

THE INFLUENCE OF DISINFECTION METHODS AND LIQUID PHASE MEDIA ON *Paphiopedilum insigne* SEEDS GERMINATION AND MEDIA SUPPLEMENTS ON MORPHOLOGICAL FEATURES OF PROTOCORMS IN TISSUE CULTURE

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ABSTRACT

Paphiopedilum is a very original orchid, which labellum resembles a slipper. It is cultivated as a cut flower or a pot plant. The aim of the presented work was to estimate the influence of a disinfection method and addition of a liquid media on germination of *Paphiopedilum insigne* seeds *in vitro*. The whole seed capsules were disinfected with the use of 0.5% of AgNO₃ for 20 min, 0.1% of HgCl₂ for 5 s, 1% NaOCl for 30 min or immersed in a 96% ethanol and burned in a direct flame. The disinfected seeds were placed on a 1/4 MS (Murashige and Skoog) solidified media on top of which a liquid phase containing 1/4 MS elements, GA₃ in concentration of 400 mg·dm⁻³ or distilled sterile water were added. The media without the liquid phase was also tested. It was noted that all methods of disinfection used effectively reduced contaminations of *Paphiopedilum insigne* seeds. The highest germination rate was observed when capsules were direct flamed and the liquid phase was added on top of the solidified media. The influence of growth regulators (BA, TDZ, KIN and 2,4-D) and casein hydrolysate added to the media on the morphological features of the obtained plants was also tested. It was observed that the most regenerated protocorms of the best quality were obtained when the media was supplemented with 1 mg·dm⁻³ of BA + 2 mg·dm⁻³ of TDZ or 5 mg·dm⁻³ of KIN + 1 mg·dm⁻³ of BA.

Key words: slipper orchid, micropropagation, *in vitro*, regeneration, casein hydrolysate, growth regulators

INTRODUCTION

Paphiopedilum Pfitzer belongs to the Orchidaceae family. The genus includes about 100 species, the most of which naturally occurs in south-west Asia [Guo et al. 2015]. Plants characterize with a very impressive flowers which shape resembles a shoe or a slipper, what makes them one of the most popular orchid in the world [Ng and Saleh 2011]. A slipper orchid is cultivated as a cut flower or as a pot plant [Zeng et al. 2016]. Fakouri Ghazani et al. [2014] state that orchids are among 10 plant species the most often cultivated as cut flowers.

Propagation of *Paphiopedilum* orchids in traditional way, through division of mother plants, is very difficult due to a slow growth [Ng and Saleh 2011]. More and more often, tissue culture is used, especially the asexual germination of seeds [Zeng et al. 2012]. Collection of seeds does not damage mother plants, however it needs a cross-pollination of plants. Pollination is made when plants are in a full blossom. Then, pollen from one specimen has to be placed on a pistil of the second one. Maturity of seed capsules has to be estimated as they have to be collected in a right moment,

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when seeds have a gray-green color, because it significantly influenced germination rate of seeds [Zeng et al. 2012]. According to the literature data, orchid seeds germinated with difficulty, they are non-endospermic and have little lipids [Fakouri Ghazani et al. 2014]. They also need some time for organogenesis and synthesis of nutrients. In case of *Paphiopedilum wardii*, it was 180 days after pollination [Zeng et al. 2012]. Other important factors affecting germination of orchid seeds intensity is a way of disinfection, especially a substance used and media content [Jevšnik and Luthar 2015]. According to Soonthornkalump et al. [2019], who studied propagation of *Paphiopedilum niveum*, in order to produce a large number of genetically homogeneous orchid seedlings commercially, genetically stable protocorm-like bodies (PLB) should be obtained, among other things. Fakouri Ghazani et al. [2014] state that supplementation of the media with the right combinations of growth regulators (PGRs) affects morphological features of the obtained seedlings. The most often used PGRs for orchids germination are benzyladenine (BA) together with NAA (naphthaleneacetic acid) [Fakouri Ghazani et al. 2014] or IBA (indole-3-butyric acid) [Khatun et al. 2010]. Sibin and Gangaprasad [2012] proved that the use of casein hydrolysate, an organic compound, positively influenced fresh weight of *Rhynchostilis retusa* protocorms as well. According to Thompson et al. [2007], use of a double phase media might also be advantageous. The authors used the double-phase media for *Disa* seeds germination.

The aim of the presented work was to estimate the influence of different methods of disinfection and a double phase media on seeds germination and growth of protocorms of *Paphiopedilum insigne*, as well as a media content on morphological features of the obtained microplants.

