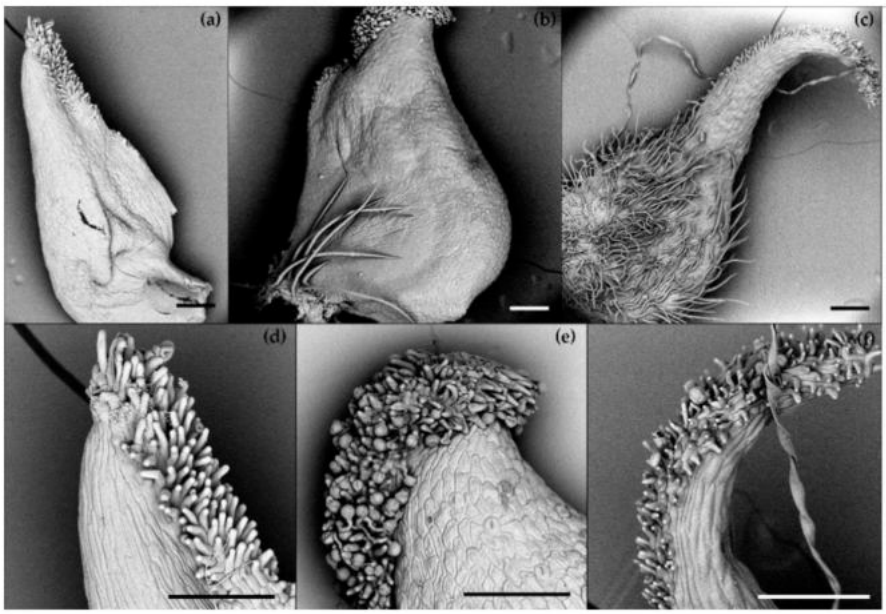

Figure 2. Pistil morphology of *Ranunculus* species, SEM; (**a**,**d**)—*R. illyricus*, (**b**,**e**)—*R. bulbosus*, (**c**,**f**)—*R. cassubicus*; bar = 200 μm.

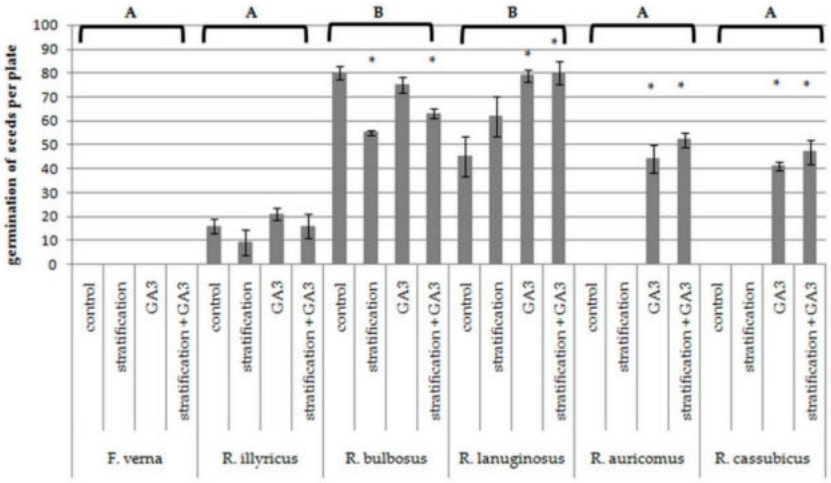

Figure 3. Ability of seeds to germinate after exposure to low temperature and GA_3 treatment. Letters indicate differences between species according to Kruskal-Wallis and Dunn's test with $\alpha = 0.05$. * indicate differences between each treatment and control according to U-Mann–Whitney test with $\alpha = 0.05$ separately for each species.

Germination did not improve in the case of *F. verna* and *R. illyricus*, while for the germination of *R. auricomus* and *R. cassubicus* L., application of GA_3 was crucial. GA_3 also significantly improved germination of *R. lanuginosus* seeds. Low temperature decreased the germination ability of *R. bulbosus* (Figure 3). The combined action of the dormancy breakers did not improve germination parameters compared to the effect of either of the factors separately.

The species exhibited a variety of features, however, in order to decide if reproduction traits allow for distinguishing separate species groups, a cluster analysis was carried out based on pollen diameter and viability per sample, number of pistils per flower, efficiency of fruit set per flower, viability of seeds, and their ability to germinate per plate.

3.3. Cluster Analysis

Our cluster analysis formed four groups at 2.0 Euclidean distance. Cluster 1 with *R. bulbosus* and *R. lanuginosus*, taxa that reproduce only sexually, is characterized by the highest viability of pollen, highest ability of seeds to germinate, highest number of pistils per flower, highest efficiency of fruit set and high viability of seeds (Table 5, Figure 4). Cluster 2, comprising *R. auricomus* and *R. cassubicus*, is distinguished by the lowest pollen viability accompanied by a low ability of seeds to germinate and a medium number of pistils per flower. The clonal species belong to separate clusters. *R. illyricus*, which forms cluster 3, had the highest number of pistils per flower and the lowest efficiency of fruit set, and developed the most viable seeds, which germinated with difficulties. Cluster 4 with *F. verna* is characterized by high pollen viability, the lowest number of pistils per flower, and set seeds of low viability, which did not germinate.

Table 5. Values of traits of generative reproduction in clusters (mean ± SE). Statistical test used is mentioned. Letters indicate differences between clusters according to statistical analysis for each parameter, separately. In the case of chi-squared analysis, it can be only stated that clusters differ from each other.

Parameter	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Test	*p* Value
	R. lanuginosus* *R. bulbosus	***R. auricomus* *R. cassubicus***	***R. illyricus***	***F. verna***		
Pollen viability (%)	88 ± 1.9 c	46 ± 2.7 a	59 ± 2.1 b	84 ± 3.1 c	ANOVA, LSD Fisher's test	0.000000
Number of pistils per flower	27 ± 0.6 b	68 ± 2.5 c	145 ± 3.2 d	12 ± 0.3 a	Kruskal–Wallis test	0.000
Efficiency of fruit set (%)	58 ± 2.3 d	34 ± 1.2 c	11 ± 1.2 a	22 ± 1 b	Kruskal–Wallis test	0.000
Viability of seeds (%)	82	42	100	7	Chi-square	0.00000
Germination of seeds per plate (%)	67 ± 2.7 b	23 ± 4.3 a	16 ± 2.1 a	0 ± 0 a	Kruskal–Wallis test	0.0000
Pollen diameter (µm)	39 ± 1.5 a	36 ± 0.4 a	36 ± 0.4 a	37 ± 0.2 a	Kruskal–Wallis test	0.4439

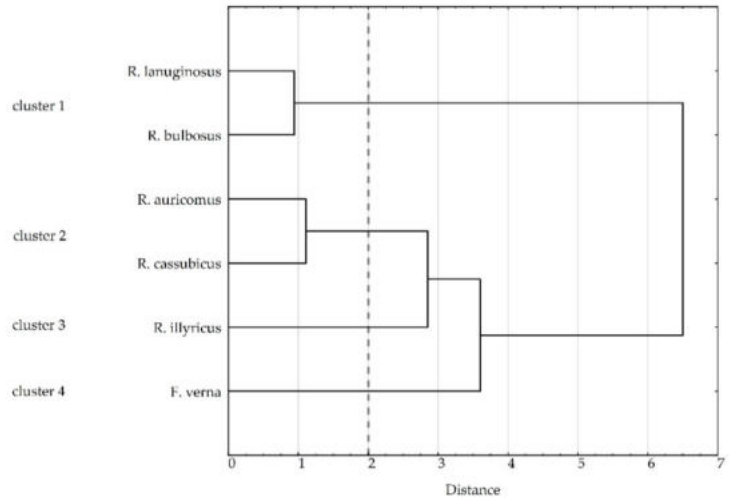

Figure 4. Cluster analysis of species based on pollen viability and diameter, number of pistils per flower, efficiency of fruit set, viability and seed germination (Euclidean distance, Ward's method). Dashed line marks cut-off point (distance 2) and separation into 4 clusters.

4. Discussion

A comparative analysis of traits affecting sexual reproduction was carried out for five *Ranunculus* and one *Ficaria* species, out of which two are clonal perennials with two reproduction modes. The hypothesis to be verified assumed that the clonal species of *Ranunculus* have a limited effectiveness of generative reproduction compared to nonclonal ones. Our results confirmed this hypothesis, and additionally, the cluster analysis revealed that the relationships were more complex than expected. As a result, four groups of species (clusters) differing in their efficiency of sexual reproduction were separated.

The separation of clonal species from nonclonal species was confirmed, however, each of the clonal species constituted a separate group characterized by a low efficiency of generative reproduction expressed in the number of seeds capable of germination. Limitations to this efficiency appeared at different stages of generative reproduction: in *R. illyricus*, as a result of the low efficiency of fruit setting, and in *F. verna*, mainly following the formation of nonviable seeds. It was reported by Perje [55] that the low efficiency of sexual reproduction of *F. verna* was due to low viability of pollen unable to germinate on the stigmas. However, our investigation showed that pollen of this species was viable, germinated on the stigma and entered the ovary, in agreement with the report by Wcisło and Pogan [32].

In both clonal species, vegetative reproduction dominates, which relates to high allocation of resources to the production of vegetative propagules. Although these two species develop underground tubers, the mode of vegetative reproduction is different. Our observations of the *R. illyricus* plants in the collection revealed that the mother cluster of bulbs develops descendant clusters of bulbs (ramets) at the end of underground rhizomes for a few consecutive years. The underground clusters of tubers of *R. illyricus* are dedicated to new progeny, while the underground tubers of *F. verna* serve as a resource storage ensuring survival of the mother plant. Propagating bulbs of this species are formed on aboveground shoots in the axils of leaves.

Among the nonclonal species, two sister groups were distinguished: the first group comprised *R. lanuginosus* and *R. bulbosus*, whereas the second group included *R. auricomus* and *R. cassubicus*. This division is very interesting, because among the nonclonal species, a group of apomictic species has been separated [14,16] which were characterized by the lowest pollen viability accompanied by a low ability of seeds to germinate and a medium number of pistils per flower.

Our cluster analysis suggests that apomictic species are closer to the group of clonal species than sexual ones. The common feature of apomictic and clonal species is uniparental offspring developing without fertilization, copying favorable genotypes. However, in the case of apomixis, this process takes less input energy: resource allocation is similar to that of sexually reproducing species with genetically diversified progeny. Although facultative apomixis restricts recombination and reduces the evolutionary potential of the species, it maintains the fertility of individuals and is advantageous for colonization [56]. Apomixis could also support the fitness of a population under unfavorable environmental conditions [57].

