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TISSUE CULTURE MULTIPLICATION OF *Paphiopedilum insigne* DEPENDING ON THE MEDIUM TYPE, GROWTH REGULATORS AND NATURAL SUPPLEMENTS

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ABSTRACT

Paphiopedilum is an ornamental orchid used mainly for interior decoration. As the division of plants is uneconomical, a fast method of propagation is needed. The aim of this study was to evaluate the influence of medium type (MS or VW), growth regulators, i.e. BA, KIN, TDZ used separately or in combinations and natural additives, i.e. coconut water, banana pulp, casein hydrolysate, on *Paphiopedilum insigne* plantlets grown *in vitro*. It was found out that BA in concentration of 0.5 mg·dm^{-3} allowed to obtain the highest multiplication rate (2.92), however the use of KIN in concentration of 1 mg·dm^{-3} resulted in formation of bigger and higher quality plantlets, It is possible to replace cytokinins with other biologically active substance, such as banana pulp. The 1/2 MS medium might be used for *Paphiopedilum insigne* tissue culture, as there was no difference in terms of multiplication rate and the obtained plantlets were of better quality, especially that it is cheaper and easier to prepare.

Key words: biological supplements, lady slipper, micropropagation, orchid, cytokinins

INTRODUCTION

Orchids are valued ornamental plants used both in floristic compositions and as decorative pot plants [Zeng et al. 2016]. *Paphiopedilum* sp. has a high position among other species in this group of plants due to a very original and showy, bilaterally symetric flowers with a cup-like lip, resembling a shoe, which is responsible for the common name Lady slipper [Ng and Salech 2011].

Propagation of *Paphiopedilum* in a traditional way, through division of mother plants, is not effective [Ng and Saleh 2011], because only a few new plant may be obtained from one mother plant. Due to high demand of the market, it seems necessary to develop a technology for the mass production of these species and tissue culture is one of the methods to obtain the highest vegetative propagation rate.

Paphiopedilum insigne (Wall. ex Lindl.) Pfitzer is a terrestrial orchid, it does not produce pseudobulbs. In natural conditions, orchids get nutrients from air, rain and bird manure, they do not tolerate urea. They have rather low nutrient requirements, especially during vegetative growth, and such growing media should be provided for *Paphiopedilum* cultivation. Plants may utilize only the amount of nutrients they need, they cannot store any for the future, therefore a continous supply of nutrients is necessary [Naik et al. 2010].

The composition of a growing medium plays an important role in plant propagation in tissue culture.

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The media used for micropropagation of most orchid species is poor in nutrients. Murashige and Skoog (MS) medium [1962] with macro- and micronutrients reduced by half was proved to have a positive effect on many of Orchidaceae species in tissue culture. It was successfully used with *Dendrobium aqueum* [Parthibhan et al. 2015], *Cymbidium ensifolium* var. *misericors*, *Epidendrum* sp., *Oncidium* sp. or *Paphiopedilum* sp. [Chang et al. 2005]. Use of the media with a reduced amount of components allows to reduce plant production costs additionally. The other medium often used for micropropagation of orchids is Vacin and Went (VW) [1949]. It was used for the tissue culture of *Vanda teres* [Sinha and Roy 2004] or *Vanda coeruela* [Jitsepakul et al. 2013].

Apart from the macro- and micronutrients, the addition of plant growth regulators (PGRs) might have a crucial influence on growth and development of plants in tissue culture. Use of PGRs is one of the most effective methods of controlling plant organogenesis *in vitro*. As for Orchideaceae species, the most often used PGRs are benzyladenine (BA) [Talukder et. al. 2003, Asghar et al. 2011, Jitsepakul et. al. 2013], thidiazuron (TDZ) [Jitsepakul et. al. 2013] and kinetin (KIN) [Asghar et al. 2011]. Some authors emphasize the role of other media supplements that might act as both source of nutrients and compounds regulating development of plants, which are biologically active substances of natural origin. Some of them were proven to have a positive effect on propagation of plants in tissue culture [Oszkinis 2004]. They include coconut water, extracts or juices from pineapple, banana, papaya, potato, tomato, yeast and malt, poplar or vine [Oszkinis 2004]. They are a valuable source of amino acids, fiber, phenols, organic acids, peptides, carbohydrates, fats, vitamins and growth regulators in various concentrations [Gnasekaran et al. 2010]. According to Parthibhan et al. [2015] the use of organic substances for supplementation of the media in the form of coconut water or banana pulp is profitable. The studies on micropropagation of *Dendrobium aqueum* showed that the applied substances induced growth of shoots, enhanced multiplication and rooting.

