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Asymbiotic seed germination of *Dactylorhiza fuchsii* as a tool for the introduction of a legally protected species into the natural environment

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Abstract

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Dactylorhiza fuchsii (Druce) Soó is a legally protected species in some countries with considerable commercial potential. The main objective of this study was to develop an efficient protocol for seed propagation using *in vitro* cultures that could serve as a basis for the successful reintroduction of regenerated plants into their natural habitats. The most effective sterilisation procedure involved immersion in a 0.8% aqueous detergent solution for 18 h, followed by treatment with a 5% aqueous sodium hypochlorite solution for 60 min. The highest germination rate was observed on *Orchimax* medium supplemented with 10 mg/L^{-1} kinetin. The resulting protocorms were regenerated on several media, whereas plant development was achieved on media lacking phytohormones. The greatest number of regenerated shoots was obtained on half-strength Murashige and Skoog medium. The results demonstrate the feasibility of propagating this species under in vitro conditions and provide a basis for its conservation and potential commercial use.

Keywords: orchid, *Orchimax* medium, regeneration, seeds, sowing.

INTRODUCTION

Dactylorhiza fuchsii (Druce) Soó is a terrestrial species of the family Orchidaceae, distributed across most of Europe, except for its southern regions, and its range also extends eastwards to Siberia and Mongolia (Piękoś-Mirkowa & Mirek, 2018; Djordjević et al., 2014). In nearly all areas of occurrence, species of the genus *Dactylorhiza* Neck. ex Nevski are protected in some countries, yet their populations are declining due to habitat degradation and collection for floristic purposes (Sumbembayev et al., 2022; Kindlmann et al., 2023). The protection of *Dactylorhiza* therefore requires not only legal measures but also efforts to restore natural habitats (Sumbembayev et al., 2022). In this context, research on *in vitro* propagation is particularly important for species conservation and for their potential use in horticulture (Fakouri Ghaziani et al., 2014; Nowak, 2020). Such propagation techniques have a long tradition: as early as 1942, Gavino Rotor published the first study on orchid seed germination in *in vitro* cultures (Yam & Arditti, 2009), and shortly thereafter, Morel & Martin (1952) reported the use of apical meristem cultures for virus elimination in cultivated plants of the genus *Dahlia* Cav.

This breakthrough soon prompted the use of analogous techniques to generate virus-free *Cymbidium* plants (Morel, 1960). In the following years, Wimber (1963) developed a complete protocol for the *in vitro* propagation of Cymbidium using meristem cultures, while Steward & Mapes (1971) refined the method and obtained protocorm-like bodies, which allowed efficient orchid propagation. As a result of these advances, micropropagation techniques are now widely applied on a commercial scale to propagate diverse orchid genera, including *Cymbidium* Sw., *Dendrobium* Sw., *Oncidium* Sw., *Odontoglossum* Kunth, *Phaius* Lour. and *Vanda* R.Br. (Sabastian et al., 2021), demonstrating their broad applicability.

In parallel with these technological achievements, it is important to emphasise that maintaining genetic variability within species is crucial for the successful reintroduction of orchids into natural habitats. Therefore, the micropropagation of *Dactylorhiza fuchsii* should be conducted using seeds collected from wild populations (Seaton et al., 2013). However, seed germination in this genus is strongly influenced by mycorrhizal associations, which play a decisive role in early plant development. In particular, fungi of the genus *Tulasnella* (Tulasnellaceae) are commonly involved in symbiosis with terrestrial orchids (Rasmussen, 1990; Jacquemyn et al., 2012). Their presence significantly enhances seed germination by providing essential nutrients and improving water availability, both of which are critical factors in the earliest stages of germination (Kauth et al., 2008). Moreover, orchids utilise fungal metabolites, such as carbohydrates produced through starch hydrolysis (Yeung, 2017) and compounds that inhibit gibberellin activity (Miura et al., 2024), while mycorrhizal fungi additionally regulate proteasome activity, lipid metabolism and other physiological processes in inoculated roots. These beneficial interactions not only promote early growth but also protect seedlings against pathogens, as demonstrated by studies showing that mycorrhizal root extracts inhibit the development of *Phytophthora cactorum* (Hampejsová et al., 2022). Thus, while *in vitro* techniques offer valuable tools for orchid propagation, the ecological role of fungal partners should not be overlooked.

