In vitro propagation of endemic *Scabiosa cinerea* Lam. subsp. *cinerea*

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Abstract: *Scabiosa cinerea* Lam. subsp. *cinerea* (syn. *Scabiosa leucophylla* Borbás) is an endemic taxon subspecies of the Balkan and Iberian Peninsulas. A tissue culture was established to prevent the extinction of this taxon and promote its use in horticulture. The germination percentage on two types of MS media supplemented with different plant growth regulator combinations was the same (60%). The highest multiplication rate was recorded on the MS media with 2 mg L^{-1} BAP, 0.4 mg L^{-1} IBA, and 0.2 mg L^{-1} GA3. 100% rooting of plantlets was recorded on the MS supplemented with IBA (1.0 and 2.0 mg L-[1]), but the formed roots were morphologically different. After four weeks, the obtained seedlings were fully acclimatized in the commercial substrate to *ex vitro* conditions. This study may provide a basis for the multiplication and sustainable use of *S. cinerea* subsp. *cinerea* in bioconservation strategy and horticulture.

Key words: Caprifoliaceae, micropropagation, *Scabiosa cinerea* subsp. *cinerea, Scabiosa leucophylla*, tissue culture

INTRODUCTION

The genus *Scabiosa* L. (Dipsacaceae, Caprifoliaceae /Reveal & Chase, 2011/) is a taxonomically very complex genus, with a distribution area throughout the Mediterranean Basin, Asia, and southern Africa. The number of described species for the genus varies, but today comprises 65 accepted taxa (Carlson et al., 2012; George & Roland, 2013; WFO, 2024). *Scabiosa cinerea* Lam. subsp. *cinerea* (syn. *S. leucophylla* Borbás) is an endemic perennial of the Western Balkans and the Iberian Peninsula (Domina 2017+; POWO 2024). In Bosnia and Herzegovina, this taxon has been evaluated against the Red List criteria as Least Concern (LC) category (Đug et al., 2013; Šoljan, 2023).

In traditional human and veterinary medicine, several *Scabiosa* species are used as a source of essential oils and various secondary metabolites, such as triterpenoid derivatives, saponins, iridoids, phenolic acids and flavonoids (Quattrocchi, 1999; Bonet & Vallès, 2007; Rigat et al., 2007; Moteetee & Kose 2016; Kilinc et al., 2023). *Scabiosa* species are also used in pharmacy and cosmetics (Besbes Hlila et al., 2013). Essential oils are known for their antibacterial, antifungal and insecticidal properties. Herbal preparations show anti-arthritic, anti-neurodegenerative, anti-inflammatory, antioxidant, anti-carcinogenic, antihyperglycemic, diuretic, hepatoprotective, antimicrobial, antifungal, and cytotoxic activity (Pinto et al., 2018; Kilinc et al., 2023; Skała & Szopa, 2023). Due to their attractive characteristics and wide range of flower colours, some species are also grown horticulturally.

Plant cell and tissue culture is an effective tool for preserving plant biodiversity and improving the cultivation of horticultural plants. According to the available literature, no *in vitro* studies of *S. cinerea* subsp. *cinerea* exist. Therefore, this investigation aimed to develop an efficient protocol for *in vitro* propagation of this endemic taxon subspecies.

MATERIAL AND METHODS

Plant material

Mature seeds of *S. cinerea* subsp. *cinerea* were harvested in the fruiting phase from natural populations on Mount Igman to establish the culture *in vitro*. The seeds were washed in tap water, surface-disinfested in 70% ethanol for 30 seconds, then in 1:4 (v/v) diluted commercial bleach solution, with constant shaking, for 20 minutes. The seeds were then rinsed with sterile distilled water under aseptic conditions, dried on sterile filter paper for 5 minutes, and then inoculated onto MS medium (Murashige & Skoog, 1962).

Media and culture conditions

To induce germination, seeds were cultured on two types of MS media: supplemented with 0.15 mg L^{-1} GA3 (Gibberellic acid), and with 1 mg L^{-1} BAP (6-Benzylaminopurine), 0.4 mg L^{-1} IBA (Indole-3-butyric acid) and 0.1 mg L^{-1} GA3, respectively. After four weeks, the roots were removed from all fully developed seedlings from both media. For multiplication induction, all epicotyls were transferred and cultivated on three types of MS media supplemented with different types and concentrations of growth regulators: MS1 (MS + 0.5 mg L^{-1} BAP, 0.2 mg L^{-1} IBA, and 0.1 mg L^{-1} GA3); MS2 (MS + 1 mg L^{-1} BAP, 0.2 mg L^{-1} IBA, and 0.1 mg L^{-1} GA3); and MS3 (MS + 2 mg L^{-1} BAP, 0.4 mg L^{-1} IBA, and 0.2 mg L^{-1} GA3).