The aim of the presented research was to estimate the optimal composition of the media ingredients, including macro- and micronutrients, PGRs and natural additives, for micropropagation of *Paphiopedilum insigne* in relation to multiplication rate and quality of plantlets.

MATERIAL AND METHODS

Plant material. *In vitro* cultures of *Paphiopedilum insigne* were initiated from microcuttings obtained as a result of asymbiotic seed germination. The plant material for all of the presented studies were plantlets of *Paphiopedilum insigne* obtained from a stabilized culture cultivated on a basic medium containing micro- and macronutrients according to Murashige and Skoog [1962] reduced by half (1/2 MS) and supplemented with vit. B_1 – 0.05 mg·dm^{-3}, vit. B_6 – 0.25 mg·dm^{-3}, vit. PP – 0.25 mg·dm^{-3}, glycine – 1 mg·dm^{-3}, myo-inositol – 50 mg·dm^{-3} and sucrose – 15 mg·dm^{-3}. The basic medium did not contain any growth regulators.

Effect of the cytokinins on the multiplication and morphological features. Explants which had three completely formed leaves of about 8–12 mm length and 1–2 roots, were placed in 300 ml Erlenmeyer flasks containing 75 ml of the basic medium and supplemented with PGRs: benzyladenine (BA) in concentrations of: 0.5, 1 or 2 mg·dm^{-3}, thidiazuron (TDZ): 0.5, 1 or 2 mg·dm^{-3}, kinetin (KIN): 1. 2.5 or 5 mg·dm^{-3}, 1 mg·dm^{-3} BA + 0.5 mg·dm^{-3} TDZ, 1 mg·dm^{-3} BA + 2 mg·dm^{-3} TDZ, 1 mg·dm^{-3} BA + 0.5 mg·dm^{-3} KIN or 1 mg·dm^{-3} + 5 mg·dm^{-3} KIN. The PGRs were obtained from Sigma-Aldrich, St. Louis, USA. The pH media was 5.7. The media was gelled with BIOCORP agar in concentration of 6.75 g·dm^{-3}. The experiment consisted of 16 treatments, in two repetitions with 3 flasks containing 7 explants each. The experiment lasted for 16 weeks. At the end of the experiment the following features were evaluated: number of leaves per plantlet, length and width of leaves (mm), multiplication rate per plantlet (calculated as a number of new plantlets obtained from one explant), percentage of rooted plantlets, number, length (mm) and fresh weight (mg) of roots

Effect of a medium type, growth regulators and natural additives on the multiplication rate and morphological features. The plant material for the research were leaf rosettes obtained from a stabilized culture cultivated on the basic medium. The explants were 4 mm high and 3 mm wide and had 2 completely formed leaves.

The explants were placed in 300 ml Erlenmeyer flasks containing 1/2 MS medium, supplemented with the same substances as in the basic medium or VW medium [1949] supplemented with myo-inositol – 50 mg·dm^{-3} and sucrose – 15 g·dm^{-3}. Both media were supplemented with PGRs and biologically active substances of natural origin in the following combinations: 5 mg·dm^{-3} KIN + 1 mg·dm^{-3} BA; 1 g·dm^{-3} casein hydrolysate (CH); 1 mg·dm^{-3} BA + 2 mg·dm^{-3} TDZ; 1 g·dm^{-3} CH + 1 mg·dm^{-3} BA, coconut water (CW) in concentrations of 15 or 20% (v/v), banana pulp (BP) in concentrations of 20 or 30 g·dm^{-3}. The pH of the media was 5.7. PGRs used in the experiment were selected on the basis of the results obtained in the previous experiment. The experiment consisted of 16 treatments with 2 replications with 3 repetitions containing 21 explants each. The study lasted for 16 weeks. At the end of the experiment, the same features were evaluated as in the first experiment.

