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GERMINATION AND PROTOCORM FORMATION OF OPHRYS SPHEGODES MILL. – *IN VITRO* PROTOCOL FOR A RARE ORCHID SPECIES

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Summary: *Ophrys sphegodes Mill. is a wild orchid species which is threatened and protected due to its pollination biology, small seed and habitat destruction. The aim of this study was to establish asymbiotic germination protocol for the purpose of ex situ conservation. Two basal media Knudson C (KC) and Malmgren (MM), supplemented with organic additives (peptone (PE), L-glutamin (A)e, folic acid, casein hydrolysate (CA)) added separately and control media KC--C and MM--C were used in the present research. All the nutrition media contained 2% sucrose, 7% agar and 1% activated carbon, while their pH was adjusted to 5.8 ± 0.02 before autoclaving at 121 ° C for 20 minutes. The seeds were examined under two illumination conditions, 0/24 light/dark (L/D) and 16/8 L/D. The presented results indicate a huge influence of illumination and nutrition media on the seed germination and protocorm formation. The seed germination was overall significantly more successful in dark conditions (0/24 L/D) than with lighting (16/8 L/D). Protocorm, rhizoids and shoot formation were achieved only on the seeds cultured on MM medium, while the KC medium caused only swelling of the embryo. Organic additives had positive influence on the germination rate. According to the obtained results, the best germination rate and seedling development were achieved on MM-PE media, cultured in dark. The presented procedure accelerates the germination period and can provide a large number of plants in a relatively short period of time so it can be used for conservation programs and mass production protocol.*

Key words: *apricots, cultivars, selections, technological properties of fruit, compote, sensory evaluation*.

INTRODUCTION

Ophrys sphegodes Mill., also known as the early spider-orchid, is a species of terrestrial orchids (family *Orchidaceae*) found on alkaline meadows, chalk or limestone. Although *O. sphegodes* is widespread across Europe and the Middle East, it is considered rare and vulnerable. In Serbia, *O. sphegodes* is a strictly protected wild species by CITES convention. On the other hand, many of the remaining populations on the British Isles consist of very few plants (Hutchings, 1989). According to the mentioned author, if the rate of decline observed over the last fifty years continues, the species will disappear from the British Isles by the end of this century. Hutchings (1989) stated that the main reasons why these orchids are threatened are climate changes together with the pollination biology of this species. *O. sphegodes* reproduces through sexual deception of male *Andrena nigroaenea* bees (pseudocopulation). Slightly warmer springs are causing the males to emerge much earlier, which means that they are less synchronized with the orchids' flowering (Davy et al., 2014). Furthermore, other extrinsic factors involved in population reduction

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are habitat extermination, often for real estate land, and plant exploitation for ornamental and edible purposes. The basic problem with terrestrial orchid propagation, in general, is a small probability of seed germination due to its extremely small size. The orchid seed has an undifferentiated embryo and lack of nutrients for growth. Hence, they form a symbiotic relationship with fungi known as orchid mycorrhiza, which provides nutrients for protocorm formation. To overcome these obstacles, and the rapid decrease of *O. sphegodes* population, *ex situ* conservation and *in vitro* asymbiotic propagation are estimated as the best solutions for conservation. Knudson (1922) pointed out that germination is possible on a substrate which contains minerals and carbohydrates, in absence of fungi. Germination depends on a large number of factors, such as temperature, light, minerals, carbohydrates, pH, vitamins, hormones (Rasmussen, 1995; Arditti, 1967; Stewart and Kane, 2006; Godo et al., 2010; Chen et al., 2015; Ponert et al., 2013; Gupta, 2016; Bozdemir, 2018). Seed usually germinates at 20-25°C and pH 4.8-5.8 and often demands Fe and Mn for germination; coconut water, pineapple juice, peptone, tryptone, yeast and salep are also used as supplements (Marić, 1995). Asimbiotic seed germination of the species belonging to the genus *Ophrys* is affected by various factors, and varies from species to species (Van Wears and Debergh 1986; Rassmusen 1995; Kitsaki et. al, 2004, Pierce et. al., 2015). Although *in vitro* germination of *O. sphegodes* was successfully carried out by Van Wears and Debergh (1986), further research is needed. Modification of nutrition media and physical conditions (e.g. illumination, temperature) could increase the germination rate and accelerate the germination period.