Interestingly, the four clusters separated here coincide with the sections described within the genus *Ranunculus* by Tutin et al. [6] (Table 1) based on morphological traits. Similarly, studies [58] on the morphological and genetic diversity of apomictic and sexual *Cenchrus* species have shown that the apomictic species were clustered together separately from the sexual species. Moreover, the genetic distinctiveness was also evident from the assessed morphological features.

4.1. Seed Germination

The seed germination behavior is one of crucial traits in the life history of the plant species. According to the data available, different species of buttercups have different requirements for treatments breaking dormancy and stimulating germination [30,34–36]. It was shown [37] that the use of a low temperature is the most effective way to interrupt

dormancy in species from the Ranunculaceae family growing in temperate climate and mountain areas. We have presented two groups of species that were distinguished in terms of the ability of seeds to germinate and their reaction to the applied factors breaking dormancy (Figure 3). One group included clonal and nonclonal facultative apomictic species, the seeds of which germinated worse than the seeds of sexual species. Neither GA_3 nor low-temperature treatments improved the germination abilities of the clonal species, while gibberellin significantly increased the germination ability of the apomictic species.

Gibberellin, a natural phytohormone, is known for its ability to reduce seed dormancy time and successfully stimulate the seed germination of other plants. Gibberellin is an antagonist of abscisic acid, and both hormones are crucial for germination control [59–61]. Their proportions, and hence the seed germination ability, may be considerably modified by external factors, such as temperature [62]. Consequently, low- or high-temperature treatments are often used to stimulate germination [63]. Low temperature is the most typical factor controlling the physiological dormancy of seeds of plants in temperate climates [60]. Prechilling has been shown on numerous occasions to be effective in breaking seed dormancy for such species [64–66].

The seeds of the remaining nonclonal species in our experiment were characterized by a much greater ability to germinate, although the response of these species to the factors used was different. *R. lanuginosus* reacted like apomictic species, and GA_3 was beneficial for seed germination. The reaction of xerothermic *R. bulbosus* seeds to stratification, which negatively affected the ability to germinate, was thought-provoking. Most likely, the plant was brought into secondary dormancy due to low temperature, a phenomenon already described for annual winter [67], some xerothermic [68,69] or desert species [70]. This mechanism may be important in preventing early germination of seeds in summer or early fall, when the conditions are not suitable for the development of new plant generations.

4.2. Pollen-Tube Development

Microscopic observations confirmed that there had been no restrictions on pollination both in the flowers from natural sites and from the collection (Figure 1d–i). Most of the pistils were pollinated in all the species under investigation and pollen germinated on stigmas profusely.

In all the species except *Ranunculus illyricus*, there were bundles of pollen tubes growing from the stigma towards the ovary where their growth was inhibited. Only for *R. bulbosus* was it possible to record a pollen tube entering the ovule (Table 4). In *Ranunculus*, which possess one ovule per carpel, a single pollen tube is sufficient for fertilization and fruit set. Our results corroborate those of Rendle and Murray [11] and Beruto et al. [20], who in the case of for *Ranunculus* also described large numbers of pollen grains capable of germinating, yet the growth of all but one pollen tube ceased and only that one tube reached the micropyle.

In the case of clonal *Ficaria verna*, for more than half of the specimens analyzed, numerous pollen tubes entering the ovary were observed. This contrasts with the results of Wcisło and Pogan [32] for this species, who reported only a few cases of single pollen tubes entering the ovary and only 7% of fruit set efficiency. The authors stated that the low efficiency of fruit set was a result of retarded growth of pollen tubes, and in consequence, a lack of fertilization in the majority of ovules. Our observations revealed a normal elongation of pollen tubes of *F. verna* corresponding to 25% of fruit set, but seed viability was only 7%. This means there must have been disturbances during the embryo development.

Different results were obtained for *Ranunculus illyricus*, in which numerous pollen grains remained ungerminated and no pollen tube entering the ovary was observed. In this case, mechanisms inhibiting pollen-tube growth probably appeared in the early stages of its growth. The relatively high number of pistils with ungerminated pollen grains of *R. illyricus* (54.5%) could also be a result of low viability of pollen (Table 3).

Ranunculus illyricus, which is a rare and endangered species in many countries, deserves special attention. Active conservation measures should take into account the fact

that the fruit set of *R. illyricus* is low in ex situ conditions, perhaps owing to geitonogamy. It cannot be ruled out that this phenomenon also affects the wild population, but the biology of *R. illyricus* has not yet been investigated.

5. Conclusions

Our comparative analysis of reproductive traits of five *Ranunulus* species and *Ficaria verna* allowed for the distinguishing of four clusters of species, reproducing in sexual (cluster 1), clonal (cluster 2 and 3) and apomictic (cluster 4) modes. The clonal species, which belong to two separate clusters, were characterized by lower efficiency of sexual reproduction compared with the nonclonal species. However, within the nonclonal species, an additional group of apomictic species was separated. The new divisional clusters coincide with classical systematic sections identified within the genus *Ranunculus* by Tutin based on morphological criteria.

The reproductive efficiency, expressed as the development of viable seeds able to germinate, as well as the susceptibility of seeds to dormancy-breaking factors, varied across the species. Gibberellin treatments improved seed germination in contrast to low temperature, which inhibited germination of *R. bulbosus* seeds.

Great potential for sexual reproduction expressed by the number of pistils in *Ranunculus illyricus* flowers was limited by the relatively low viability of pollen and its poor performance in the style. The fruit set efficiency in *R. illyricus* was low, however, seeds were viable. It is important that methods of dormancy breaking should be developed to support future conservation efforts.

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Publikacja 2

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Reproductive Biology of Dry Grassland Specialist *Ranunculus illyricus* L. and Its Implications for Conservation

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Simple Summary: The Ranunculus illyricus—Illyrian buttercup—is threatened with extinction in many countries and measures should be taken to protect it. In order to increase the effectiveness of such measures, it is necessary to know the methods of propagation and to evaluate their efficiency. *R. illyricus* reproduces generatively by seed and vegetatively by clusters of progeny tubers. The method and potential of vegetative propagation are described here for the first time and compared with the potential and actual effectiveness of generative propagation. Both the generative and vegetative propagation methods should be used to strengthen existing populations and create replacements.

Abstract: *Ranunculus illyricus*, a component of xerothermic grasslands, is a declining species and deserves active conservation treatments in many countries preceded by studies on the biology of its reproduction. So far, our knowledge of *R. illyricus*, a species with two modes of reproduction, has been fragmentary. The purpose of the studies presented here was to describe the annual development cycle of *R. illyricus* with particular emphasis on the production of underground tuber clusters that serve as vegetative propagation. Based on three-year-long observations in an ex situ collection, the efficiency of vegetative propagation was estimated and compared with the efficiency of generative propagation. It was found that in 3 years the best clones could produce up to 57 progeny clusters followed by flowering specimens in the first season. Meanwhile, the high potential for generative reproduction was suppressed by many limitations including fruit setting, the germination capacity of seeds, seedling survival rate, and additionally, the first flowering plant was observed only in the third year. It seems that the efficiency of vegetative propagation of this species can be higher than the efficiency of generative propagation. Moreover, vegets bloomed in the first year after emergence, whereas the first plant of generative origin was observed to bloom only after 3 years. A large proportion of individuals of vegetative origin can negatively affect the genetic diversity of the population but their survival rate against competing plants is higher. To enhance the existing populations or to create new ones, it would be best to use plants derived from clonal propagation of genets carried out in ex situ conditions.

Keywords: tubers; progeny plants; *Illyrian buttercup*; clonal plants

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1. Introduction

Anthropogenic pressure in the environment leads to the loss and fragmentation of natural habitats, invasion of alien species, overexploitation of resources, environmental pollution, and climate change. As a result, more and more species are becoming extinct. According to various studies, it is estimated that about 8% [1] up to one-third of plant species are at risk of extinction, including most of those that have not yet been described

given their limited ranges and local rarity [2,3]. In many cases, it is necessary to implement active conservation programs, and their effectiveness depends on the extent to which the biology of the species and threats in the environment are recognised [4]. Reproductive biology directly affects wild populations because reproductive success and certain life cycle traits (longevity, offspring recruitment, and survival) determine population survival and growth, whereas the life form and mode of reproduction affect the level of genetic diversity and distribution within and among populations [5].

Currently, natural and semi-natural grasslands are highly threatened plant communities as a result of conversion to cropland, afforestation, spontaneous succession, and urbanisation. It is estimated that at least 50% of all grasslands in Central and Eastern Europe have disappeared in the last 200 years. These communities are extraordinary refuges of biodiversity. In Europe, 29% of all bird species are associated with grassland habitats, calcareous grasslands and steppes are home to 63% of butterfly species, and among Europe's >6000 endemic vascular plant species, grassland species make up the second-largest group (18.1%) [6,7].

One of the species inhabiting xerothermic grasslands, steppes, and dry sunny slopes is *Illyrian buttercup* (*Ranunculus illyricus*), a clonal herb in the family Ranunculaceae, section Ranunculastrum, subgenus Ranunculus (*Ranunculus*) [8]. It is classified as a dry grassland specialist and referred to as an indicator species of "flowery, meadow steppes" [9–11]. It also occurs in synanthropic habitats, such as burial mounds [12] or old graveyards surveyed in the Pannonian ecoregion [13,14].

The main range of occurrence covers most of the countries of the Balkan Peninsula, central Italy, Romania, Ukraine, the European part of Russia (in the south), and Turkey. Scattered populations occur in many European countries, such as Sweden, Germany, the Czech Republic, Slovakia, Hungary, and Slovenia, where it is most often considered an endangered species [15,16]. In Poland, it is a rare and critically endangered species subject to legal protection and currently occurs only in two locations [17,18]. The resources of this species, closely linked to xerothermic grasslands, are decreasing throughout its whole range.

The *Illyrian buttercup* is easy to recognize due to strong, grey-white hairs on the aboveground parts, also on its three-petaled leaves. It is a perennial classified as a geophyte with a relatively short growing season from April to June, and a flowering period typically between May and June; yet, soon after fruiting, the plant dries up. It is a monoecious, bisexual, and insect-pollinated plant, which reproduces by seeds or vegetatively using underground stolons at the base of the shoot [16,19]. Flowers typical of the genus *Ranunculus*, with the calyx sepals characteristically bent downwards, are self-incompatible [19]. The fruits of this species are one-seeded achenes, whose morphology and anatomical details were described by Mourad et al. [20] and Gherghişan [21].