Cultivation conditions. The flasks with explants were placed in a growth room at 28°C ±2°C during the day, 24°C ±2°C at night and 16-hour photoperiod. The light source were Fluora fluorescent lights with the light intensity of approximately 30 µmol·m^{-2}·s^{-1}.

Statistical analysis. The obtained data was analyzed statistically according to the one-way orthogonal analysis of variance in the first experiment and two-way orthogonal analysis of variance in the second experiment with the use of Statistica 13 software (StatSoft). 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

Effect of the cytokinins on the multiplication rate and morphological features. The cytokinins used in the presented experiments had a significant effect on multiplication rate and morphological features of *Paphiopedilum insigne* cultivated in tissue culture (Tab. 1, Fig. 1).

The highest multiplication rate was obtained on the media supplemented with BA 0.5 mg·dm^{-3} (2.92) in comparison to KIN 2.5 or 5 mg·dm^{-3} (1.38 and

Table 1. Influence of cytokinin on the multiplication rate and morphological features of *Paphiopedilum insigne in vitro*

Treatment (mg·dm^{-3})	Multiplication rate	Number of leaves	Length of leaves (mm)	Width of leaves (mm)	Presence of roots (%)	Number of roots	Length of roots (mm)	Fresh weight of plants (mg)
BA 0.5	2.92 a*	3.12 bc	7.52 e	3.33 e	73 bc	3.06 a-c	6.26 d	171.22 b
BA 1	1.83 ab	4.09 a	11.14 bc	4.86 ab	88 bc	2.68 a-d	7.58 cd	225.79 ab
BA 2	1.72 ab	3.64 a-c	9.63 c-e	4.10 b-e	89 a	2.18 b-d	6.39 cd	139.60 b
TDZ 0.5	1.57 ab	3.21 a-c	12.10 a-c	4.47 b-d	83 bc	2.20 b-d	6.27 cd	207.32 ab
TDZ 1	1.91 ab	3.20 a-c	11.42 bc	4.14 b-e	78 bc	2.26 a-d	6.58 cd	162.58 b
TDZ 2	1.88 ab	3.75 ab	12.83 ab	3.99 c-e	69 bc	1.39 d	8.00 a-d	252.80 ab
KIN 1	1.53 ab	3.27 a-c	8.69 de	3.67 de	97 a	3.84 a	10.93 ab	246.54 ab
KIN 2.5	1.38 b	3.03 bc	10.77 b-d	5.08 ab	97 a	3.64 ab	9.01 a-d	256.24 ab
KIN 5	1.00 b	3.93 ab	14.47 a	5.79 a	100 a	2.97 a-c	9.20 a-c	399.33 a
BA 1 + TDZ 0.5	1.39 b	3.24 a-c	8.49 de	3.53 de	32 c	1.30 d	5.08 d	111.18 b
BA 1+ TDZ 2	1.90 ab	2.74 c	11.74 bc	4.28 b-d	74 bc	1.59 cd	5.91 d	270.02 ab
BA 1 + KIN 0.5	1.59 ab	3.29 a-c	10.82 b-d	4.53 b-d	59 bc	2.12 b-d	11.62 a	246.92 ab
BA 1 + KIN 5	1.48 ab	3.50 a-c	9.57 c-e	4.05 b-e	30 c	1.29 d	6.33 cd	131.31 b