After four weeks of cultivation, the number of explants that produced lateral shoots, the number of lateral shoots per explant, and the number of necrotized explants were recorded. To determine the degree of multiplication, multiplication percentages and the multiplication index were calculated for each treatment. The multiplication percentage represents the percentage of explants that produced lateral shoots on a given treatment, while the multiplication index represents the number of lateral shoots per explant. For root induction, 2–3 cm long healthy shoots were cultured on MS medium supplemented with different concentrations of IBA: MSR1 (1.0 mg L^{-1}) and MSR2 (2.0 mg L^{-1}). All media were adjusted to pH 5.8 before sterilization and autoclaved at 121°C for 20 mins. Cultures were incubated in controlled climate conditions (16h photoperiod; 25±2°C). The experiments were repeated in triplicate, with 30 explants per treatment. Acclimatization to *ex vitro* conditions was carried out by transferring plantlets with a well-developed root system to a commercial substrate (Florahum-S, KS Commerce d.o.o. Široki Brijeg, B&H), and keeping them in controlled ambient conditions (RH 60-80%; 24°C; 16h photoperiod) for four weeks.

Data analysis

All obtained data was statistically analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test and Pearson's correlation coefficient. The significance level of $p<0.05$ is considered significant.

RESULTS

Germination of *S. cinerea* subsp. *cinerea* seeds on both types of treatment were observed after three weeks. No statistically significant difference in the number of germinated seeds was observed between the treatments used. The percentage of germinated seeds was approximately 60% in both cases.

The multiplication rate of *S. cinerea* subsp. *cinerea* shoots was evaluated using three different treatments. Non-parametric analysis showed significant differences in the number of adventitious shoots between all treatments (Tab. 1). In the MS1 treatment, there was no multiplication (Fig. 1a), while treatments MS2 and MS3 gave significantly better results.

Table 1. The effect of growth regulators on the multiplication of ***Scabiosa cinerea*** subsp. ***cinerea*** shoots

Treatment	Total number of shoots	Multiplication percentage (%)	Multiplication index
MS1	0.00	0.00	1.00[a]
MS2	86.00	38.89[a]	17.20[b]
MS3	131.00	47.14[b]	24.25[c]
Note: The values are expressed as means. Treatments that do not share the same letter are significantly different, at $p<0.05$.			

Figure 1. a) Shoot multiplication of ***S. cinerea*** subsp. ***cinerea*** on the MS1 media, and **b)** on the MS2 media. **c)** Rhizogenesis induced on MSR1 media (left) and MSR2 media (right). **d)** Plants acclimatized to ***ex vitro*** conditions.

A total of 86 lateral shoots were produced on the MS2 medium, and their number ranged from 1 to 5 shoots per explant. The percentage of multiplication was 38.89%, and the multiplication index was 17.20 (Tab. 1; Fig. 1b). Increasing the plant growth regulator concentrations (MS3) had a positive effect expressed through a total number of lateral shoots of 131, while their number ranged from 1 to 10 per explant, and 10 explants necrotized. The multiplication per-

centage in this treatment was 47.14%, and the multiplication index was 24.25 (Tab. 1).

A Pearson's correlation test showed a positive correlation between the increase in BAP concentration and the number of formed adventitious shoots of *S. cinerea* subsp. *cinerea*.

In the rooting phase, rhizogenesis was achieved on both media, but a significant morphological difference was observed in the formed roots. On the MSR1 medium, a larger number of thinner roots developed (Fig. 1c-left), in contrast to the MSR2 medium, where only one – albeit significantly thicker – root without lateral roots developed (Fig. 1c-right). In the acclimatization phase, 100% adaptation to *ex vitro* conditions was accomplished after four weeks (Fig. 1d).

DISCUSSION

Seeds are the ideal choice for preserving biodiversity and maintaining healthy plant material. For some species, dormancy is a common problem that can be successfully overcome by *in vitro* culture and treatment with gibberellin GA3, which is used to stimulate seed germination in many plant species (George, 1996). Literary data on the germination of *Scabiosa* species is sparse, and does not provide sufficient insight into the degree and conditions of germination. Although more data on the propagation of *Scabiosa* taxa could be found on nursery sites, the germination of seeds of different origins under controlled conditions was relatively poor and variable. Thus, it was observed that the seeds of *S. ochroleuca* from various localities had different germination percentages (Ishmuratova & Tyrzhanova, 2020). However, a significant increase in germination was observed after the treatment of *S. atropurpurea* seeds with KNO^3 (Ponte-e-Souza et al., 2012) and the stratification of *S. ochroleuca* seeds (Tyrzhanova et al., 2022). Similar problems were also observed in the *in vitro* culture of various *Scabiosa* species. Kheloufi et al. (2022) obtained the highest germination of *S. stellata* seeds *in vitro* at a temperature of 25°C. Panayotova et al. (2008) observed that germination of *S. argentea* seeds is very weak or impossible on media with the addition of growth regulators naphthaleneacetic acid (NAA) and/or BAP at different concentrations. The treatments used in this work resulted in 60% germination of seeds after three weeks on both media, indicating that the amount of exogenously added hormones was most likely optimal.