The *Ranunculus* genus comprises species rich in alkaloids with essential oils containing a high percentage of fatty acid (mainly hexadecanoic acid), phytol, and hydrocarbons [22–24]. The herb of *R. illyricus* is used for medicinal baths because it contains coumarins (umbelliferone) with antibacterial properties [25].

Although *R. illyricus* is known to develop stolons and storage roots, there is no information available on the developmental biology of its underground parts and the recruitment mode of vegetative progeny. As an endangered species in most of its range and as a potentially useful plant (medicinal and ornamental), *R. illyricus* deserves a more detailed understanding of its reproductive biology. The objectives of this study are (A) to describe the biology of the species, and (B) to assess and compare its ability to reproduce generatively and vegetatively in order to verify the hypothesis that the two reproduction modes of this species are equally effective. Understanding the plant's reproduction system can help conservation and management strategies because plant populations may be greatly impacted by limitations related to generative or vegetative reproduction. Reproductive output is defined as the number of individuals produced in the following growing seasons,

i.e., seedlings in generative reproduction (genets) and clusters of progeny tubers (PC) in vegetative reproduction (ramets).

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The research was carried out on an ex situ population of *Illyrian buttercup* (*R. illyricus*) grown in a collection at the Faculty of Biotechnology and Horticulture of the University of Agriculture in Kraków. The cultivated plants represented the population coming from Miernów—one of the Polish natural populations [16,17]—and the plant material was received from the Botanic Garden of the Jagiellonian University in Kraków.

Individual tagged plants were grown in pots of a 7 cm diameter containing peat substrate and deacidified peat (1:1). That was the standard soil used throughout the experiment. Plants were cultivated in open-field conditions, naturally covered by snow during winter. The pots with the plants were watered and over the years of observations, the data describing the thermal conditions in the collection were recorded. The experiment was established in the autumn of 2016 and observations of aboveground and underground parts were conducted for three consecutive seasons (in 2017 and 2018 for all clones, whereas in 2019 for randomly selected clones) (Figure 1). The observations allowed describing the annual development cycle under ex situ conditions.

Figure 1. Labelled *Ranunculus illyricus* specimens growing in the collection: (**a**) vegetative aboveground shoots (20 April 2019); (**b**) flowering (28 May 2019); (**c**) ripening of fruit and die-back of aboveground shoots (24 June 2019).

2.2. Vegetative Reproduction

In autumn 2016, 39 tuber clusters were planted. Each year, during the dormancy period (September/October), the plants were taken out of the pots and the development of the underground parts was evaluated: the number of progeny tuber clusters (PC), the fresh matter (FM) of the mother (MC), and progeny tuber clusters (PC), as well as the number of tubers in the cluster. Each cluster was then replanted separately in new pots and labelled so that the clones derived from a single parent plant could be tracked throughout their development. The data collected in this way allowed us to not only describe the vegetative development of individual clones but also to compare the underground organs (tuber clusters) produced during three consecutive years in each age group of clusters: new, one-year, and two-year clusters of tubers. The clusters labelled "new" were those that were produced as new progeny in a given season, e.g., the clusters planted in the autumn of 2016 had produced entirely "new" clusters by the autumn of 2017. Those replanted in the

autumn of 2017, in turn, were "one-year" clusters in 2018. Throughout the manuscript, a cluster of tubers represents one individual (plant). What is meant by vegetative offspring is a progeny cluster of tubers (PC)—ramets—produced by the mother cluster of tubers (MC).

2.3. Generative Reproduction

During flowering, the proportion of flowering individuals in the population, the height of flowering stems, and the number of flowers per plant were assessed (2018, 2019); in the tagged flowers (n^{2019} = 20), the stamens and pistils (n^{2017}= 25; n^{2018} = 24; n^{2019} = 72 flowers) were counted followed by the fruit set. The efficiency of seed formation was calculated as the ratio of the number of mature achenes to the number of all pistils, expressed as a percentage.

2.3.1. Pollen Quality (Production and Viability)

The anthers were isolated from the breaking buds of the non-tagged plants (Figure 2h) to evaluate the quality of the pollen. The viability of the pollen was assessed on the day of harvest by the indirect staining Alexander method [26], in which red-stained pollen is considered viable and green is considered unviable. Data were collected during three growing seasons. Assessments were made in three replicates, each prepared as a pollen mixture from 10 flowers. The viability of 300 pollen grains from each replicate was assessed using a Zeiss Axio Imager M2 microscope (Carl Zeiss, Jena, Germany). Photographs were taken using an EOS 450D Digital Camera (CANON, Tokyo, Japan).

Pollen production was estimated for the stamens that were sampled before anther dehiscence (Figure 2h). A mixture of 10 stamens from 10 flowers was placed in a 1.5 mL Eppendorf tube and dried at room temperature. After dehiscence, 1.0 mL of distilled water was added and the content was vortexed immediately prior to counting pollen grains using a Bürker haemocytometer [27]. The number of pollen grains in one anther was calculated according to the following formula:

$$A = (X \times Ve)/(Vc \times n)$$

$$A = (X \times 1000\ \mu L)/(0.1\ \mu L \times 10)$$

where:

A—number of pollen grains per anther,
X—average number of pollen grains per counting field,
Ve—total volume of the pollen grain solution in a 1 mL (1000 μL) Eppendorf tube,
Vc—volume of the counting field of 0.1 mm^3 (0.1 μL),
n—number of stamens in the Eppendorf tube.

2.3.2. Seed Viability and Ability to Germinate

The viability was assessed for 30 seeds using the tetrazoline method [28]. The achenes were soaked in water for 24 h, then the pericarp was removed and seeds were soaked in tetrazolium for another 24 h. The seeds whose endosperm and embryo were completely red were considered to be alive, whereas those that were partly red or white were treated as dead.

Ripe one-seeded achenes were tested for their ability to germinate using the blotter test in Petri dishes in conditions of a 16/8-hr day/night photoperiod [29]. The influence of the factors interrupting the seed dormancy was evaluated. In 2017, the effects of germination temperature (10 °C or 20 °C) and warm stratification were evaluated immediately after harvest (soaking in a 50 °C bath for 2 min prior to sowing). The impact of low-temperature stratification (4 °C for a period of four weeks) and pre-sowing conditioning with gibberellic acid (1.0×10^{-3} mM, for 24 h) was evaluated in 2018. The results were recorded after four weeks. The experiment was repeated four times for each factor (combination) using four Petri dishes with five seeds for each combination.

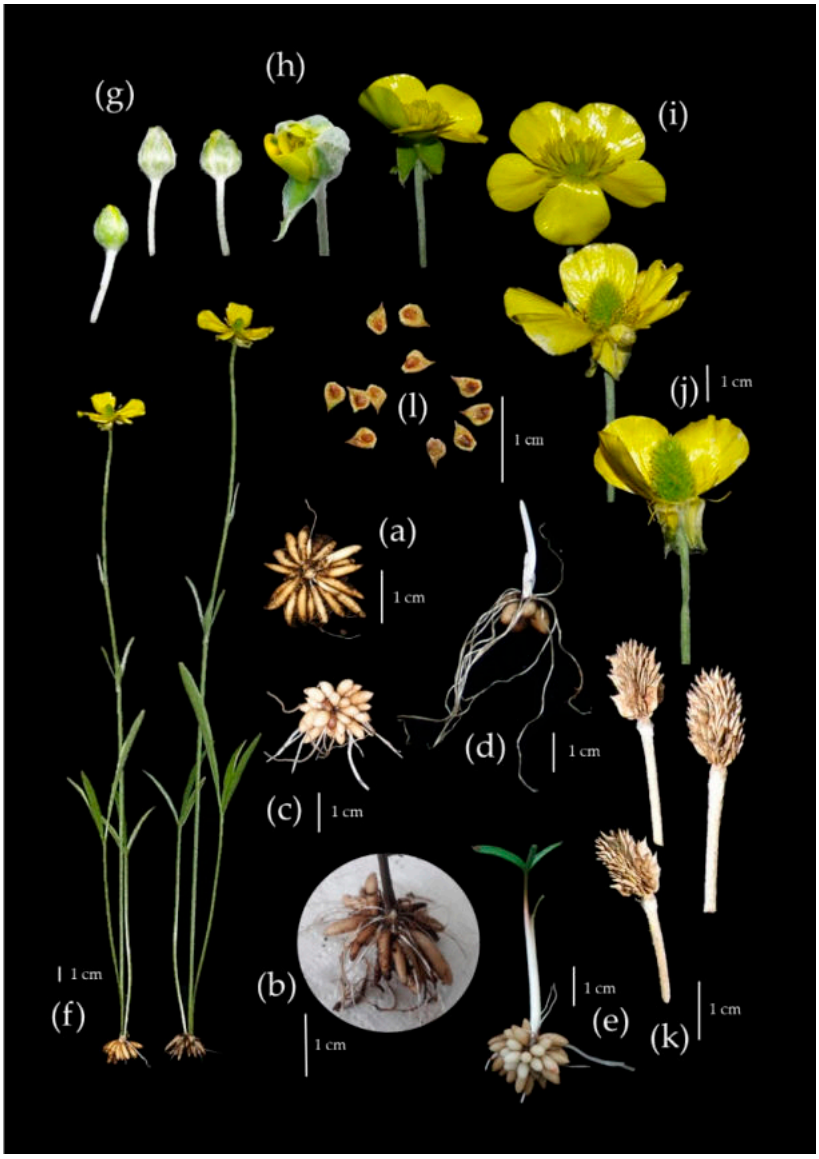

Figure 2. Annual developmental cycle of *Ranunculus illyricus*: (**a**) dormant cluster of tubers, (**b**–**d**) development of underground parts and (**e**) shoot with leaf after dormancy, (**f**) flowering plants, (**g**–**j**) developmental stages of flower, (**k**) receptacle with achenes, (**l**) ripe fruits.

2.3.3. Development and Survival of Seed-Derived Plants under Ex Situ Conditions

A month after sowing (18 July 2018), 25 seedlings with cotyledons obtained from the blotter test were planted individually into pots in the same soil as the mother plants. They were cultivated for 4 weeks in a Sanyo vegetative chamber (Sanyo-Onoda, Japan), under a 16/8-hr day/night photoperiod and photon flux density of 45 μmol m^{-2} s^{-1}, a temperature of 24 ± 2 °C, and humidity of approximately 60%. The plants were then moved to an unheated greenhouse and in spring, after overwintering, transferred to a field collection. The survival rate and development of aboveground and underground organs of the seed-derived plants were observed in subsequent growing seasons (2018–2021) and the number of clusters and tubers per cluster were recorded.