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

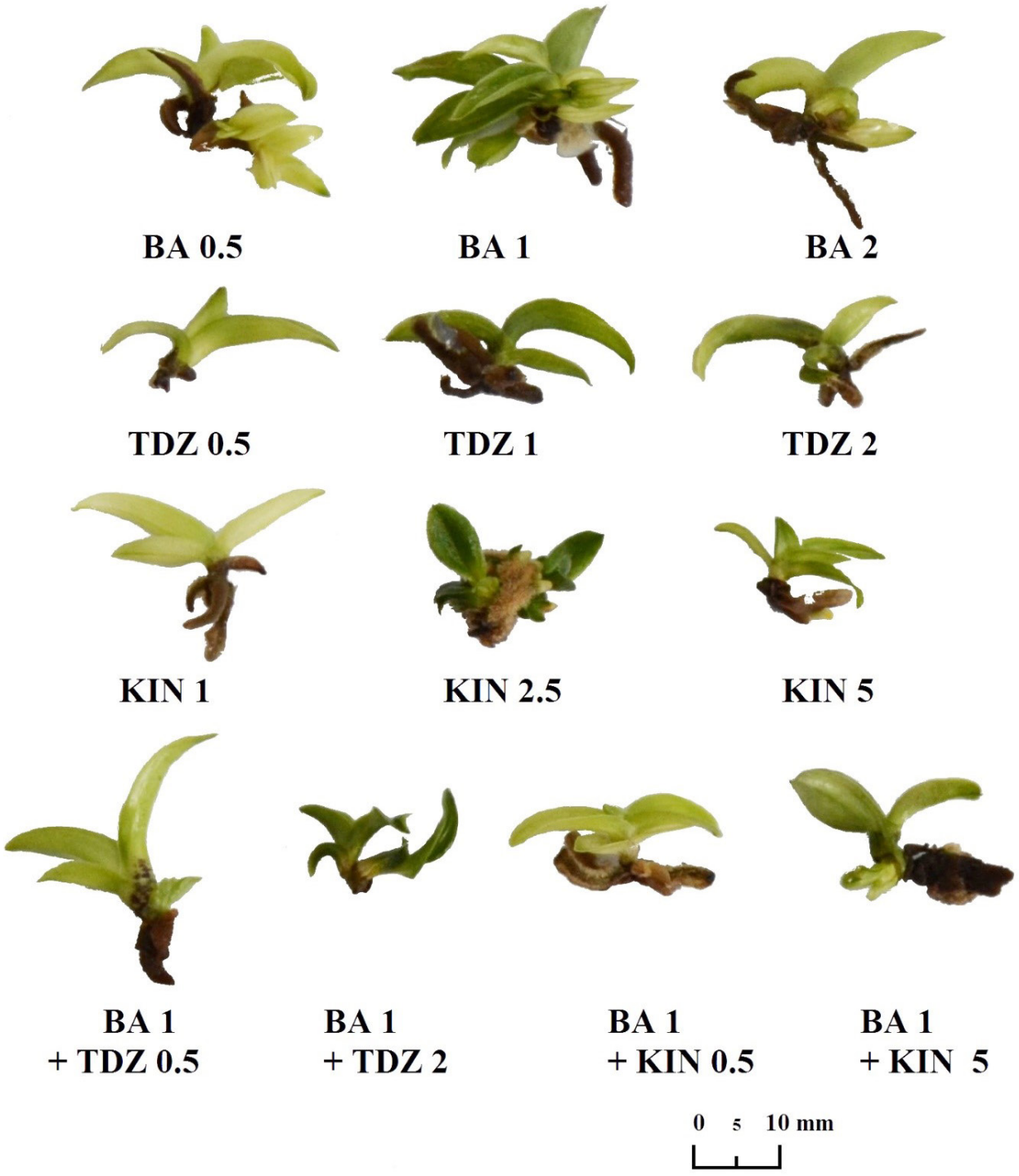

Fig. 1. Influence of cytokinins on growth and morphological features of *Paphiopedilum insigne in vitro*

1.0 respectively) and BA 1 mg·dm^{-3} + TDZ 0.5 mg·dm^{-3} (1.39). A tendency was observed that the use of BA in higher concentration lowered multiplication rate, what confirmed the research conducted by Jitsepakul et al. [2013]. The authors obtained the highest multiplication rate of *Vanda coeruela* in presence of BA, however the concentration above 3 mg·dm^{-3} reduced the number of new shoots. A positive effect of BA was also proved by Roy and Banerjee [2002] in case of *Geodorum densiflorum*, Talukder et al. [2003] in *Dendrobium* and Asghar et al. [2011] in *Dendrobium nobile* var. Emma White. On the other hand, Parthibhan et al. [2015] noted a disadvantageous effect of BA at concentration of 1, 3, 5, 7 or 10 mg·dm^{-3} on *Dendrobium aqueum* multiplication of shoots *in vitro*. According to Parthibhan et al. [2015], BA inhibits protocorms conversion into plants therefore it is not recommended to use high concentrations of this PGR. Another cytokinin often used in tissue culture is kinetin. Hong et al. [2008] observed the positive influence of KIN on micropropagation of *Paphiophedilum* 'Alma Gavaert', while Nisayan et al. [2010] noted a negative effect of KIN on multiplication of *Paphiopedilum* 'Delrosi'.