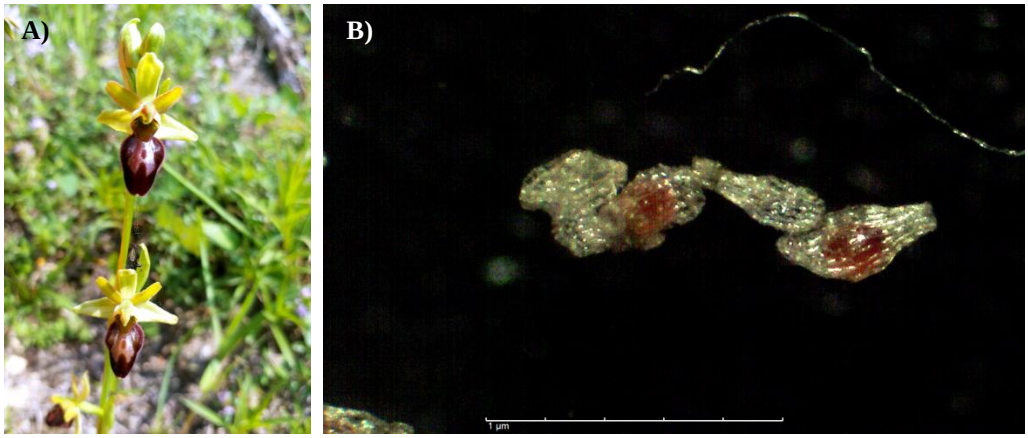
Thus, the aim of this study was to establish asymbiotic germination protocol for the purpose of *ex situ* conservation of the endangered orchid species *O. sphegodes*.

MATERIAL AND METHODS

Plant material

Maturate capsules (Fruška Gora locality 45.190898˚N 19.709237˚E 256.82m) were sampled in late June of 2017 (Image 1). Extracted seeds were dried at 26˚C for 48h to eliminate excess moisture, placed in Petri dishes over silica gel and stored at 4˚C in continuous darkness until use.

The seed surface was sterilized with 7% calcium hypochlorite containing 0.1% Tween 20 for 20 minutes. The seeds were further rinsed three times with distilled water. Sterilization was accomplished in laminar flow hood with 70% ethanol for 2 minutes, followed by triple rinsing in sterile distilled water.



Picture 1. Plant material: A) Mother plant; B) Viable red colored seeds

Cultivation media composition and culture conditions

Two basal media – KC (Knudson C, 1946) and MM (Malmgren, 1996), supplemented with different organic additives except for the control media (KC-C and MM-C) were applied. Basal medium Knudson C was enriched with casein hydrolysate 400 mg l^{-1} with folic acid 0.5 mg l^{-1} (KC-CA) and L-glutamine 100 mg l^{-1} with folic acid 0.5 mg l^{-1} (KC-A) while Malmgren medium was enriched with peptone 2 g l^{-1} (MM-PE) and L-glutamine 100 mg l^{-1} (MM-A). All nutrition media contained 2% sucrose, 7% agar and 1% activated carbon, while their pH was adjusted to 5.8 before autoclaving at 121 ° C for 20 minutes. After successful sterilization the seeds were spread on the medium followed by wrapping of Petri plates with a single layer of parafilm (Parafilm M, Menasha, Wisconsin, US) and stored at a temperature of 23 ± 2 ° C. Five replicate plates were used for every germination medium treatment, and each plate contained between 50 and 100 seeds. To examine the effects of light conditions on the seed germination and protocorm formation, Petri dishes were placed under two illumination conditions – 0/ 24 light/dark (L/D) and 16/8 (L/D) of approx. 40μmol $m^{-2}s^{-1}$ provided by white fluorescent lamps. Counting of germinated seeds

was carried out eight and twelve weeks after sowing. Also, the measurements of the embryo and the protocorm size were performed and included the height and width of embryo and protocorm, as well as protocorm shoot height. All parameters were measured using binocular digital stereo microscope Motic 39C N9GO.