2.4. Statistical Analysis

All statistical analyses were performed with STATISTICA v. 13.3. The normality of data in groups was tested with the Shapiro–Wilk test. The homogeneity of variance in the groups was tested by employing the Levene test. When comparing the groups with a

normal distribution and homogeneity of variance, parametric tests (ANOVA and Tukey or Student *t*-test) were used. However, when comparing the groups characterised by the lack of a normal distribution or the lack of homogeneity of variance, nonparametric tests (Kruskal–Wallis and Dunn's test or -Mann–Whitney U test) were applied. A correlation analysis was also performed where the Pearson correlation coefficient r was determined. Details of the tests used are provided in the captions of tables or figures. The significance level was $\alpha = 0.05$.

3. Results

3.1. Annual Development Cycle

Under experimental conditions, *R. illyricus* develops a system of fibrous roots underground, some of which accumulate storage materials (starch) and develop into tuberous roots (Figure 2b–e). The unmodified roots are annual, whereas the storage roots form perennial tuber clusters with a perennating bud in the central part. In addition, the stolons, which are the organ of vegetative reproduction of this species, can form at the base of the developing bud in autumn (Figure 3).

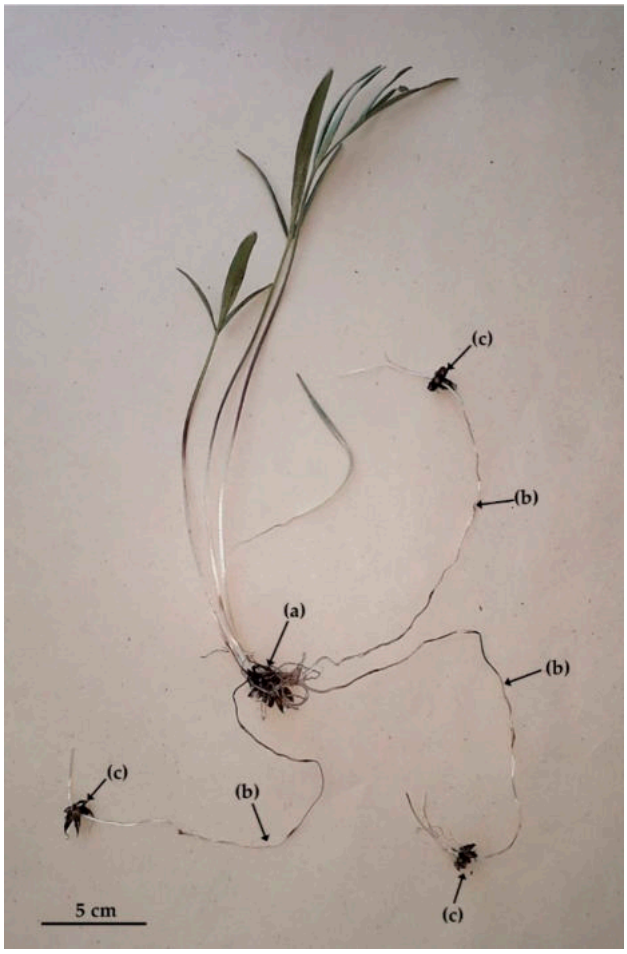

Figure 3. Vegetatively reproducing individual of *Ranunculus illyricus*: (**a**) mother cluster of tubers with aboveground shoot, (**b**) underground stolons, (**c**) progeny clusters of tubers.

At the end of June, the whole aboveground part, the unmodified roots, and the underground stolons connecting the clusters of mother tubers with the progeny die. The plant takes the form of a cluster of tubers with a dormant perennating bud on top (Figure 2a). Dormancy occurs in summer until the beginning of autumn (July–September) and lasts for at least three months. At the end of September, new roots emerge at the base of the regenerating bud, followed by underground shoots. The dormant bud meristem also

becomes active and starts to grow (Figure 2b,c). One month later (in October) the roots reach about 10 cm. At this time, the stolons are shorter than the roots (up to 5 cm), and are thicker and more rigid (Figure 2c,d). In November, the tuber clusters have a developed abundant root system and several-centimetre-long shoots—greenless and hidden under the soil surface (Figure 2d).

In some seasons, the shoots develop leaves above the soil surface and thus overwinter (Figure 2e). The further development of the aboveground parts proceeds in parallel with the further development of the stolons and the formation of progeny clusters. At the end of April, the aboveground vegetative shoots are developed and the stolons end in clusters of progeny tubers underground. The stolons reach a length of 15–20 cm and are divided into 4–5 internodes. At the nodes, single, reduced leaves are visible in the form of scales. Clusters of progeny tubers are formed at the top of the stolon (always one cluster on one stolon). The progeny cluster has only one regenerating bud in the central part, opposite the stolon (Figure 3). The growth of inflorescence shoots occurs in May, whereas the beginning of flowering falls in the third week of May (Figure 2f). The flowering of the population lasts about 3 weeks (to the beginning of June), and the flowering of a single flower, from the opening of the bud to the falling of the perianth and stamens, takes 6–8 days (Figure 2g–j). After ripening and drying of the fruit (Figure 2k), the whole aboveground part dies and the plant starts the summer dormancy period again.

3.2. Vegetative Reproduction

Labelled tuber clusters and their annual monitoring allowed us to track the life history of individual clones and to estimate their ability to reproduce vegetatively in subsequent years. Of the 39 tuber clusters planted in 2016, 90% had survived by 2017, 70% by 2018, and 64% by 2019. This shows that *R. illyricus* ramets can survive at least 3 years under ex situ conditions.

The potential for vegetative propagation was detailed using the example of the clone designated "11" over three years (Figure 4). Each season, an average of 3.3 progeny clusters were produced per plant, with a maximum of 5. During this time, a total of 57 progeny clusters (ramets) were produced from a single mother cluster (clone 11). We observed that each ramet could produce progeny clusters for at least two years (Figure 4).

However, the clones differed in vegetative potential. Some of the plants died off without forming progeny clusters. In general, in the plants that had reproduced vegetatively, an average of 8 (between 3 and 19, depending on the clone) progeny clusters were formed from a single cluster after 2 years, and after 3 years 13–51.

It was not only the clone that affected the number of PC developed but also the age of the cluster. The age of the cluster also determined its FM, the number of tubers in the cluster, and the number of flowers per plant. One-year-old clusters had the highest number of tubers, which was much lower in the two-year-old clusters as was the FM of the cluster (Table 1). Younger tuber clusters also produced more flowers, although not all flowers in both age groups set fruit (Table 2).

Table 1. The fresh matter (FM) of tuber clusters, the number of tubers in one cluster, and number of progeny clusters (PC) for different age clusters in the years 2017–2019.

Age of Cluster	Number of Clusters	FM of Tuber Cluster ± SE [mg]	Number of Tubers in One Cluster ± SE	Number of PC * Produced by One MC **
New	377	573.5 ± 16.9 b ***	13.9 ± 0.21 a	Have not produced PC yet
One year	85	682.3 ± 43.0 b	22.1 ± 0.99 b	2.23 b
Two years	32	392.3 ± 46.5 a	16.5 ± 1.15 a	1.78 a
		Kruskal–Wallis and Dunn's test	Kruskal–Wallis and Dunn's test	Student *t*-test

* PC—progeny cluster, ** MC—mother cluster, *** a, b—values within a column followed by the same letter are not significantly different at $p = 0.05$.

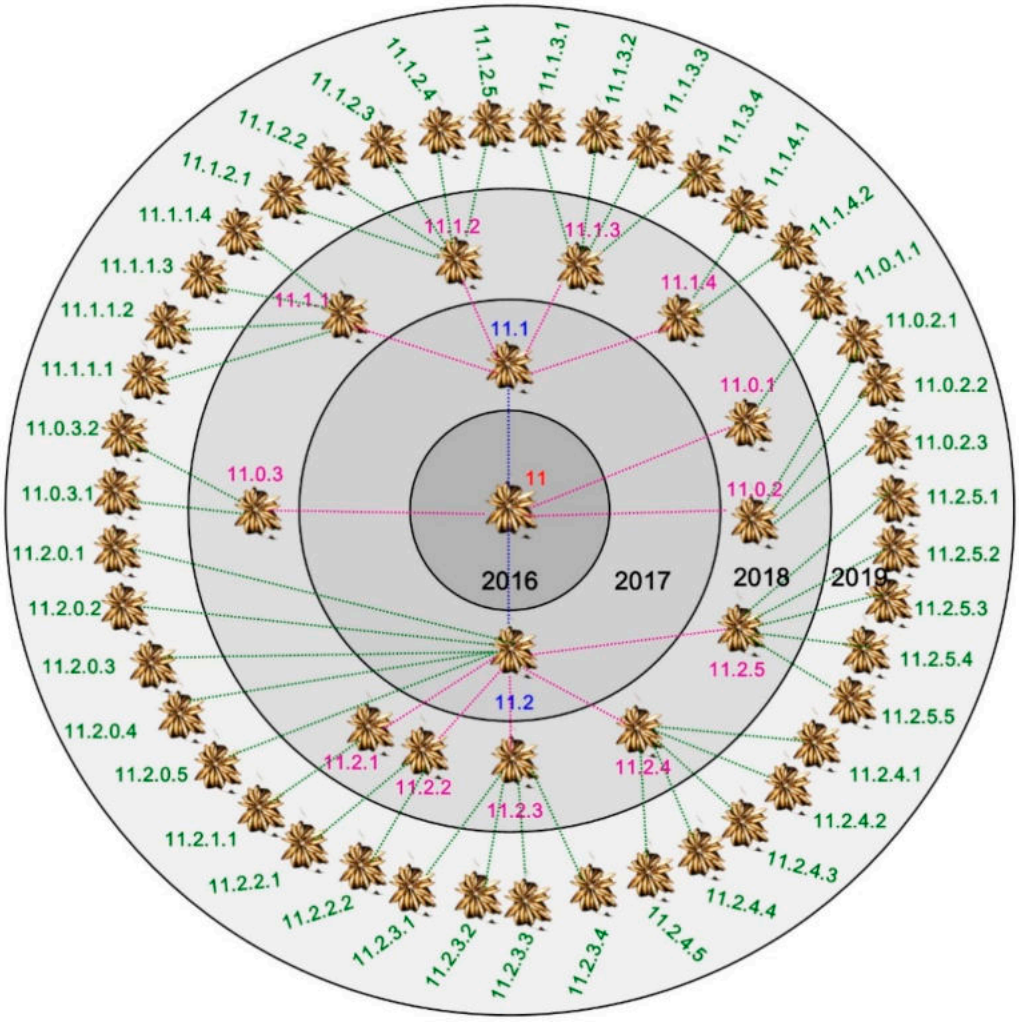

Figure 4. Vegetative development of one *Ranunculus illyricus* clone during a three-year period. Example: 11–mother cluster planted in 2016 produced two progeny clusters in 2017 (11.1 and 11.2) and three in 2018 (11.0.1, 11.0.2 and 11.0.3). In 2018, individual 11.1 produced four progeny clusters of tubers (11.1.1–11.1.4).