MATERIAL AND METHODS

Plant material were seeds of *Paphiopedilum insigne* obtained from a hand cross-pollination. The seed capsules were collected 292 days after pollination, when they were fully browned.

The capsules were washed under a running tap water with a Ludwik detergent, then they were shaken in a solution of the Topsin fungicide in concentration of

2 mg·dm⁻³ for 30 min and immersed in a 70% ethanol for 5 s. The capsules were then disinfected in the following solutions: 1% of sodium hypochlorite (NaOCl) for 30 min, 0.5% of silver nitrate (AgNO₃) for 20 min or 0.1% of mercuric chloride (HgCl₂) for 5 s. The disinfected capsules were washed in a sterile distilled water in a laminar flow cabinet. Additionally, one seed capsule was immersed in a 96% ethanol and directly flamed in a laminar flow cabinet. The capsules were then opened and seeds were taken out.

The disinfected seeds were placed in a 100 ml Erlenmeyer flasks containing 25 ml of the 1/4 MS [Murashige and Skoog 1962] media supplemented with vitamin B₁ – 0.025 mg·dm⁻³, vit. B₆ – 0.125 mg·dm⁻³, vit. PP – 0.125 mg·dm⁻³ and glycine – 0.5 mg·dm⁻³, inositol – 25 mg·dm⁻³ and sucrose – 7.5 g·dm⁻³. The media was gelled with BIOCORP agar in concentration of 6.75 g·dm⁻³ and supplemented with 0.5 mg·dm⁻³ of NAA (1-naphthaleneacetic acid) and 1 g·dm⁻³ of AC (activated charcoal). pH of the medium was adjusted to 5.7 with the use of 1M NaOH and 1M KCl. The media was autoclaved in the temperature of 121°C and 1 MPa for 21 min. The disinfected seeds were placed on the solid media and then a liquid phase containing sterile distilled water, 1/4 MS medium or gibberelic acid (GA₃) solution in concentration of 400 mg·dm⁻³ was added. The treatment without the liquid phase was also used. The experiment consisted of 16 treatments with 25 repetitions (flasks) containing 300 seeds each. The scheme of the experiment is presented in Table 1.

Flasks with *Paphiopedilum insigne* seeds were placed in a growing room in a temperature of 28 ± 2°C and 16-hour photoperiod. The light source were Fluora fluorescent lights with the light intensity of 30 μmol·m⁻²·s⁻¹.

The experiment lasted for 16 weeks and the number of the obtained protocorms was estimated. During the experiment the systematical observations were done and from the moment of seeds germination the photographs were done with the use of Eduko SE 220 stereoscopic microscope.

The obtained protocorms were placed on the fresh media. The influence of the media content on morphological features and survival rate of the protocorms were studied. The obtained *Paphiopedilum insigne* protocorms were transferred on the 1/2 MS media supplemented with the following substances:

Table 1. Scheme of the experiment on influence of the disinfection method and double phase media on *Paphiopedilum insigne* seeds germination in tissue culture

Disinfection solution	Liquid phase media			
	none	sterile wather	liquid 1/4 MS	GA ₃ 400 mg·dm ⁻³
0.5% AgNO ₃ (20 min)	×	×	×	×
0.1% HgCl ₂ (5 s)	×	×	×	×
1% NaOCl (30 min)	×	×	×	×
96% ethanol (5 s) + direct flame	×	×	×	×

5 mg·dm⁻³ TDZ (thidiazuron) + 1 mg·dm⁻³ 2,4-D (2,4-dichlorophenoxyacetic acid), 5 mg·dm⁻³ KIN (kinetin) + 1 mg·dm⁻³ BA (benzyladenine), 1000 mg·dm⁻³ CH (casein hydrolysate), 1 mg·dm⁻³ BA + 2 mg·dm⁻³ TDZ, 1000 mg·dm⁻³ casein hydrolysate + 1 mg·dm⁻³ BA. Each treatment was set up in ten replications with 20 protocorms in each. After 8 weeks, the number of protocorms that started to grow was counted and the following features were evaluated: number of leaves, height and width (mm), fresh weight (mg).

The obtained data was analyzed statistically according to the one-way or two-way orthogonal or non-orthogonal independent analysis of variance. The percentage values were transferred into Bliss angles [Bliss 1938]. The significance of differences between the means was estimated with the Tukey's confidence intervals at the level of significance $\alpha = 0.05$.