Data processing and statistical analysis

Germination percentage and seedling development were calculated by dividing the number of the germinated seeds with the total number of the seeds observed. The data were subjected to analysis of variance (ANOVA) and the mean values of protocorm size were separated using Duncan's Multiple Range Test (DMTR). The collected data was analyzed in Statistica 13 (StatSoft, DELL) software.

RESULTS AND DISCUSSION

The presented results indicate a huge influence of illumination and nutrition media on the seed germination and protocorm formation. The seed germination was overall significantly more successful in dark conditions (0/24 L/D) than with lighting (16/8 L/D). This result coincides with the results of Godo et al. (2010), who found that light conditions have an inhibitory effect on terrestrial orchid *Calanthe tricarinata* Lindl. germination. The results showed that MM medium has a considerably better impact on the germination and protocorm formation, while on KC medium the seeds stay in swelled condition while their further development is stopped. This result can be explained by the composition of the nutrition media. While MM medium contains only an organic form of nitrogen (glycine and casein hydrolysate) KC medium contains an inorganic form of nitrogen, which in some cases has been shown to have an inhibitory effect on the seed germination and protocorm formation (Ponert et al., 2013, Sgarbi et al. 2009). This result also coincides with the result of Van Weas and Debergh (1986), confirming that asymbiotic germination of this species is promoted by organic nitrogen.

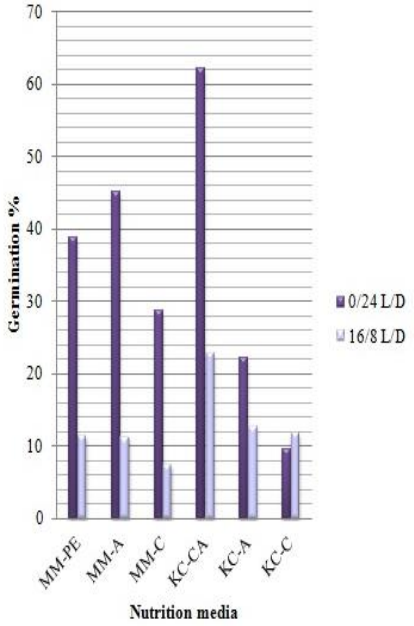


Figure 1. Measurements 8 weeks after sowing – full germination

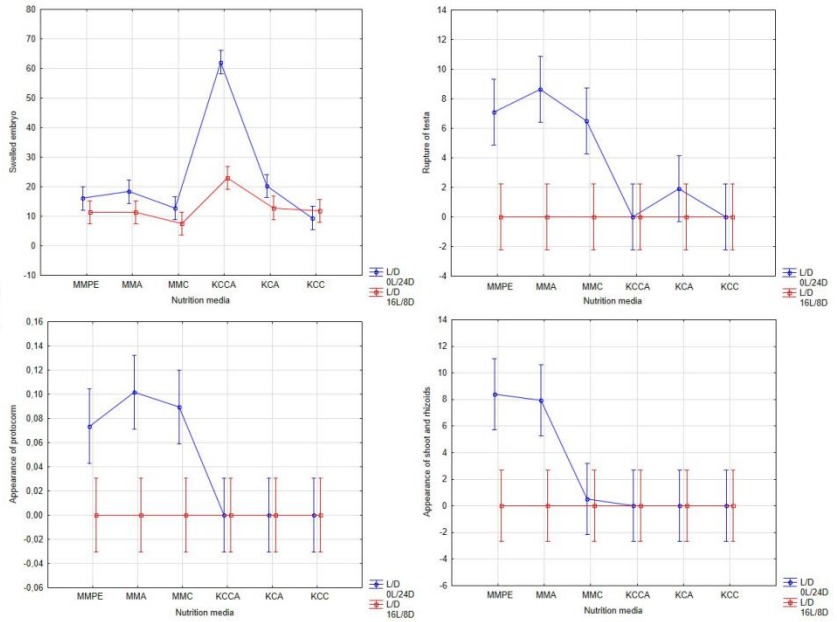


Figure 2. Influence of illumination and nutrition media 8 weeks after sowing: A) Swelled embryo; B) Rupture of testa; C) Appearance of protocorm; D) Appearance of shoot and rhizoids