Table 2. Effect of tuber cluster age on the number of flowers produced.

Age of Cluster	Number of Clusters	Number of Flowers per Cluster ± SE
New	0	Have not flowered yet
One year	284	1.7 ± 0.08 b *
Two years	105	1.3 ± 0.13 a
		Mann–Whitney U test

* a, b—values within a column followed by the same letter are not significantly different at $p = 0.05$.

To determine whether the size of planted clusters can determine the mode of reproduction in the next season, the plants were evaluated in two age groups in 2018. The plants indicated as "new" in 2017 ($n = 64$) are "one-year-old" in 2018, whereas the "one-year-old" plants in 2017 ($n = 31$) are "two-year-old" in 2018 (Table 3). It turned out that most individuals reproduced both vegetatively and generatively in 2018 (VG in Table 3). In the group "one-year-old" in 2018, 63% reproduced this way, whereas in the group "two-year-old" in 2018, it was 49%. However, there were some that reproduced only vegetatively (36 and 35%, respectively), or only generatively (3 and 16%, respectively).

Table 3. The size of tuber clusters and the reproduction mode for two age groups of clusters in the year 2018 and their effect on selected traits related to the efficiency of generative and vegetative propagation.

Mode of Reproduction in 2018	VG *	V	G	VG	V	G
Assessed Parameter	**Clusters**					
	"New" 2017 (n = 64)			**"One-Year" 2017 (n = 31)**		
Number of tubers in cluster in 2017	16.0 a **	15.0 a	15.5 a	26.9 a	24.1 a	26.2 a
Fresh matter of clusters in 2017 [mg]	1020 ab	700 a	1100 b	1740 a	1150 a	1010 a
	"One-year" 2018			**"Two-year" 2018**		
Number of flowers in 2018	2.2 a	×	1.5 a	1.7 a	×	1.4 a
Percentage of flowers setting fruits in 2018	79.1 a	×	50 a	56.1 a	×	50 a
Height of flowering stem in 2018 [cm]	30.4 a	×	19.9 a	25.7 a	×	25.2 a
Number of tubers in clusters in 2018	26.7 b	19.9 a	×	16.9 a	14.7 a	×
Fresh matter of clusters in 2018 [mg]	850 b	560 a	×	430 a	300 a	×

* VG—vegetatively and generatively, V—vegetatively, G—generatively ** a, b—values in rows for the same age followed by the same letter are not significantly different according to Kruskal–Wallis and Dunn's test as well as Mann-Whitney U test and p = 0.05.

It turned out that the FM of one-year tuber clusters did not co-vary the mode of reproduction in the following year. However, the FM of the younger clusters—"new" clusters—did. In that case, clusters with a higher FM produced individuals that reproduced only generatively (G), and clusters with a lower FM produced individuals that reproduced only vegetatively (V)—Table 3. Moreover, next year in this age group, the tuber FM of the VG individuals was significantly higher than that of the V individuals, which may be due to differences in the FM of the clusters planted a year earlier. However, the VG plants also produced more clusters of tubers (2.7) than the plants reproducing only clonally (1.6). It is puzzling why flowering individuals produced more clusters of higher FM. It could be expected that the allocation of resources to the organs of generative reproduction will have a negative effect on the tuber FM. However, it seems that the additional photosynthetic area of leaves on a flowering shoot meets the needs of generative reproduction as well as the accumulation of storage materials. Plants that reproduce only vegetatively develop only a rosette of leaves.

The height of the inflorescence shoot, the number of flowers, and the percentage of fruit-bearing flowers were the same regardless of the mode of reproduction (G or VG) in both age groups (Table 3).

Temperatures in the months when the development of the aboveground part took place (March, April, and May) affected the FM of the clusters. Higher temperatures in March positively influenced their FM, but higher temperatures in April and May decreased the tuber FM (Table 4, Figure 5). The data suggest that March can be the main month for tuber formation. Assuming optimum plant watering, the negative effects of higher April and May temperatures can be explained by the intensification of developmental processes other than the accumulation of storage materials, for example, faster shoot growth or more numerous flower buds.

Table 4. Correlation matrix of mean temperatures and size of tuber cluster (its FM and number of tubers).

	Mean Temperatures			
	March	**April**	**May**	**June**
Number of tubers in cluster	r = 0.2551 p = 0.000	r = −0.2560 p = 0.000	r = −0.2448 p = 0.000	r = 0.0016 p = 0.975
Fresh matter of cluster	r = 0.4120 p = 0.000	r = −0.4624 p = 0.000	r = −0.3812 p = 0.000	r = −0.1722 p = 0.000

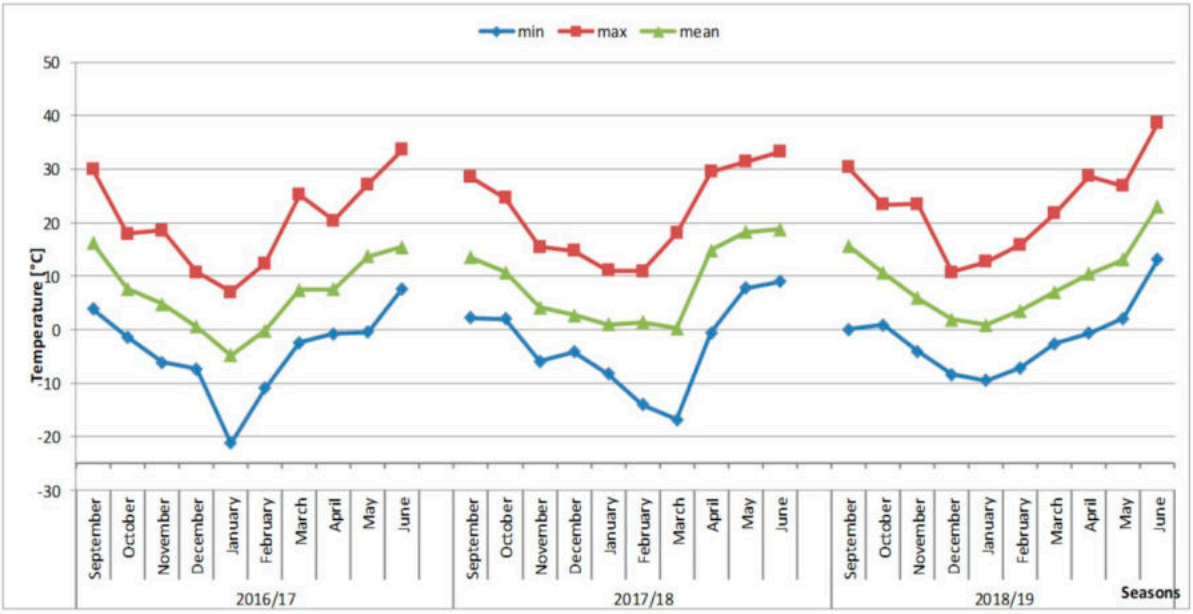

Figure 5. Daytime temperatures during the growing season of *R. illyricus* (September–June) in the years 2015–2019.

3.3. Generative Reproduction

3.3.1. Flowering and Fruit Setting

During the 2018 growing season, 57.6% of the individuals flowered, whereas in 2019, 85.0% of the individuals flowered; the non-flowering individuals developed only a rosette of leaves. The flowering plants had a single stem with one to four flowers, yet some of the flowers did not set fruit—Table 3. On average, the number of stamens was 66, whereas the number of pistils was 147 (138–156), without seasonal variations. The viability of pollen was 53.6–68.5% depending on the year (Table 5). The diameter of the alive (red) pollen grains was larger than that of the unstained, dead pollen grains (Figure 6a). The production of pollen was abundant as almost 140 thousand pollen grains were obtained from one flower (Table 5).

The species is characterised by a high potential for generative reproduction due to the high number of developed pistils in the flower, the number of stamens, and numerous pollen grains with relatively high viability varying across seasons. On the other hand, the seed-setting efficiency, although varying from season to season, was low (the highest was 12.8%) and therefore a small number of fruits formed from numerous pistils (Table 5).

3.3.2. Seed Germination

The seeds of the *Illyrian buttercup* were 100% viable (Figure 6b) but germinated with difficulty. The best germination rate was obtained when the seeds were germinated at a reduced temperature (10 °C), and also those that were subjected to cold stratification germinated relatively well (Tables 6 and 7).

3.3.3. Development of Seedlings under Ex Situ Conditions

R. illyricus seeds germinated between 10 to 18 days after sowing. The seeds germinated epigeically and one month after sowing most of the seedlings had cotyledons (Figure 7a,b). In the first year, plants produced aboveground a rosette of juvenile leaves (3–12 leaves) (Figure 7c,d) and a fibrous root system belowground (Figure 7e). In some cases, the formation of elongated tuberous storage roots was observed in autumn (Figure 7f). In the first year (2018), the mortality of seedlings was high; only 50% survived the first winter (Figure 7g).

Table 5. The production and viability of pollen and efficiency of fruit setting of *Ranunculus illyricus*.