RESULTS AND DISCUSSION

In the presented experiment, all disinfection methods used were efficient as 100% of *Paphiopedilum insigne*. cultures were free of contaminations. Pindel and Pindel [2004] state that the percentage of microbiological infections depends on an orchid species. The authors proved that disinfection of seed capsules of *Cypripedium calceolus*, *Dactylorhiza maculata*, *Epipactis helleborine*, *Goodyera repens* and *Gymnadenia conopsea* in 0.1% of mercuric chloride for 5 min was effective [Pindel i Pindel 2004]. In case of *Bletilla striata* disinfection was sufficient when 0.2% of HgCl₂ was used [Kulpa and Katroń 2012]. A low contamination rate might be an evidence that the in-

side of seed capsules together with seeds is sterile and microbiological contaminants are situated on the surface. Therefore, the outer side of seed capsules should be disinfected before being used for tissue culture initiation [Oszkinis 2004].

The use of different substances for disinfection and addition of the liquid media as a second phase significantly influenced the number of germinating seeds of *Paphiopedilum insigne in vitro*, as shown in Table 2.

It was observed that the most seeds germinated when the seed capsules were immersed in 96% ethanol for 5 seconds and directly flamed. A positive influence of this method of disinfection might come from it's phytotoxic activity and short time of use. The advantageous effect of ethanol applied for 2 min was proven in case of *Anacamptis morio* [Ponert et al. 2011]. However, Ponert et al. [2011] state that lengthening the time of disinfection with alcohol reduced the number of germinating seeds *Anacamptis morio*. In the presented study it was noted that when capsules had been treated with 0.1% HgCl₂, only 3.5 seeds of *Paphiopedilum insigne* germinated on average. Different result were obtained by Kaur and Bhutani [2016] who obtained satisfactory while disinfecting *Paphiopedilum venustum* with 0.05% of that substance for 2–3 min.

The presented research proved that disinfection of seeds in 0.5% of AgNO₃ and 1% of NaOCl resulted in very poor germination of seeds (0.03 and 0.05 seeds respectively), what might resulted from too high concentrations of those substances or too long time of disinfection, what was confirmed by Zeng et al. [2012] and Katsalirou et al. [2017]. Katsalirou et al. [2017]

Table 2. The influence of the disinfection method and a liquid phase on the number of germinated seeds of *Paphiopedilum insigne* in tissue culture

Disinfection solution	Double phase media				mean A
	none	sterile water	liquid medium 1/4 MS	GA ₃ 400 mg·dm ⁻³	
0.5% AgNO ₃ (20 min)	0.04 c	0.04 c	0.00 c	0.12 c	0.05 C
0.1% HgCl ₂ (5 s)	4.48 c	4.16 c	3.32 c	2.12 c	3.52 B
1% NaOCl (30 min)	0.12 c	0.00 c	0.00 c	0.00 c	0.03 C
70% ethanol (5sec) + direct flame	31.00 b	39.36 a	42.52 a	38.40 a	37.82 A
Mean B	8.91 B	10.89 A	11.46 A	10.16 A	1.03

Means followed by the same letter do not differ significantly at $\alpha = 0.05$

showed a relation between the activity of substances used for disinfection and permeability of seed coatings. The authors scarified seeds of *Anacampsis laxiflora* for a few minutes in 1% NaOCl. Similarly, Zeng et al. [2012] informed that concentration and time of disinfection influence germination ability of seeds. They proved that in case of *Paphiopedilum wardii* the most seeds germinated when they had been disinfected with NaOCl containing 1% of active chlorine for 40 min. The positive effect of sodium hypochlorite at a concentration of 3% on the viability and germination of *Paphiopedilum insigne* seeds was also demonstrated by Diengdoh et al. [2017]. Studies conducted by Alvarez-Pardo et al. [2006], show that together with increasing the concentration and duration of NaOCl activity, germination of south Brazilian orchids decreased. In case of *Cypripedium macranthos* it was proved that sodium hypochlorite damaged cell walls integrity and therefore eased adsorption of nutrients, what positively influenced germination [Bae et al. 2010]. On the other hand, negative effect of NaOCl was observed in tissue cultures of *Dactylorhiza fushii*, *Dactylorhiza majalis* initiated from seeds [Ponert et al. 2011] and *Dactylorhiza incarnata* subsp. *serotina*, in case of which embryos were damaged what limited germination of seeds [Vejsedova 2006]. There is very few information on the influence of silver nitrate on seeds germination. Kutas and Ogrodnik [2011] confirm usefulness of AgNO₃ in concentration of 0.1% on germination of *Rhododendron* sp. seeds.