At the same time, the development of asymbiotic seed germination methods, first introduced by Knudson (1922) using sugar-containing media, represented a true breakthrough in orchid biology and enabled large-scale *in vitro* propagation during the 20th and 21st centuries (Jolman et al., 2022). Thanks to tissue culture techniques, seeds can be used as initial explants, thereby ensuring genetic diversity and creating opportunities to introduce propagated plants into natural habitats. The effectiveness of seed-based *in vitro* propagation has been confirmed in numerous orchid species, including C*ymbidium giganteum* Wall. ex Lindl., *Catasetum fimbriatum* Lindl., *Cyrtopodium paranaense* Schltr., *Dactylorhiza hatagirea* (D.Don) Soó, *Laelia speciosa* (Kunth) Schltr., *Platanthera bifolia* (L.) Rich., *Cymbidium elegans* Lindl. and *Rhynchostylis retusa* (L.) Blume (Szot et al., 2015), showing that this approach is both versatile and widely applicable.

In *Dactylorhiza fuchsii*, Jakobsone (2008) has demonstrated that *in vitro* propagation significantly shortens the relatively long time required for complete plant development under natural conditions. Nevertheless, her study does not provide quantitative data on germination efficiency or the number of plants obtained, leaving an important knowledge gap (Jakobsone, 2008). This study aimed to develop an efficient and verifiable protocol for asymbiotic propagation of *Dactylorhiza fuchsii* from seeds by selecting conditions that simultaneously ensure high effectiveness of surface disinfection and reduced contamination, identifying the medium that most effectively induces protocorm formation, and determining the culture medium variant that promotes regeneration of plantlets from protocorms, thereby producing plants suitable for subsequent acclimatisation and conservation applications.

MATERIALS AND METHODS

Plant material

Seeds of *Dactylorhiza fuchsii* used in this study were obtained from plants purchased from the Ogrodywodne® plant nursery (Gorzycko Stare near Międzychód, Poland). The plants were hand-pollinated, and seed capsules were collected approximately 60 days (eight weeks) after pollination; upon reaching maturity, seeds were collected for use in the experiments.

Seed sterilisation

In the experiments, both closed seed capsules and extracted seeds were sterilised. Closed capsules were immersed in 96% ethanol for five minutes, then flame-dried over a gas burner. This type of sterilisation was designated as "T." in the present experiment. Subsequently, the sterilised capsules were cut open with a scalpel, and seeds were aseptically transferred onto culture media.

In the next experiment, mature seed capsules were cut open with a scalpel, and the seeds were transferred into 50 mL test tubes containing the respective sterilisation solutions. Four sterilisation variants differing in treatment duration and chemicals were tested:

A – 0.8% aqueous solution of Fairy detergent (Procter & Gamble) for 18 hours, followed by 5% sodium hypochlorite for 40 minutes.

B – 0.8% Fairy for 18 hours, followed by 5% sodium hypochlorite for 60 minutes.

C – 0.8% Fairy for 12 hours, followed by 5% sodium hypochlorite for 40 minutes.

D – 0.8% Fairy for 12 hours, followed by 5% sodium hypochlorite for 60 minutes.

A subset of sterilised seeds was inspected under a stereomicroscope after rinsing; no obvious embryo damage was observed.

Sterilisation was performed in closed Falcon tubes, and the liquid was removed using a pipette with sterile tips. Seeds were rinsed three times with sterile distilled water aseptically on a table with laminar airflow and transferred to Petri dishes containing culture medium. On average, 1730 seeds were placed per dish, which were then sealed with parafilm to maintain sterility.

The number of seeds per dish was estimated by counting seeds in three small aliquots of a well-mixed seed suspension using a stereomicroscope and extrapolating to the final plated volume.

The efficiency of each sterilisation method was assessed based on the percentage of contamination-free plates, the proportion of seeds developing embryos, and the number of protocorms obtained. In the sterilisation experiment, seeds from each sterilisation variant were sown on ¼ Murashige and Skoog (Murashige & Skoog, 1962) and modified Orchimax medium (OM1) (Bektaş et al., 2013) media (Fig. 1).