	Year of Evaluation	Mean ± SE	Test
Number of pollen grains in one anther [pcs]	2019	2106.3 ± 990.8	-
Number of stamens in one flower [pcs]	2019	66.3 ± 1.6	-
Number of pollen grains in one flower [pcs]	2019	139,644 ± 69,822	-
Viability of pollen grains [%]	2017	61.8 ± 1.2 ab *	ANOVA and Tukey test $p = 0.05$
	2018	68.5 ± 2.1 b	
	2019	53.6 ± 4.9 a	
Number of pistils in one flower [pcs]	2017	138 ± 7.6 a	ANOVA and Tukey test $p = 0.213$
	2018	154.7 ± 8.3 a	
	2019	148.0 ± 3.4 a	
Number of achenes in one flower [pcs]	2017	18.9 ± 3.0 b	Kruskal–Wallis and Dunn's test $p = 0.0000$
	2018	6.0 ± 1.6 a	
	2019	13.3 ± 1.8 b	
Effectiveness of fruit set [%]	2017	12.8 ± 1.7 c	Kruskal–Wallis and Dunn's test $p = 0.0000$
	2018	3.7 ± 0.9 a	
	2019	8.9 ± 1.1 b	

* a, b, c—values within a column for one assessed feature followed by the same letter do not differ significantly.

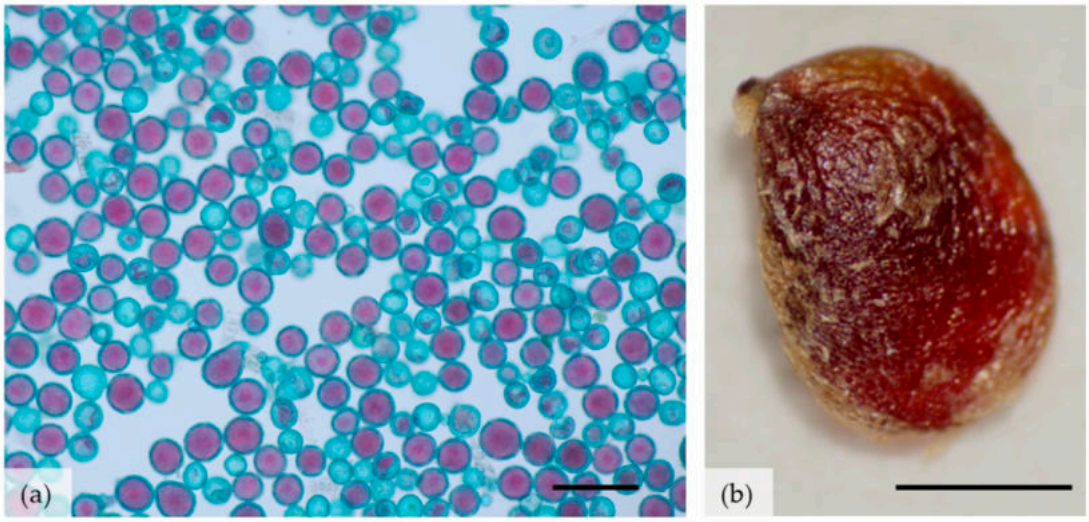

Figure 6. Viability of pollen and seeds of *Ranunculus illyricus*, (**a**) pollen after Alexander staining: red pollen grains are viable; (**b**) seed after tetrazolium staining—living tissue is red. Scale bars: (**a**) 100 μm, (**b**) 1 mm.

Table 6. The effect of hot stratification and temperature on germination ability of *Ranunculus illyricus* seeds [% per plate ± SE].

Temperature of Germination	Hot Stratification		Means for Temperature of Germination
	Without Hot Stratification	Hot Stratification (50 °C)	
20 ± 2 °C	0.0 ± 0.0 a *	0.0 ± 0.0 a	0.0 ± 0.0 *A*
10 °C	40.0 ± 8.1 c	20.0 ± 8.1 b	30.0 ± 6.5 *B*
Means for stratification	20.0 ± 8.5 A	10.0 ± 1.8 A	

* a, b, c, A, *A*, *B*—values within columns and rows followed by the same letter do not differ significantly for $p = 0.5$ and Tukey test; two-way ANOVA was performed: one factor: hot stratification (upper case), second factor: temperature of germination (upper case italics), interaction: hot stratification × temperature of germination—lower case.

Table 7. The effects of cold stratification and gibberellin application on germination ability of *Ranunculus illyricus* seeds [% per plate ± SE].

Application of GA_3	Cold Stratification		Means for GA_3 Application
	Without Stratification	Cold Stratification (4 °C)	
$-GA_3$	0.0 ± 0.0 a *	15 ± 9.6 ab	7.5 ± 6.9 *A*
$+GA_3$	5.0 ± 5 a	30 ± 12.9 b	17.5 ± 11.25 *A*
Means for stratification	2.5 ± 3.5 A	22.5 ± 11.3 B	

* a, b, A, B, *A*—values within columns and rows followed by the same letter do not differ significantly for $p = 0.5$ and Tukey test; two-way ANOVA was performed: one factor: cold stratification (upper case), second factor: GA_3 application (upper case italics), interaction: cold stratification × GA_3 application—lower case.

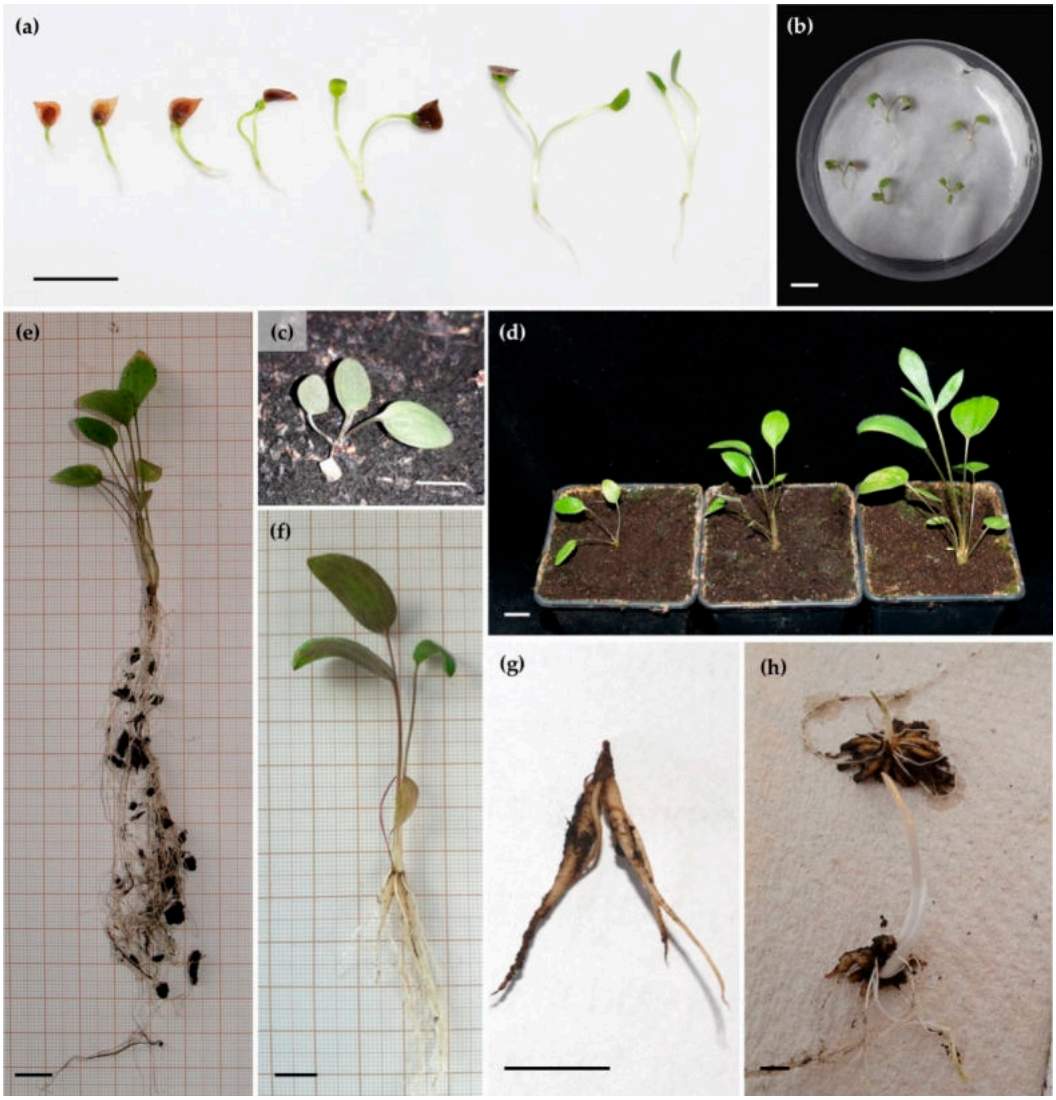

Figure 7. Seed germination and seed-derived plant development of *R. illyricus* under ex situ conditions: (**a**) epigeic seed germination between 10–20 days after sowing, (**b**) seedlings with cotyledons before potting, (**c**) two-month-old seedling with dried cotyledons and juvenile leaves, (**d**) three-month-old seed-origin plants, (**e**) morphological structure of above- and belowground organs of a seed-origin plant at the end of the first growing season, (**f**) individual of generative origin with some tuberous roots at the end of the first growing season, (**g**) cluster of tubers after the first overwintering, (**h**) three-year-old plant obtained from seed (top) and its vegetative progeny (bottom) before winter. Scale bar = 1 cm.

In the spring of the second year (2019), the active plants produced leaves but none of the plants flowered. The plants had thickened roots underground and half of them produced stolons terminated with progeny clusters of tubers but only one per plant.

In the third year, generative reproduction occurred for the first time but only one seed-derived plant flowered. All three-year-old seedlings produced progeny clusters with 5–15 tubers (Figure 7h). In the next growing season, the number of tubers in clusters was higher, on average 15 for three- or four-year-old clusters and 9.5 for two- and one-year-old clusters.

4. Discussion

This article presents *R. illyricus*, a species with a wide geographic range throughout Europe and also Asia. However, in many countries, this species, associated with xerothermic grasslands, is rare and to various degrees under threat of extinction. So far, however, little is known about its reproductive biology and the available data are only fragmentary [30–32].

In order to carry out effective active conservation measures, it is necessary to recognize the threats not only to individual habitats but also to the biology of plant reproduction, which will fully reflect the existing causes of population decline [4,33,34]. In Poland, this species is an extremely rare plant considered to have been extinct for several decades (EX category) [16]. All the more valuable are the two populations discovered later: one is located in a steppe reserve (the Skorocice reserve) and the other on a kurgan in an agricultural landscape (the village of Miernów) [17,18]. The second population is particularly exposed to all adverse changes observed in such a type of habitat: progressive succession, eutrophication, invasion of alien species, intensive agrotechnical treatments, and isolation [35,36]. The observations described here were made on an ex situ collection representing Polish natural resources of *R. illyricus* from the Miernów site.