The positive effect of the liquid phase on the number of germinated *Paphiopedilum insigne* seeds was observed in the presented work (1/4 MS – 10.46, distilled water – 10.89 and gibberellic acid solution – 10.16, in comparison to no liquid – 8.91). Borkowska [1997] stated that addition of the second, liquid phase, might increase availability of nutrients, which amount is low in solidified media. According to Chmiel and Jędrzejuk [2015], germination of seeds depends on environmental conditions, among which the most important is possibility to water uptake through seed coat, what allows seeds to swell and germinate.

The second phase in a form of a liquid 1/4 MS medium positively influenced seeds germination. According to Borkowska [1997], this solution contains both nitric and ammonium nitrogen. There comes to a chemical balance between solidified and liquid phases. The process works in the same way even when the second liquid phase is differs from the solidified medium [Borkowska 1997]. Many orchid species naturally grow in soils poor in nitrogen, therefore enrichment of medium in nitric and ammonium nitrogen enhances germination of seeds, what was proven by Zaniecka and Łojkowska [2004], in case of such orchids as *Cypripedium calceolus*, *Dactylorhiza majalis* and *Epipactis palustris*. Thompson et al. [2007] confirm a positive influence of a second phase in a form of a liquid MS medium added after 7 days from the placing seeds on the solidified medium on germination of *Disa* sp. The use of liquid gibberellic acid solu-

tion as a second phase might break seed dormancy. In case of *Compantia falcata* it was observed that GA_3 increased the percentage of germinated seeds and advantageously affected development of plants [Pedroza-Manrique et al. 2005]. According to Borkowska [1997], the liquid phase might also dilute and immobilize toxic substances that are present in media.

On the basis of the statistical analysis it was proven that the least seeds germinated when the liquid phase was not added. The studies confirm research by Jevšnik and Luthar [2015] conducted on *Epidendrum nocturnum*, *Prosthechea garciana* i *Zygopetalum*. The authors showed, that in case of seeds placed on

the media gelled with agar the delay in seeds germination and growth of protocorms was observed.

Regeneration of *Paphiopedilum insigne* protocorms is presented on Figure 1.

In the presented work, addition of various supplements to the media, such as growth regulators and casein hydrolysate, had a positively influence on morphological features of the regenerating protocorms, as shown in Table 3.

It was observed that supplementation of the medium with $1 \text{ mg} \cdot \text{dm}^{-3}$ BA and $2 \text{ mg} \cdot \text{dm}^{-3}$ TDZ resulted in the highest regeneration of protocorms (73%). Satisfactory effects were also obtained when the media