The cytokinins used in the research influenced the number of formed leaves, their length and width (Tab. 1, Fig. 1). It was noted that there were more leaves per rosette, when explants were cultivated in presence of BA 1 mg·dm^{-3} (4.09 pcs.), in comparison to BA 0.5 mg·dm^{-3}, KIN 2.5 mg·dm^{-3}, and BA 1 + TDZ 2 mg·dm^{-3} (3.12; 3.03 and 2.74 pcs. respectively). The leaves were the biggest in presence of KIN 5 mg·dm^{-3} (14.47 mm long and 5.79 mm wide), while the smallest ones were observed on the media supplemented with BA 0.5 mg·dm^{-3} (7.52 mm long and 3.33 mm wide). The positive effect of KIN on length of leaves was observed in the reserach of Roy and Banerjee [2002] on micropropagation of *Geodorum densiflorum* and Asghar et al. [2011] on *Dendrobium nobile* var. Emma White, while Talukder et al [2003] observed that leaves of *Dendrobium* were longer in presence of BA.

The cytokinins used in the research influenced rooting of *Paphiopedilum insigne* plantlets in tissue culture (Tab. 1). In presence of 5 mg·dm^{-3} KIN, 100% of rosettes formed roots. Higher percentage of rooting was noted also on media supplemented with KIN 1 or 2.5 mg·dm^{-3} (97%). A good rooting in presence of KIN was earlier observed by Parthibhan et al. [2015] in *Dendrobium aqueum*. The least rooted rosettes were noted in presence of BA 1 with KIN 5 mg·dm^{-3} (30%) or with TDZ 0.5 mg·dm^{-3} (32%) what confirmed the results observed by Wattanawikkit et al. [2011] who proved that in order to obtain rooting in *Paphiopedilum callosum* TDZ should be eliminated and the concentration of BA should be low. The positive effect of KIN on morphological features of *Paphiopedilum insigne* plantlets might result from its participation in accelerating cells and increasing their volume [Rogozińska 1969].

The number of roots was also dependent on the cytokinins used. The most roots were obtained on the media supplemented with KIN 1 mg·dm^{-3} (3.84). Similar number was noted in presence of BA 0.5 and 1 mg·dm^{-3} (3.06 and 2.68 respectively), TDZ 1 mg·dm^{-3} (2.26), KIN 2.5 and 5 mg·dm^{-3} (3.64 and 2.97). Similar results were noted in case of *Dactylorhiza hatagiera* [Warghat et al. 2014] and *Paphiopedilum* 'Alma Gavaert' [Hong et al. 2008]. The negative effect of BA on rooting was proved by Parthibhan et al. [2015] on *Dendrobium aqueum.* In the presented research low amount of BA had a positive effect on number of roots formed what confirms the results obtained by Talukder at al. [2003] who noted the biggest number of roots of *Dendrobium* in presence of BA.

The cytokinins influenced the fresh weight of plantlets. Rosettes with the highest fresh weight were obtained on the media supplemented with 5 mg·dm^{-3} of KIN in comparison to BA in concentration of 0.5 or 2 mg·dm^{-3}, TDZ 1 mg·dm^{-3} and BA 1 used together with 0.5 mg·dm^{-3} TDZ or 5 mg·dm^{-3} KIN. It was also noted that the increase of the KIN concentration increeased the fresh weight of plantlets as well. A positive effect of KIN on the studied feature was proved by Ket et al. [2004] on *Anoectochilus formosanus*, while Asghar et al. [2011] obtained a high fresh weight of *Dendrobium nobile* 'Emma White' plantlets *in vitro* in presence of 1-2 mg·dm^{-3} BA.