Multiplication of shoots under *in vitro* conditions mainly depends on cytokinins and their concentrations (Jelaska, 1994). Hosoki & Nojima (2004) observed that a higher concentration of BAP gave the best multiplication of *S. caucasica* cv. Caucasica Blue adventitious shoots regenerated from nodal or internodal explants and leaf blades. Wang et al. (2013) also had similar results for regeneration of adventitious shoots of *S. tschiliensis*.

In our study, the most efficient multiplication of *S. cinerea* subsp. *cinerea* was on the MS3 medium, while on the MS2 medium multiplication was lower; this is likely attributable to the combination and concentrations of plant growth regulators. It is known that shoot multiplication is influenced not only by exogenous plant growth regulators, but also by endogenous hormone content. Different tissues can have different levels of endogenous hormones, so the type of explant has a critical effect on the success of regeneration.

Procedures for shoot elongation were not necessary, because the shoots on the MS3 multiplication medium reached the appropriate height for rooting. 100% rooting of plantlets was achieved on both MSR media, although morphological differences were visible. Similar results (73.85%) were obtained by Wang et al. (2013) in *S. tschiliensis* on the MS medium supplemented with 2.0 mg L^{-1} IBA. However, contrary data exists for *S. caucasica* cv. Caucasica Blue (Hosoki & Nojima, 2004), where rooting was weak on the MS medium with 0.98 mg L^{-1} IBA, which may be related to a high nitrogen concentration (Haruki et al., 1996).

In our study, root formation also occurred in the multiplication phase on the MS3 medium, although the roots were poorly developed and without branching. Root formation in this phase may be due to the presence of cytokinin (Nemeth, 1979; Datta et al., 1983; Jelaska, 1994; Soh et al., 1998).

Acclimatization is among the most sensitive phases of micropropagation. The main reasons stated for this include an unformed or incompletely formed cuticle (the reason for the leaves drying quickly), or the transition of the plants from the conditions of complete heterotrophy to the impoverished soil substrate. Hosoki & Nojima (2004) state that 85–90% of rooted plants of *S. caucasica* cv. Caucasica Blue were successfully acclimatized and stabilized in their soil substrate. Although very slow, maximum acclimatization of *S. cinerea* subsp. *cinerea* was achieved in this study.

CONCLUSION

This article, for the first time, presents an effective *in vitro* micropropagation protocol for the taxon *Scabiosa cinerea* subsp. *cinerea* suitable for conservation and extensive use in horticulture. Due to the insufficient information on this endemic plant, it is necessary to deepen research in order to identify the secondary metabolites present, as well as their activity and potential use.

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SAŽETAK

In vitro razmnožavanje endema _Scabiosa cinerea_ Lam. subsp. _Cinerea_

Scabiosa cinerea Lam. subsp. _cinerea_ (syn. _Scabiosa leucophylla_ Borbás) endemičan takson na Balkanskom i Pirenejskom poluotoku. Kako bi se spriječio gubitak ove svojte i promovirala njena upotreba u hortikulturi, uspostavljena je kultura tkiva. Postotak klijavosti na dvije vrste MS podloga, uz dodatak različitih kombinacija regulatora rasta biljaka, bio je isti (60%). Najveća stopa multiplikacije zabilježena je na MS podlozi s 2 mg L^{-1} BAP, 0,4 mg L^{-1} IBA i 0,2 mg L^{-1} GA3. 100% zakorjenjivanje biljčica zabilježeno je na MS podlozi s dodatkom IBA (1,0 i 2,0 mg L^{-1}), ali su formirani korijeni bili morfološki različiti. Dobivene presadnice u komercijalnom supstratu u potpunosti su se aklimatizirale na ***ex vitro*** uslove nakon četiri sedmice. Ovo istraživanje može pružiti temelj za razmnožavanje i održivo korištenje ***S. cinerea*** subsp. ***cinerea*** u strategiji biokonzervacije i hortikulturi.

Ključne riječi: Caprifoliaceae, kultura tkiva, mikropropagacija, _Scabiosa cinerea_ subsp. _cinerea_, _Scabiosa leucophylla_