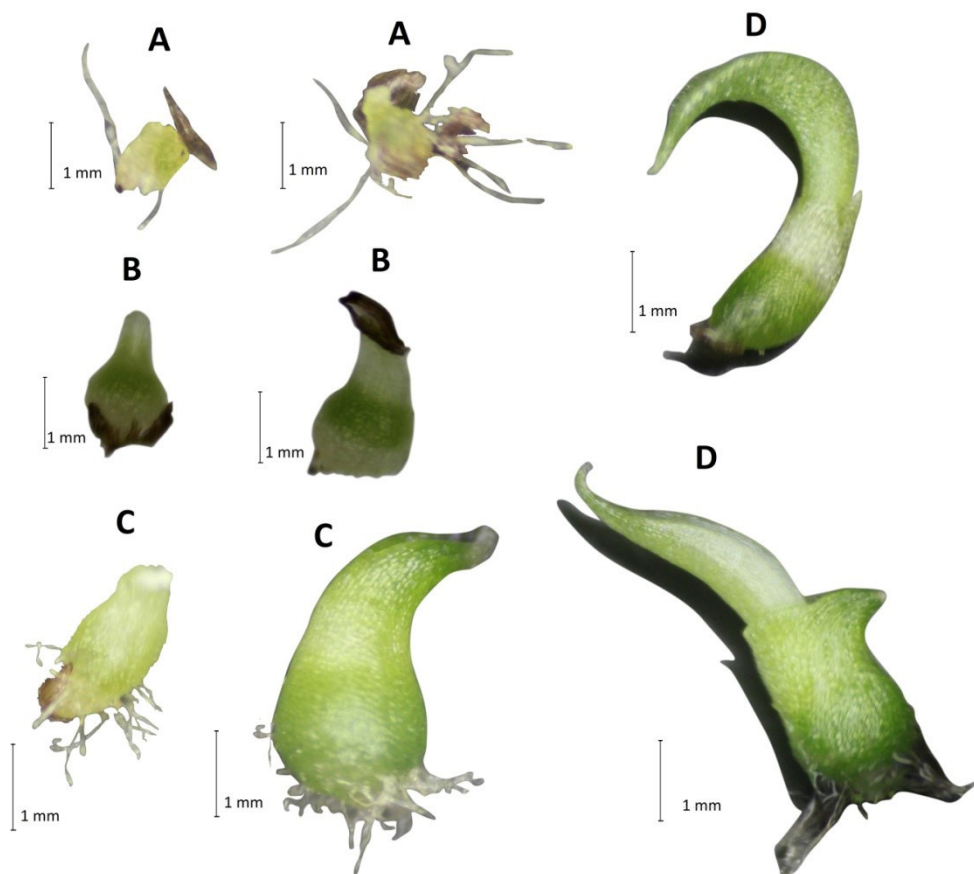


Fig. 1. A. *Paphiopedilum insigne* protocorm after 35 days from the placement on the media. B. Protocorm *Paphiopedilum insigne* after 50 days from the placement on the media. C. Protocorm with a visible leaf bud and root hairs after 70 days from the placement on the media. D. Micro-plant of *Paphiopedilum insigne* with visible leaves and small root hairs after 110 days from the placement on the media

Table 3. The influence of the media content on regeneration and morphological features of *Paphiopedilum insigne* protocorms in tissue culture

Media supplements (mg·dm ⁻³)	Percentage of regenerated protocorms	Percentage of protocorms that formed leaves	Number of leaves	Hight of regenerants (mm)	Width of regenerants (mm)	Fresh weight of regenerants (mg)
1 BA + 5 KIN	67 ab	99 a	1.77 a	4.93 a	3.91 a	9.07 a
1 BA + 2 TDZ	73 a	99 a	1.75 ab	5.39 a	4.27 a	5.94 ab
1 BA + 1000 CH	63 ab	90 ab	1.64 ab	3.99 b	2.74 b	3.90 b
1000 CH	57 bc	78 b	1.58 ab	3.36 b	2.73 b	3.82 b
5 TDZ + 1 2,4-D	50 c	57 c	1.32 b	3.47 b	2.28 b	4.72 ab

BA – benzyladenine, KIN – kinetin, TDZ – thidiazuron, CH – casein hydrolysate, 2,4-D – 2,4-dichlorophenoxyacetic acid
Means followed by the same letter do not differ significantly at $\alpha = 0.05$

was supplemented with 1 mg·dm⁻³ BA with 1 mg·dm⁻³ KIN (67%) and 1 mg·dm⁻³ BA with 1000 mg·dm⁻³ of casein hydrolysate (63%). The least protocorms, only 50%, regenerated when 5 mg·dm⁻³ TDZ with 1 mg·dm⁻³ 2,4-D were added to the media. The positive influence of benzyladenine on protocorms regeneration was proven by Fakouri Ghaziani et al. [2014] in case of *Orchis catasetum*. However, authors emphasise that this growth regulator works better in combination with NAA. Kumar et al. [2002] confirmed, that the use of BA in low concentrations advantageously affects development of *Rhynchostilis retusa* and *Cymbidium elegans* into plants. The authors noted that the concentration of BA should be lowered below 0.23 mg·dm⁻³. In the presented research the least regenerated protocorms were obtained when the media was supplemented with 2,4-D, what confirms studies conducted on *Aerides crispum* by Sheelavanthmath et al. [2005].

In the presented work the most protocorms of *Paphiopedilum insigne* formed leaves in presence of 5 mg·dm⁻³ KIN with 1 BA mg·dm⁻³ and 1 mg·dm⁻³ BA with 2 mg·dm⁻³ TDZ (99%), in comparison to addition of casein hydrolysate in concentration of 1000 mg·dm⁻³ (78%) or 5 mg·dm⁻³ TDZ with 1 mg·dm⁻³ 2,4-D (57%). An important factor deciding about a good quality of plants of *Paphiopedilum insigne* is a number of formed leaves. It was observed that the most leaves developed when protocorms were cultivated on the medium supplemented with 1 mg·dm⁻³ BA with 5 mg·dm⁻³ KIN (1.77) in comparison to