Ranunculus illyricus is a species with potentially two modes of reproduction: vegetative and sexual. An analysis of data collected over three seasons of observations allowed us to describe the full annual vegetative cycle of *R. illyricus* with particular emphasis on the development of underground vegetative organs. The underground-forming rhizomes ending in clusters of tubers are used for vegetative reproduction and as storage resources.

The number of PC produced is a measure of the efficiency of vegetative propagation. It was found that a single cluster can live for at least three years and produce PC each year. The number of clusters produced varied across clones but also depended on the age of the MC. One-year-old clusters had the highest reproductive potential and produced on average about 2.3 progeny clusters. Two-year-old tuber clusters were already characterised by a lower reproductive capacity, which may be due to their declining storage resources: they counted fewer tubers and had lower FM and they also flowered less (Tables 1–3). It has also been shown that external factors can indirectly affect the efficiency of vegetative propagation. The number and FM of tubers were positively correlated with March temperatures. Warm spring months stimulate earlier development of the aboveground vegetative part, which accumulates the produced resources at that time in the formed underground organs. Presumably, water resources could also modify the efficiency of vegetative reproduction (and this is probably the case in natural populations); however, the ex situ collection was regularly watered and observations were conducted under optimal watering conditions.

The potential efficiency of generative reproduction depends on numerous factors related to the development of the plant, such as the number of viable pollen grains formed and the number of developed pistils in the flower, as well as the number of developed flowers, among others. In the case of *R. illyricus*, numerous pistils and a large number of pollen grains with fairly high viability were formed, but this potential was not fully exploited. Although fruit-setting efficiency varied in successive growing seasons, it was always low and did not exceed 12.8%. The low fruit set may be a consequence of the lack of effective pollinating insects, but it may also be a result of a low genetic variation of the plants in the collection. The collection contained clones that reproduced vegetatively—no individuals of generative origin appeared. Since *R. illyricus* is a self-incompatible species [19], the genetic

homogeneity of the population is a factor that significantly limits the efficiency of generative reproduction. In future, it should be verified what the efficiency of the generative reproduction of this species is and what the proportion of individuals of generative versus vegetative origin would be in natural populations. In the course of our observations, other limitations to generative reproduction have also been noted. Not all developed flower buds opened (Table 3) and set fruit. It is difficult to judge whether this is the result of environmental factors or perhaps a programmed dying of lateral flower buds, which has been described for *Ranunculus bungei* as well as other species in the family Ranunculaceae [37] at early stages of inflorescence differentiation.

Further limitations to generative propagation arise from the difficulty in germinating seeds that enter dormancy. Of the dormancy-interrupting factors tested, the best results were observed with a low (10 °C) germination temperature or the interaction of stratification with GA_3 application. Based on this, it can be concluded that favourable conditions for germination in the wild are created by a cool autumn in the year of seed shedding or by the spring of the following year. A low temperature is a factor that contributes to the breakdown of abscisic acid, which is a germination inhibitor. GA_3 is a plant-growth regulator, an antagonist of abscisic acid, and has been repeatedly used to break seed dormancy [38–41] for *R. asiaticus.* A high temperature, which positively stimulated the germination of *R. asiaticus* seeds [42], proved to be an ineffective factor in breaking the *R. illyricus* dormancy in our studies.

Assuming the most optimistic parameters affecting the efficiency of generative reproduction in ex situ conditions: the number of seeds per flower—19, and the germination capacity of the seeds—40%, it takes seeds from more than three flowers (3.3) to obtain one flowering individual after 3 years. In turn, in the example of clone 11, after one year, on average 3.3 PC develop from one MC, and after three years up to 57. In addition, plants derived from vegetative propagation take up growth and flower already in the first year after formation. With such assumptions, it can be concluded that most individuals, also in natural populations, are of vegetative origin.

The vegetative way of reproduction ensures the survival of vegetative progeny among strong competitors (i.e., grasses). In xerothermic grassland populations, vegetative reproduction is dominant [43]. Seedling development is rare because of strong competition [44,45]. Vegetative reproduction is expensive, as evidenced by the high allocation of mass to the production of vegetative progeny but ensures almost 100% reproduction success. This situation is a classical illustration of the trade-off rules—greater investment in the progeny increases their chances of survival [46]. It also happens that the efficiency of the generative reproduction of xerothermic grassland species is very high, but their populations decline in the face of strong competition and changes in habitat use [40]. In this case, the introduction of grazing or mowing on xerothermic grasslands with the removal of green matter could positively affect the survival of seedlings on natural sites and at the same time increase the genetic diversity of the population.

Two other species with similar biology can be used as a reference point to evaluate reproductive processes: *R. asiaticus* and *Ficaria verna* (i.e., *R. ficaria*). *R. asiaticus* occurs naturally on the Mediterranean coast and is widely cultivated as an ornamental plant [47]. *F. verna*, on the other hand, is a component of the spring undergrowth of deciduous forests native to Europe and Asia, whereas in North America it is an eradicated invasive species [48]. All three species can be defined as perennial geophytes with monocarpic aboveground stems adapted to seasonal climatic changes. *F. verna* was also found to exhibit low efficiency of generative reproduction [32], which does not prevent it from occurring in large numbers thanks to its efficient vegetative reproduction. It is known that in undisturbed communities this mode of reproduction dominates and effectively ensures the survival of plants, e.g., the perennial undergrowth of deciduous forests [49,50] or the steppe perennial in the temperate zone [43].

The development of the underground organs of *R. illyricus*—tuberous roots and stolons—is somewhat reminiscent of *R. asiaticus*, which also undergoes a period of dor-

mancy during hot and dry summer months. During wet and cool months, the plant goes through a generative phase and develops stolons and tuberous roots [31,47,51]. However, the vegetative buds of this species are located in the external leaves of the rosette and give rise to the growing point of the tuberous roots. The tuberous roots can be divided but the annual multiplication rate is only 2–5 [47] making vegetative reproduction of this species less efficient than generative reproduction. The flowers of *R. asiaticus* with over 30 stamens and circa 660 pistils are capable of producing almost 500 achenes for some cultivars and none for others. Therefore, the efficiency of generative reproduction in this species depends on the breeding system of the cultivar [52] and poses a challenge to horticultural production rather than species conservation.

Our research has shown that *R. illyricus* can be successfully propagated under ex situ conditions and plant material can be obtained for active conservation treatments. The plants grown can be used depending on whether they are needed to enhance existing populations or to establish new ones for replacement populations. The vegetative propagation of the species is very efficient and produces a large number of progeny plants in a short time (each season, up to 3–4 progeny plants could be produced per mother plant). Although generative propagation is limited by low fruit-setting efficiency and low seed-germination capacity, it is also possible to obtain progeny plants in this way. *R. illyricus* is described as a self-incompatible species, so it is worth making such an effort to increase genetic diversity within the population and to facilitate cross-pollination between individuals representing different genotypes. Mature achenes can be collected from natural sites or a conservation collection, but it is doubtful that their direct sowing on natural sites can bring satisfactory results. Rather, we recommend that collected seeds should be further handled under ex situ conditions and stratified and/or germinated in low temperatures. The resulting seedlings and later adult plants and their progeny should be carefully labelled and cultivated as separate clones (progeny of one individual of generative origin). Such a procedure requires at least a few years; in our study, the first progeny clusters were formed in two-year-old plants obtained from seed, whereas all three-year-old plants were vegetatively propagating. The first plant obtained from seed also flowered only after three years. It is important to maintain an ex situ plant collection over a long time with the aim of gradually increasing genetic diversity (by obtaining seedlings), and at the same time some plants representing the different clones can be used to feed natural populations. Currently, we have 17 clones obtained from seed in our collection. Based on the observations of the seasonal cycle of *R. illyricus*, the optimal time for conservation measures involving the introduction of plants into the environment may be the dormancy period, which lasts about three months from July to September. It is recommended to plant dormant clusters of tubers in plots cleared of turf, a dozen or so at a time, mixing plants belonging to different clones.

The method of active conservation of *R. illyricus* proposed in the manuscript can be applied to different populations of this species, both in Poland and throughout its geographical range, where the species is losing natural resources. In our opinion, it may also be useful for various species requiring conservation and, in particular, to plants that pursue two modes of reproduction. Rare species having rapidly declining populations undergo a loss of genetic diversity that can have demographic consequences. Therefore, it is preferable to reintroduce plants using seedlings as it is most beneficial for enriching the genetic variability of the population being enhanced or restored [53,54]. However, this is not always easy due to the limitations of generative reproduction, e.g., lack of a partner for mating in self-incompatible plants, low pollination success, or low germination rates [4,55–57]. For example, in the clonal species *Lysimachia asperulifolia*, an effective population feeding treatment was carried out using rhizomes (ramets) [58]. In this case, it can be assumed that if plants were obtained from seed at an earlier stage and then propagated vegetatively, the use of such material would not only contribute to an increase in population size but also to n enhanced genetic diversity. However, each species and even population requires individual treatment and prior recognition of biology and threats [4].

5. Conclusions

1. This is the first time the annual developmental cycle of *R. illyricus* has been described, allowing us to present its ability to reproduce both generatively and vegetatively.
2. The efficiency of vegetative propagation ex situ depended on the age of the tuber (clone) and indirectly on weather conditions. After three years, the best clones could produce up to 57 progeny clusters, which flowered in the first vegetative season, but the regeneration potential of the tubers started to decrease in the case of the two-year-old tubers.
3. The high potential of *R. illyricus* for generative reproduction was limited by low seed-setting efficiency under ex situ conditions and difficulties with seed germination and seedling survival. In addition, the first flowering plant of seed origin was observed in the third year after planting.
4. Vegetative reproduction was more effective than generative reproduction because more progeny clusters could be obtained during one season and they were able to propagate through both reproduction modes in the following season.
5. The best way to increase the natural resources of this species would be ex situ generative propagation followed by vegetative propagation of the resulting plants.

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Publikacja 3

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ORIGINAL ARTICLE

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 genus aconite (monkshood) *Aconitum* L. belongs to 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 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, 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), *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 (Belwal et al. 2016), A. *chasmanthum* (Rafiq et al. 2021)d *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* 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 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 ($HgCl_2$) 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 (Gamborg 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.

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:

$$\mathrm{CFM\%} = \frac{\mathrm{FMf} - \mathrm{FMi}}{\mathrm{FMi}} \times 100\%$$

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 $m^{-2}s^{-1}$.

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.