Seed germination

Seed viability before sowing was not assessed separately; vitality was evaluated during culture as the proportion of seeds developing embryos.

For germination experiments, seeds were sterilised using the most effective method (method B). They were sown on two media: MS and modified Orchimax medium (Table 1). The ¼ MS medium, which contained 25% of standard MS, was solidified with 8 mg/L^{-1} agar and adjusted to pH 5.8. The modified Orchimax me-

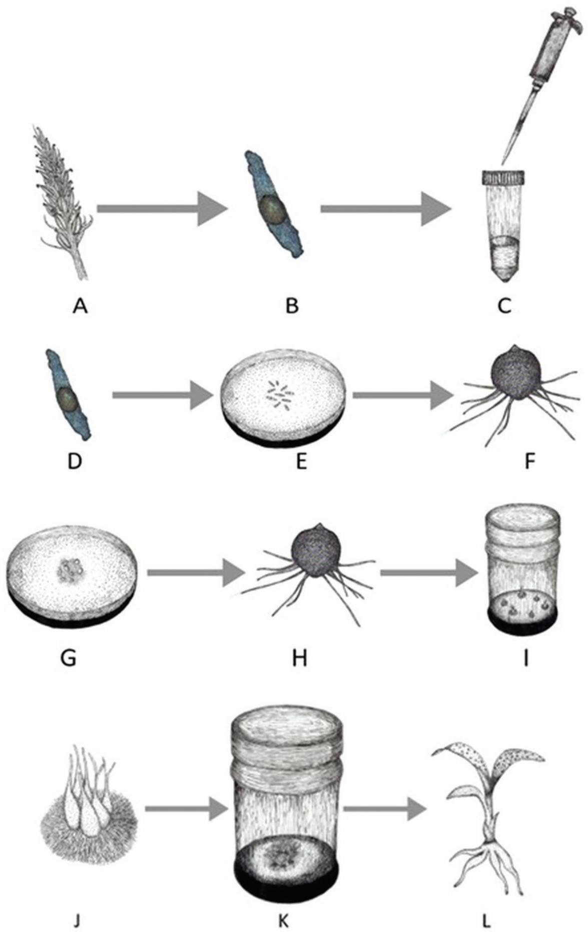

Fig. 1. Seed-based asymbiotic germination of *Dactylorhiza fuchsii* in tissue culture: a – inflorescence of the donor plant; b – mature seed capsule; c – sterilisation of seeds extracted from the capsule in sodium hypochlorite solution (test tube); d – disinfected seeds; e – sterile seeds sown on Petri dish with culture medium; f – seeds forming protocorms; g – petri dish with developing protocorms; h – developed protocorm; i – protocorm-like bodies forming on medium; j – protocorm-like bodies on regeneration medium; k – protocorm-like bodies regenerating into plantlets; l – fully regenerated *in vitro* plants.

dium had a pH of 5.8 (commonly used for terrestrial orchids), contained 20 mg/L^{-1} sucrose (Table 1), and was solidified with 5 mg/L^{-1} agar (Fig. 1d, e, f).

Media (¼ Murashige and Skoog and Orchimax medium) were supplemented with 10 mg/L^{-1} kinetin and sterilised by autoclaving (121 °C, 20 min, 103 kPa overpressure). Petri dishes containing seeds were sealed with parafilm and incubated in darkness at 21°C. After 27 days, the number of viable seeds capable of further development was recorded. On day 48, germinating protocorms were counted (Fig. 2a). Incubation continued for 69 days, after which the developed protocorms were transferred to jars and placed in a phytotron at 25 ± 2°C under a 16/8 h (light and dark) photoperiod. Illumination was provided by cool-white lamps (fluorescent tubes or equivalent light-emitting diodes at a photosynthetic photon flux density of 40–50 µmol m^{-2} s^{-1}.

Regeneration

Protocorms were transferred to regeneration media in 330 mL jars (Fig. 1). The effect of different media on plant regeneration from protocorms was evaluated. In this experiment, Murashige and Skoog (1962) medium and base medium (Calevo et al., 2017) were used (Table 1).