5 mg·dm⁻³ TDZ with 1 mg·dm⁻³ 2,4-D (1.32). A positive influence of benzyladenine on leaves development was proven in studies of Talukder et al. [2003] on *Dendrobium* sp. and Fakouri Ghazani et al. [2014] on *Orchis catasetum*. Talukder et al. [2003] noted that plants formed the most leaves when the media was supplemented only with 2.5 mg·dm⁻³ BA, while Fakouri Ghazani et al. [2014] observed the same reaction when BA was combined with NAA in concentration of 0.5 mg·dm⁻³ each. Research conducted by Nisayan et al. [2010] observed good growth of *Paphiopedilum* leaves when kinetin in concentration of 1 mg·dm⁻³ was used together with 1 mg·dm⁻³ 2,4-D. The combination of those two growth regulators positively affected formation of leaves in the presented work as well.

In the presented research it was observed that the growth regulators used significantly influenced hight and width of leaf rosettes of *Paphiopedilum insigne*. The best parameters characterized microplants cultivated on the media supplemented with 1 mg·dm⁻³ BA together with 2 mg·dm⁻³ TDZ (5.39 mm and 4.27 mm respectively) and 1 mg·dm⁻³ BA with 5 mg·dm⁻³ KIN (4.93 mm and 3.91 mm respectively). A positive effect of benzyladenine use on hight of plants is described in many works. Fakouri Ghaziani et al. [2014] applied 0.5 mg·dm⁻³ together with 0.5 mg·dm⁻³ NAA in cultivation of *Orchis catasetum*. Kumar et al. [2002] stated, that the influence of BA on height of plants depends on its concentration. The authors observed that height of *Rhynchostilis retusa* and *Cymbidium el-*

egans increased with lowering the concentration of BA. Asghar et al. [2011] noted that in presence of BA *Dendrobium nobile* ‘Emma White’ formed shorter plants and the increase of height was noted only when the medium was supplemented with kinetin. Sibin and Gangaprasad [2012] in the studies on *Rhynchosytilis retusa* proved an advantageous influence of the media supplementation with casein hydrolysate, what was not confirmed in the presented work, as the substance used did not significantly affected the width of the protocorms.

Moreover, it was noted that the highest fresh weight characterized plants obtained on the media supplemented with $5 \text{ mg} \cdot \text{dm}^{-3}$ KIN with $1 \text{ mg} \cdot \text{dm}^{-3}$ BA (9.07 mg) in comparison to $1 \text{ mg} \cdot \text{dm}^{-3}$ BA with $1000 \text{ mg} \cdot \text{dm}^{-3}$ casein hydrolysate (3.90 mg). The obtained results confirm the studies conducted by Asghar et al. [2011]. The authors showed that plants of *Dendrobium nobile* ‘Emma White’ with the highest fresh weight were obtained in presence of $2 \text{ mg} \cdot \text{dm}^{-3}$ of BA. Similar results were also observed by Khatun et al. [2010] in case of *Dendrobium*, who obtained protocorms of the highest fresh weight when the media was supplemented with $1 \text{ mg} \cdot \text{dm}^{-3}$ BA together with $1 \text{ mg} \cdot \text{dm}^{-3}$ IBA. Sibin and Gangaprasad [2012] proved the usefulness of 0.05% of casein hydrolysate for regeneration of *Rhynchosytilis retusa* protocorms characterizing with the high fresh weight, however it was not confirmed in case of *Paphiopedilum insigne* in the presented work, as less protocorms regenerated in presence of casein hydrolysate and they characterized with smaller size and fresh weight.

Suming up, it might be stated that in the presented research, benzyladenine used together with TDZ or kinetin, positively influenced most of the studied morphological features of *Paphiopedilum insigne* in tissue culture.

CONCLUSIONS

1. Seed capsules might be used as a source of sterile seeds for tissue culture initiation of *Paphiopedilum insigne* and the disinfection effectiveness does not depend on the method of disinfection used.

2. Disinfection of seed capsules in 96% of ethanol and direct flaming results in contamination free cultures and the highest germination rate of *Paphiopedilum insigne* seeds.

3. The addition of the liquid phase to the solidified media positively influences germination rate of *Paphiopedilum insigne* seeds *in vitro*.

4. Supplementation of the media with $1 \text{ mg} \cdot \text{dm}^{-3}$ BA + $2 \text{ mg} \cdot \text{dm}^{-3}$ TDZ or $5 \text{ mg} \cdot \text{dm}^{-3}$ KIN + $1 \text{ mg} \cdot \text{dm}^{-3}$ BA allows to obtain good quality microplants of *Paphiopedilum insigne* in tissue culture.

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