After the first measurement, the seeds cultured in continual darkness on KC-CA medium had the highest full germination percentage (62%) (Figure 1). However, the appearance of protocorm was notable on the seeds sown on MM-PE, MM-A, and MM-K, while shoots and rhizoids appeared only on MM-PE and MM-A media, cultured in continual darkness (Figure 2). The results presented in this paper show that KC medium promotes only the swelling of the seeds without protocorm, shoots and rhizoids formation.

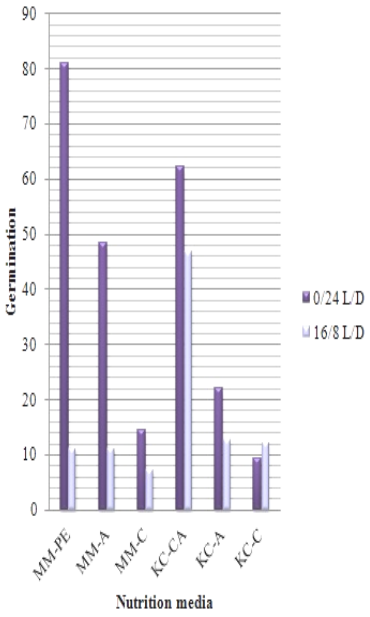


Figure 3. Measurements 12 weeks after sowing – full germination

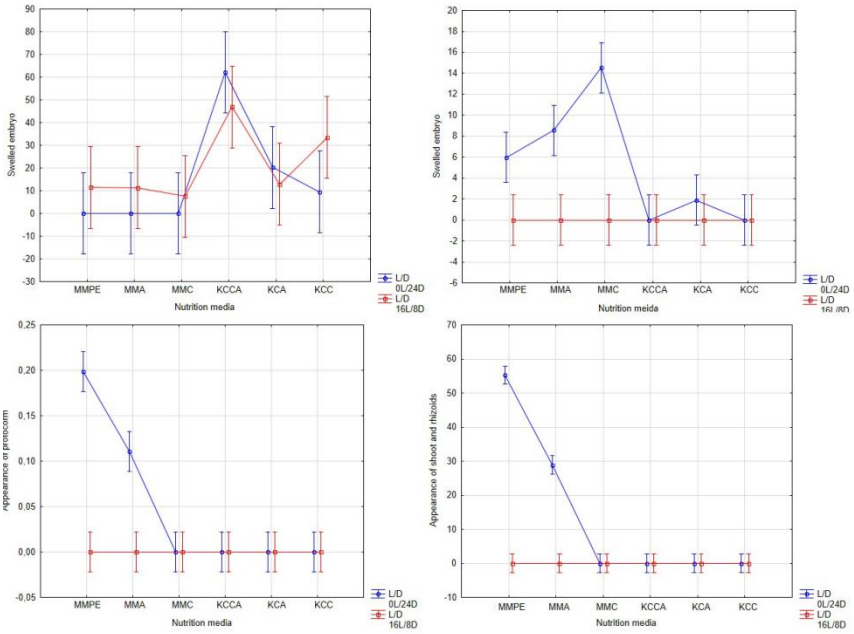


Figure 4. Influence of illumination and nutrition media 12 weeks after sowing: A) Swelled embryo; B) Rupture of testa; C) Appearance of protocorm; D)Appearance of shoot and rhizoids

The second measurement showed that on KC media the seeds remained swelled. Protocorm formation, shoots and rhizoids appearance were achieved only on MM-PE and MM-A media, in continual darkness (Figure 3, 4.). This result indicates a positive influence of organic supplements on the germination rate. With light, no growth was achieved at all.