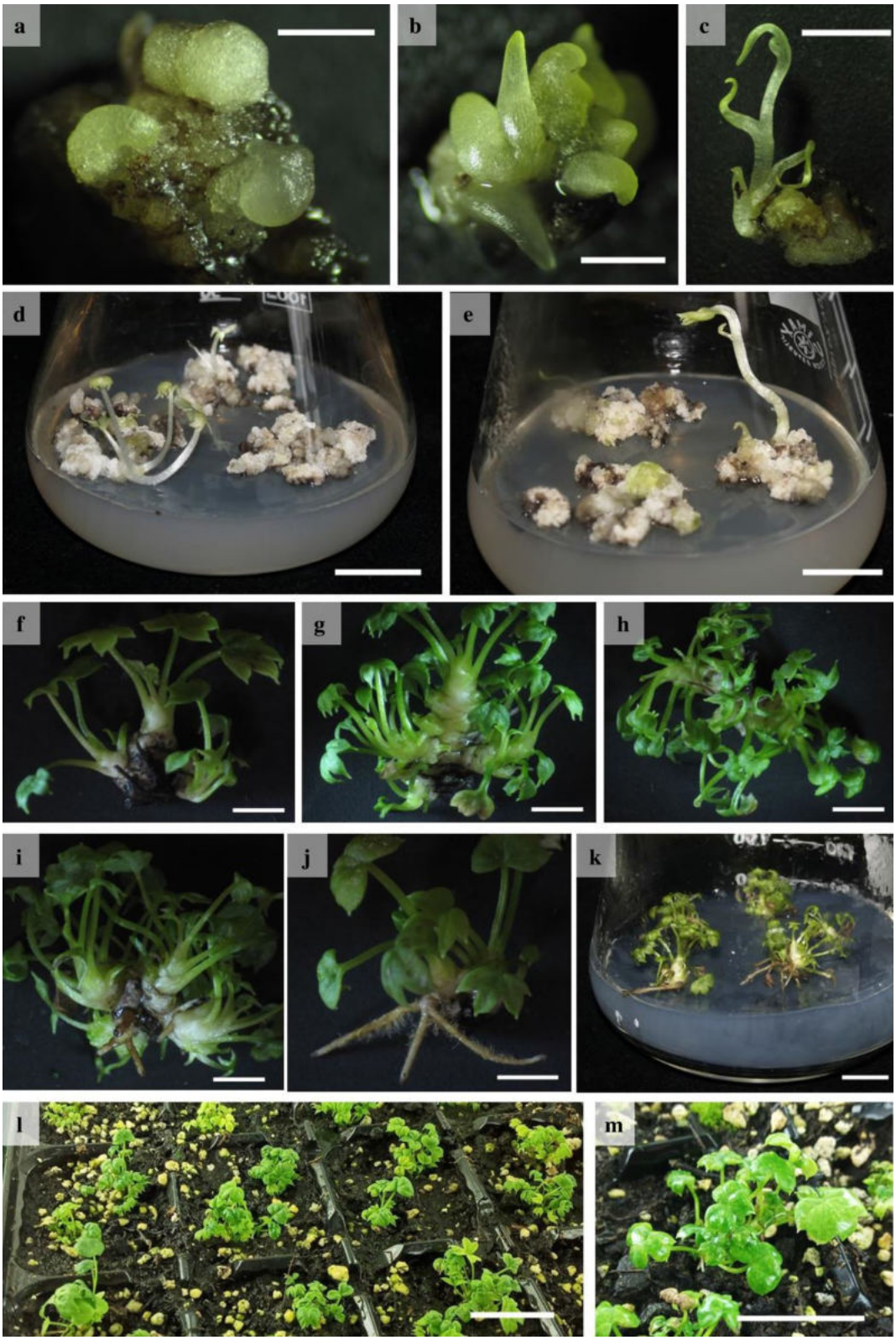
Eighty-six rooted shoots were used for acclimatization and were planted into pots filled with a 1:2 mixture of perlite and commercially available potting soil AURA® from Agaris Poland. They were cultivated for 8 weeks in Sanyo vegetative chambers (San-Yoonoda, Japan), under 16/8-h day/night photoperiod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

◂**Fig. 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 (4–6 weeks of cultivation); **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 8 weeks. **a**–**c**, **f**–**j**: bar = 5 mm; **d**, **e**, **l** and **m**: bar = 2 cm; **k**: bar = 1.5 cm

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_2O, 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 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 $P \leq 0.05$.

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

Table 1 The increase in callus fresh matter (CFM%) and the number of regenerated shoots (pcs 1 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}	
	Shoots	0.84^{A}	0.97^{A}	1.11^{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})	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}	9.3^{A}	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 letteritalics), 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^{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 $P \leq 0.05$; one-way ANOVA was performed

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). 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

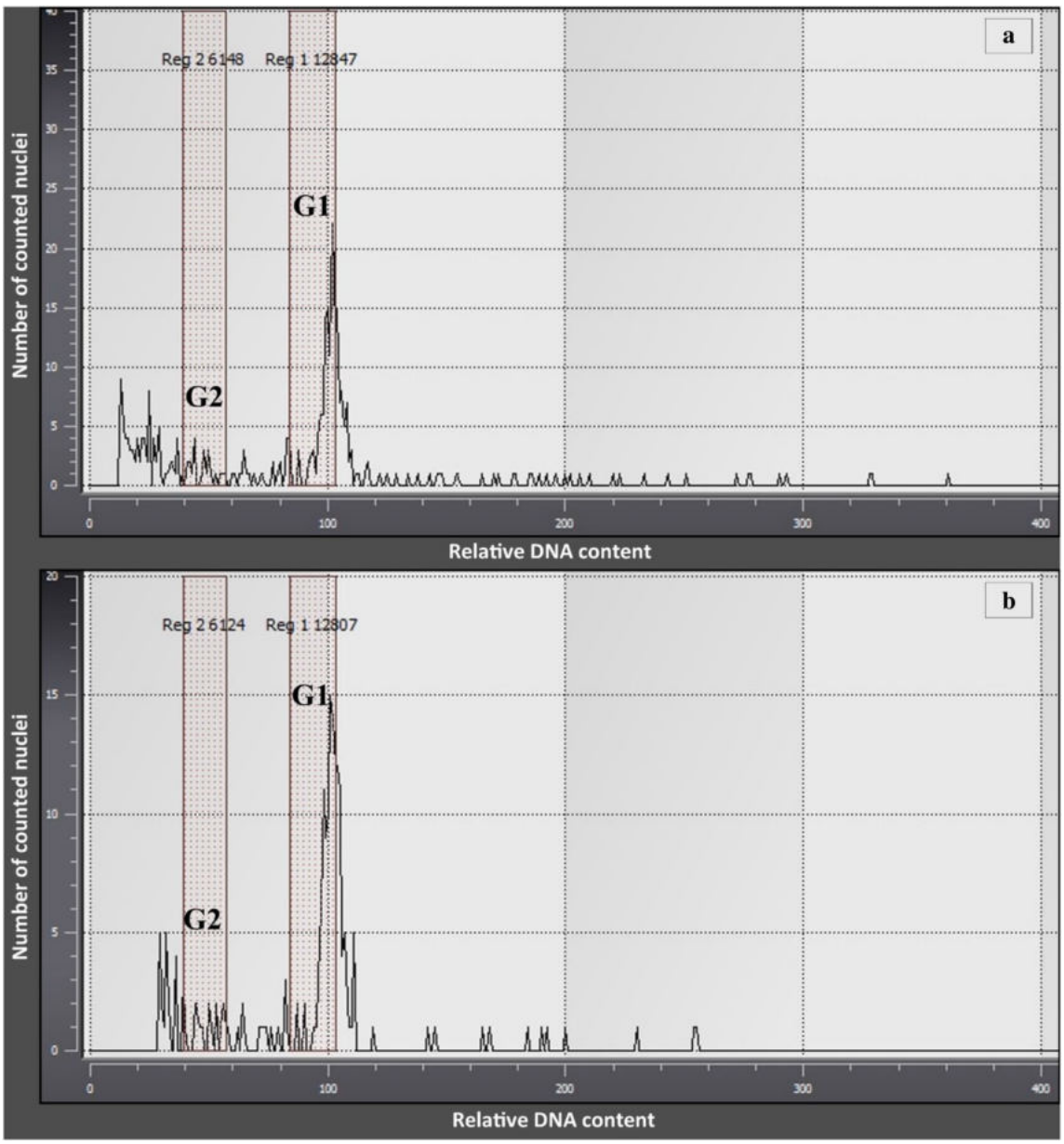


Fig. 2 Histograms of relative DNA content in the nuclei of *Aconitum bucovinense* leaf cells: **a** control seed-origin plant; **b** plant originating from in vitro (indirect organogenesis); G1, G2—phases of cell life

Table 4 Peroxidase activity in rooted shoot cultures of *A. bucovinense* (μ 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^{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}	

*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 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. bucovinense* 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. bucovinense* from leaf explants by indirect organogenesis through an intermediate callus stage.

Given the difficulty of germinating the few seeds *A. bucovinense* 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. 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 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. baicalense*—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. bucovinense* 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. bucovinense*, 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. bucovinense* 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

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et al. 2016; Gondval et al. 2016; Deb and Langhu 2017; Singh et al. 2020; Rafiq et al. 2021; Mou et al. 2022). 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. chasmanthum* 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.

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 *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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Sitek E. – koncepcja badań, metodologia, zbieranie i opracowywanie danych, analiza danych, przygotowanie manuskryptu, opracowanie graficzne, weryfikacja ostatecznej wersji, nadzorowanie

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Dawid Kocot (55%)	Dawid Kocot
Barbara Nowak (20%)	Barbara Nowak
Ewa Sitek (10%)	Ewa Sitek
Anna Starzyńska-Janiszewska (10%)	Anna Starzyńska-Janiszewska
Józef Mitka (5 %)	

Kocot D. – koncepcja i projekt doświadczeń, przeprowadzenie eksperymentów i zebranie danych, analiza danych, napisanie manuskryptu i weryfikacja ostatecznej wersji

Nowak B. – koncepcja i projekt doświadczeń, analiza danych, napisanie manuskryptu i weryfikacja ostatecznej wersji

Sitek E. – napisanie manuskryptu i weryfikacja ostatecznej wersji

Starzyńska-Janiszewska A. – przeprowadzenie eksperymentów i zebranie danych, analiza danych

Mitka J. – koncepcja i projekt doświadczeń