Murashige and Skoog-based media modifications included:

a) ½ Murashige and Skoog medium containing 50% of the macro- and microelement salts of the standard Murashige and Skoog medium.

b) ½ Murashige and Skoog supplemented with 3 mg/L^{-1} thidiazuron.

c) Murashige and Skoog medium with 2 mg/L^{-1} 1-naphthaleneacetic acid.

d) Murashige and Skoog medium with 2 mg/L^{-1} 1-naphthaleneacetic acid and 1 mg/L^{-1} 6-benzylaminopurine.

e) base medium (Mm1).

f) base medium with 100% increased mineral salt content (Mm2).

g) base medium with increased coconut water content (100 mL/L^{-1}) (Mm2).

The pH of Murashige and Skoog medium and base medium was adjusted to 5.8. All regeneration media contained 20 g/L^{-1} sucrose and were solidified with 8 g/L^{-1} agar. Protocorms were incubated in a phytotron at 23°C, with a 16-hour photoperiod (light intensity 25 µmol·m^{-2}·s^{-1}) or in continuous darkness. Observations of regeneration were initiated after the jars had been in the phytotron for 21 days of culture.

Plants were considered regenerated if they produced at least one main root and either leaves or complete rosettes with green colouration (Fig. 2 b, c, d). These criteria indicate that plants cultured on the media were autotrophic and capable of acclimatisation under *ex vitro* conditions.

Statistical analysis

Each Petri dish containing plated seeds was considered a single replicate at both the sterilisation and germination stages. On average, 1730 seeds were

Table 1. Modifications of media used in conducted experiments. Abbreviations: ½ MS and ¼ MS – media based on Murashige and Skoog formulation, containing 50% and 25% of the standard Murashige and Skoog medium macro- and microelement composition, respectively; OM1 – modified Orchimax medium (Bektaş et al., 2013); Mm1 – base medium; Mm2 – base medium with mineral salts increased by 100% (doubled); Mm3 – base medium supplemented with coconut water (100 mL L^{-1})

Component	Type of the medium				
	½ MS and ¼ MS	OM1	Mm1	Mm2	Mm3
	Quantity (L^{-1})				
Glycine	4 mg	2 mg	–	–	–
Casein hydrolysate	1 g	1 g	–	–	–
Myo-inositol	100 mg	100 mg	–	–	–
Pyridoxine HCl	0.5 mg	0.25 mg	–	–	–
Thiamine	0.1 mg	0.05 mg	–	–	–
Niacine	0.5 mg	0.25 mg	–	–	–
Sucrose	20 g	20 g	10 g	10 g	10 g
Activated carbon	–	1 g	0.5 g	0.5 g	0.5 g
Coconut water	–	–	50 mL	50 mL	100 mL