Table 1. Mean values of embryo size during the first and second measurement

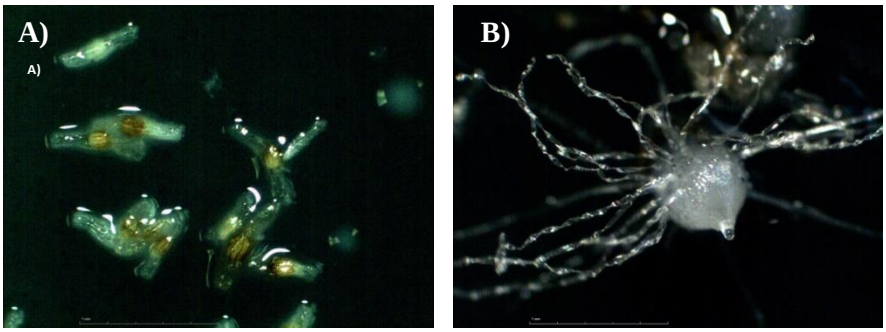
Illumination	Nutrition media	8 weeks			12 weeks		
		Height (mm)	Width (mm)	Shoot height (mm)	Height (mm)	Width (mm)	Shoot height (mm)
0L/24D	MM-PE	0.70±0.02^{a}	0.62±0.06^{a}	0.16±0.10^{a}	0.81±0.19a^{a}	0.75±0.18^{a}	0.32±0.08^{a}
	MM-A	0.64±0.10^{b}	0.56±0.05^{b}	0.14±0.10^{a}	0.77±0.2^{a}	0.65±0.16^{b}	0.24±0.22^{a}
	MM-C	0.58±0.06^{c}	0.51±0.05^{c}	0.06±0.07^{b}	0.55±0.09^{b}	0.50±0.08^{c}	0.06±0.06^{b}
	KC-CA	0.17±0.03^{d}	0.10±0.01^{d}	0.00±0.00^{c}	0.17±0.02^{c}	0.11±0.01^{d}	0.00±0.00^{b}
	KC-A	0.16±0.01^{d}	0.10±0.02^{d}	0.00±0.00^{c}	0.16±0.02^{c}	0.10±0.02^{d}	0.00±0.00^{b}
	KC-C	0.15±0.02^{d}	0.10±0.02^{d}	0.00±0.00^{c}	0.16±0.01^{c}	0.10±0.02^{d}	0.00±0.00^{b}
16L/8D	MM-PE	0.14±0.02^{d}	0.11±0.02^{d}	0.00±0.00^{c}	0.14±0.02^{c}	0.11±0.02^{d}	0.00±0.00^{c}
	MM-A	0.16±0.02^{d}	0.13±0.01^{d}	0.00±0.00^{c}	0.16±0.02^{c}	0.13±0.01^{d}	0.00±0.00^{c}
	MM-C	0.14±0.02^{d}	0.10±0.02^{d}	0.00±0.00^{c}	0.14±0.02^{c}	0.10±0.02^{d}	0.00±0.00^{c}
	KC-CA	0.17±0.02^{d}	0.11±0.03^{d}	0.00±0.00^{c}	0.17±0.02^{c}	0.11±0.03^{d}	0.00±0.00^{c}
	KC-A	0.15±0.01^{d}	0.10±.02^{d}	0.00±0.00^{c}	0.15±0.01^{c}	0.10±.02^{d}	0.00±0.00^{c}
	KC-C	0.15±0.01^{d}	0.10±0.02^{d}	0.00±0.00^{c}	0.15±0.01^{c}	0.10±0.02^{d}	0.00±0.00^{c}

*Means ± standard deviation within a column followed by the same letters are not significantly different using DMTR at $p \leq 0.05$

The measurements of the embryo size confirmed that darkness and MM medium supplemented with additives promote the germination and protocorm formation of *O. sphegodes* (Table 1.). The highest embryo size during the first measurement was achieved on MM – PE (height 0.70 mm; width 0.62 mm; shoot height 0.16mm) and on MM – A (height 0.64 mm; width 0.56 mm; shoot height 0.14mm). During the second measurement increased embryo size

was achieved only on MM-PE (height 0.81 mm; width 0.75 mm; shoot height 0.32mm), MM-A (height 0.77 mm; width 0.65 mm; shoot height 0.24mm) and MM-C (height 0.55 mm; with 0.50 mm; shoot height 0.06 mm), which indicates a positive influence of MM medium and darkness on the germination. During the second measurement, there were no changes in embryo size on the seeds cultured on 16L/8D illumination.