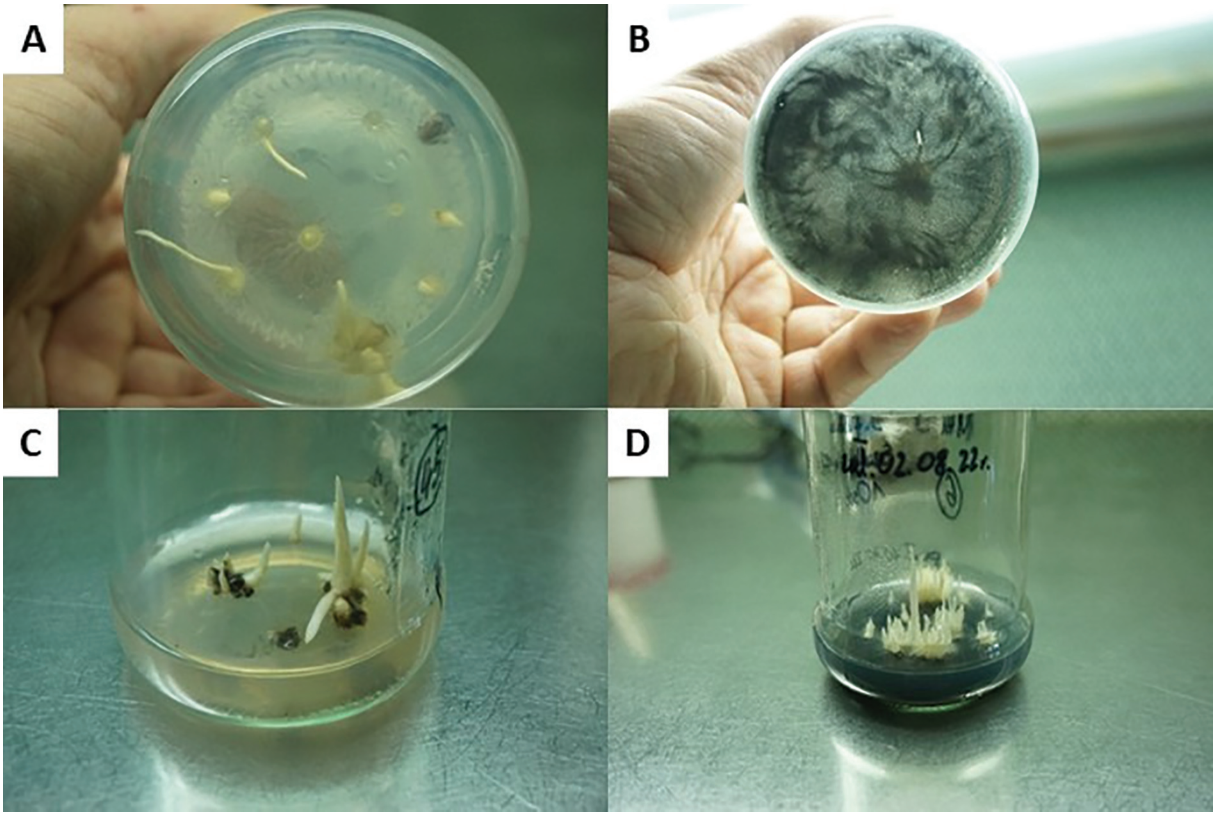

Fig. 2. Germination and *in vitro* development of *Dactylorhiza fuchsii*: a – developing roots emerging from protocorms on ½ Murashige and Skoog medium; b – developing roots emerging from protocorms on modified Orchimax medium; c – regenerating plants on ½ Murashige and Skoog medium; d – regenerating plants on modified Orchimax medium.

placed on each dish. For the regeneration experiments, protocorms were transferred to regeneration media, and each jar containing 10 protocorms was considered a replicate. The number of replicates varied across the study, depending on the availability of plant material. The obtained data were analysed using multivariate ANOVA models and nonparametric tests, specifically the Kruskal–Wallis test, with a significance threshold of $\alpha = 0.05$. All statistical analyses were performed using Statistica 8.0 (StatSoft Inc., Tulsa, USA) for Windows.

RESULTS

A critical stage in introducing plant material to *in vitro* conditions is sterilisation. Seeds of *Dactylorhiza fuchsii* are enclosed in seed capsules; therefore, in the present experiment, the method of seed sterilisation involving 96% ethanol followed by flaming of entire capsules was applied. However, this method proved ineffective: no sterile cultures were obtained, and all seeds failed to survive (Table 2). The most effective sterilisation method was treatment with 5% sodium hypochlorite for one hour. All seeds treated with this method remained sterile and, importantly, survived the procedure. Moreover, more than 36% of seeds across all sterilisation variants developed protocorms (Table 2).

Another key factor influencing germination efficiency is the culture medium. In our experiments, a relatively high germination rate of about 40% was achieved. This level of germination was obtained on modified Orchimax medium (Table 3).

In *Dactylorhiza* species, seeds first germinate into protocorms, which subsequently regenerate into plants under favourable conditions. In our experiment, protocorms were visible 27 days after seeds were placed on media supplemented with kinetin.

In our study, *Dactylorhiza fuchsii* regenerated most efficiently (37.5%) on ½ Murashige and Skoog medium without hormones (Table 4). A slightly lower percentage (35.4%) was observed on ½ Murashige and Skoog medium supplemented with thidiazuron,

Table 2. The efficiency of *Dactylorhiza fuchsii* seed sterilisation methods. For abbreviations of sterilisation methods, see Materials and Methods. Different superscript letters indicate significant differences ($p < 0.05$) according to the results of the Kruskal–Wallis post hoc test

Sterilisation method –	Petri dishes			Seeds		Obtained protocorms
	Total number	Clean		Total number	Viable (%)	
		number	%			
T	5	2	40	8650	0	0.0[a*]
A	5	3	60	8650	77.6	0.03[b]
B	3	3	100	5190	100	36.78[d]
C	3	1	33	5190	26.33	11.00[c]
D	3	3	100	5190	33.21	14.20[c]

Table 3. The influence of the media on the efficiency of seed germination of the orchid *Dactylorhiza fuchsii* under *in vitro* conditions. Different superscript letters indicate significant differences ($p < 0.05$) according to the Kruskal–Wallis post hoc test. Abbreviations: ¼ MS + KIN – medium based on Murashige and Skoog formulation, containing 25% of the standard Murashige and Skoog medium supplemented with kinetin; OM1 + KIN – modified Orchimax medium supplemented with kinetin

Medium	Total number of seeds	Viable seeds		Protocorms	
		n	%	*n*	%
¼ MS + KIN	10316	8481	82.2	283	3.3[a]
OM1 + KIN	10434	3838	36.8	1524	39.7[b]

Table 4. The influence of the medium on the efficiency of seed regeneration of the orchid *Dactylorhiza fuchsii* under *in vitro* conditions. Different superscript letters across the same column indicate significant differences ($p < 0.05$) according to the results of the Kruskal–Wallis post hoc test. Abbreviations: ½ MS – medium based on Murashige and Skoog formulation, containing 50% of the standard Murashige and Skoog medium macro- and microelement composition; ½ MS – the same medium supplemented with thidiazuron; Mm2 – base medium with mineral salts increased by 100% (doubled); Mm3 – base medium supplemented with coconut water (100 mL L^{-1}); MS + NAA – Murashige and Skoog medium supplemented with 1-naphthaleneacetic acid; MS + BAP, NAA – Murashige and Skoog medium supplemented with 1-naphthaleneacetic acid and 6-benzylaminopurine

Medium	Protocorms			Viable			
	Total number of protocorms	Non-viable		Not regenerated into plants		Regenerated into plants	
		n	%	*n*	%	*n*	%
½ MS	96	31	32.3[a]	29	30.2[a]	36	37.5[a]
½ MS + TDZ	127	62	48.9[a]	20	15.7[a]	45	35.4[a]
Mm3	40	0	0.0[b]	29	72.5[b]	11	27.5[ab]
Mm2	40	0	0.0[b]	30	75.0[b]	10	25.0[ab]
MS + NAA	46	46	100.0[c]	0	0.0[c]	0	0.0[c]
MS + BAP, NAA	49	49	100.0[c]	0	0.0[c]	0	0.0[c]

although the difference was not statistically significant. No regeneration was observed in our study on media supplemented with 1-naphthaleneacetic acid and 6-benzylaminopurine.

Another observation from our experiments was the lack of statistical differences between the percentage of protocorms regenerating into plants on base medium. Although the base medium supplemented with coconut water contained twice as many mineral salts as the base medium, regeneration efficiency remained identical. Similarly, no statistically significant differences were noted among media based on ½ Murashige and Skoog (Murashige & Skoog, 1962), although numerical differences in regeneration percentages were evident.

DISCUSSION

Seeds of *Dactylorhiza fuchsii* are enclosed in seed capsules; therefore, some researchers chose not to extract the seeds but instead sterilised the entire capsules in 96% ethanol followed by flaming (Kauth et al., 2008; Jakobsone, 2008). Although the above-mentioned authors did not report the efficiency of capsule sterilisation by burning, they obtained sufficient seed sterility to enable orchid regeneration *in vitro*. In contrast, in our study, this method proved ineffective, and the most effective approach was treatment with 5% sodium hypochlorite for one hour, which ensured both sterility and survival (Table 2). In our study, we also observed that seeds treated with sodium hypochlorite for 60 minutes germinated more effectively than those treated for 40 minutes.

Although longer exposure to sodium hypochlorite increased germination in our conditions, prolonged treatment may also increase the risk of tissue damage; therefore, this step should be optimised carefully for each seed lot.

However, recent protocols also report effective surface sterilisation of capsules or seeds without flaming.