This study presents a procedure for the *in vitro* asymbiotic germination and protocorm formation of *O. sphegodes* that can be used for conservation programs and mass production protocol. Furthermore, because of the robust and attractive character of *O. Sphegodes,* as well as its long-living flowers, the presented research could provide an impulse for using this species as a garden or potted plant. In nature, germination of terrestrial orchid seeds is conditioned by the establishment of symbiosis with mycorrhizal fungi and this process can take up to several years (Rasmussen, 1995). The presented procedure accelerates the germination period and can provide a large number of plants in a relatively short period of time.



Picture 2. Seed germination: A) Swelled embryo; B) Protocorm with shoot and rhizoids

CONCLUSION

This paper presents successful *in vitro* germination of mature seeds of a terrestrial orchid species. Both MM and KC media caused swelling of the embryo, but protocorm and shoots and rhizoids were formed only on MM media (Image 2) Also, during this research, a positive influence of organic additives on the germination rate was achieved. Remarkably better results were obtained in dark conditions (0/24 L/D), which was previously confirmed by several other authors. After 12 weeks, significant results were achieved on MM-PE and MM-A media, especially on MM-PE media. The best protocol for *Ophrys sphegodes*, therefore, is culturing seeds in dark conditions, on MM-PE media.

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IN VITRO KLIJANJE SEMENA UGROŽENE VRSTE TERESTRIČNE ORHIDEJE OPHRYS SPHEGODES MILL

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Izvod: Biologija oprašivanja, veoma sitno seme i uništavanje prirodnog staništa Ophrys sphegodes Mill. predstravljaju razloge zbog kojih se ova vrsta smatra ugroženom i nalazi se pod visokim stepenom zaštite. Cilj ovog rada bio je da se utvrdi protokol za ex situ konzervaciju metodom asimbiotskog klijanja semena u in vitro uslovima. Ispitan je uticaj dve hranljive podloge (KC i MM) obogaćene sa različitim organskim suplementima (pepton, L-glutamin, folna kiselina, kazein) kao i kontrolni medijum (KC-C i MM-C). Sve hranljive podloge su sadržale 2% saharoze, 7% agara i 1% aktivnog uglja, dok im je pH vrednost podešena na 5.8 ± 0.02 pre autoklaviranja na 121 ° C u trajanju od 20 minuta. Ispitan je uticaj dva režima svetlosti na klijanje semena: 0/24 svetlost/mrak i 16/8 svetlost/mrak. Rezultati dobijeni u ovom istraživanju ukazuju da su klijanje semena i formiranje protokorma pod direktnim uticajem hranljive podloge i osvetljenja. Znatno veći stepen klijavosti postignut je kod semenki koje su postavljene u uslove konstantnog mraka (0/24 L/D). Formiranje protokorma, rizoida i vršnog izbojka uspostavljeno na MM hranljivoj podlozi, dok je KC podloga inicirala samo bubrenje embriona. Takođe, ovim istraživanjem je ustanovljen pozitivan uticaj organskih suplemenata. Analizom dobijenih rezultata došlo se do zaključka da hranljiva podloga MM obogaćena sa peptonom (MM-PE) i uslovi konstantnog mraka predstavljaju uslove koji najpovoljnije utiču na klijanje semena i razvijanje sejanca vrste Ophrys sphegodes. Procedura koja je prezentovana u ovom radu može u relativno kratkom vremenskom periodu da obezbedi veliki broj sejanaca neophodnih za potrebe konzervacije.

Ključne reči: In vitro, Ophrys sphegodes, protokorm, rizoidi

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