Other authors (Park et al., 2023) have sterilised mature seeds of several orchid species by immersing them for 15 minutes in a 1.5% calcium hypochlorite solution placed in filter paper boxes and investigated the effect of this treatment on germination. In their study, more than 30% of the seeds germinated on the orchid seed- sowing medium, consistent with our results. The authors emphasised the important role of NaClO in breaking down germination inhibitors, consistent with the higher effectiveness we observed with longer sodium hypochlorite treatment.

Regarding culture media, the relatively high germination rate of about 40% achieved in our experiments on modified Orchimax medium (Table 3) was comparable to, or higher than, results reported by other authors. Park et al. (2023) have reported slightly lower germination (37%) on orchid seed sowing medium supplemented with 6-benzyladenine. Similarly, Bektaş et al. (2012) have found that Orchimax medium is the most effective for *Orchis coriophora*, with 27% of seeds germinating. In contrast, lower germination rates are obtained on Orchimax medium, Knudson C medium, Lindeman medium, and Phytamax orchid multiplication medium. The highest reported germination percentage in the literature (95.5%) has been achieved for *Anacamptis longicornu* on Orchimax medium (Arcidiacono et al., 2021). Furthermore, Arcidiacono et al. have demonstrated that for several orchid species, including *Ophrys panormitana*, seed germination is only successful when sown on Orchimax medium.

Regarding developmental timing, in earlier studies, protocorms were observed three weeks after culture initiation on Lindemann medium (Warghat et al., 2014). In our experiment, protocorms were visible 27 days after seeds were placed on media supplemented with kinetin. This cytokinin is widely used in orchid cultures to stimulate germination and development (Ochowicz, 2001; Aktar et al., 2007; Asghar et al., 2011). Wotavová-Novotná et al. (2007) have demonstrated high regeneration efficiency in *Dactylorhiza incarnata* and *Dactylorhiza majalis* on kinetin-containing media and showed that regeneration responses are genotype-dependent. In *Dactylorhiza incarnata*, the highest number of shoots was obtained on a medium containing 2-isopentenyladenine, whereas in *Dactylorhiza majalis* the best results were achieved on a medium without growth regulators.

In our study, *Dactylorhiza fuchsii* regenerated most efficiently on ½ MS medium without hormones (Table 4), whereas thidiazuron produced a slightly lower regeneration percentage (not statistically significant). Bhattacharyya et al. (2014) have investigated the effect of thidiazuron concentration on the regeneration of *Dendrobium nobile* protocorms. They found that the same concentration we applied did not induce regeneration, whereas reducing the dose by half (1.5 mg/L^{-1}) was most effective. These results confirm that regeneration efficiency in orchids is genetically determined and highly dependent on medium composition. Supporting this conclusion, no regeneration was observed in our study on media supplemented with 1-naphthaleneacetic acid and 6-benzylaminopurine, although other authors have reported that these regulators successfully enhanced shoot regeneration in *Phalaenopsis*, *Dendrobium*, and *Oncidium* (Kosir et al., 2004; Puchooa, 2004; Dohling et al., 2012).

Finally, the absence of statistical differences between base medium (Malmgren, 1996) and Mm3, despite the doubled mineral salt concentration in base medium supplemented with coconut water, and the lack of statistically significant differences among media based on ½ Murashige and Skoog medium, suggest that the macro- and microelement composition of ½ Murashige and Skoog medium was the most favourable for efficient regeneration of *Dactylorhiza fuchsii* plants from protocorms in tissue culture. Further research is underway to confirm this conclusion.

In summary, the study identifies an effective seed sterilisation approach and demonstrates that asymbiotic seed germination on modified Orchimax medium supports protocorm formation and subsequent regeneration. These findings provide a practical *ex situ* framework for producing planting material to support conservation-oriented reinforcement and reintroduction efforts.

CONCLUSIONS

1) Optimal sterility and viability were obtained with 0.8% Fairy (18 h) followed by 5% NaOCl (60 min).
2) Modified Orchimax medium supported the highest protocorm development under the tested conditions.
3) The established workflow can be applied for *ex situ* production of planting material to support local conservation actions.

Author contributions. WK and BD contributed to the study conception and design. WK performed material preparation, data collection and analysis. MB wrote the first draft of the manuscript. All authors commented on and contributed to the interpretation of the results and explanations in the discussion of previous versions of the manuscript. All authors read and approved the final manuscript.

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