Effect of media medium type, PGRs and natural additives on the multiplication rate and morphological features. The content of macro- and micronutrients as well as addition of growth regulators and natural origin additives had a significant effect on growth and multiplication of *Paphiophedilum insigne* plants *in vitro*.

The regeneration rate was the highest when BP at concentration of 30 g·dm^{-3} was added to the VW medi-

Table 2. Influence medium type, PGRs and biological supplements on the number of regenerated microcuttings and multiplication rate of *Paphiopedilum insigne in vitro*

Medium type	Supplements	Regeneration			
		regeneration rate (%)	mean	multiplication rate	mean
½ MS	5 mg·dm^{-3} KIN + 1 mg·dm^{-3} BA	81	69	1.05 c	1.31 A
	1 mg·dm^{-3} BA + 2 mg·dm^{-3} TDZ	75		1.02 c	
	1 g·dm^{-3} CH + 1 mg·dm^{-3} BA	67		1.21 bc	
	1 g·dm^{-3} CH	88		1.73 a-c	
	CW 15%	78		1.10 c	
	CW 20%	46		1.98 ab	
	BP 20 g·dm^{-3}	6		1.13 bc	
	BP 30 g·dm^{-3}	63		1.41 bc	
VW	5 mg·dm^{-3} KIN + 1 mg·dm^{-3} BA	85	65	1.05 c	1.41 A
	1 mg·dm^{-3} BA + 2 mg·dm^{-3} TDZ	62		1.07 c	
	1 g·dm^{-3} CH + 1 mg·dm^{-3} BA	35		1.18 bc	
	1 g·dm^{-3} CH	52		2.47 a	
	CW 15%	72		1.60 bc	
	CW 20%	53		1.31 bc	
	BP 20 g·dm^{-3}	73		1.42 bc	
	BP 30 g·dm^{-3}	93		1.12 bc	

* means followed by the same letter do not differ significantly at p = 0.05

um (93%) (Tab. 2). A lot of regenerated plantlets were also obtained in presence of 1 g·dm^{-3} of casein hydrolysate (88%) on the 1/2 MS medium or 5 mg·dm^{-3} KIN + 1 mg·dm^{-3} BA on the VW medium (85%). The least regenerated plantlets were observed when casein hydrolysate in combination with BA were added to the VW medium (35%).

The highest multiplication rate was obtained when explants were cultivated in presence of 1 g·dm^{-3} of CH on the VW (2.47) or 1/2 MS (1.73) medium and on 1/2 MS with addition of 20% coconut water (1.98) (Tab. 2, Fig. 2). In case of Orchideaceae family, natural additives were more often observed to have a negative effect on propagation *in vitro*. The negative effect of 1/2 MS media with CW on multiplication of *Dendrobium aqueum* shoots was noticed by Parthibhan et al. [2015]. Zhang et al. [2013] reported that CW limited the multiplication of *Cypripedium macranthos*. The negative effect of 20 or 30% coconut water on *Phalaenopsis violacea* regeneration was shown by Gnasekaran et al. [2010], however with the use of lower concentration, 10%, it was positive. Nambiar et al. [2012] also proved that CW added to the MS medium in concentration of 30% limited regeneration of *Dendrobium* 'Alaya Pink' PLBs. Similar effect was proved on many other species, like *Phalaenopsis violaceae* [Gnasekaran et al. 2010] or *Dendrobium* 'Alaya Pink' [Nambiar et al. 2012].

The positive effect of casein hydrolysate in concentration of 1 g·dm^{-3} observed in the presented research (Tables 2 and 3), confirmed the studies conducted by Sinha and Roy [2004] on *Vanda teres*. The authors reported that the use of that substance to supplementation of the medium significantly influenced the elongation of plants.

There was no difference in multiplication rate, when the types of media were compared only. VW medium is commonly used in cultivation of orchids in tissue culture, for example it was used for micropropagation of *Vanda teres* [Sinha and Roy 2004], *Van-*

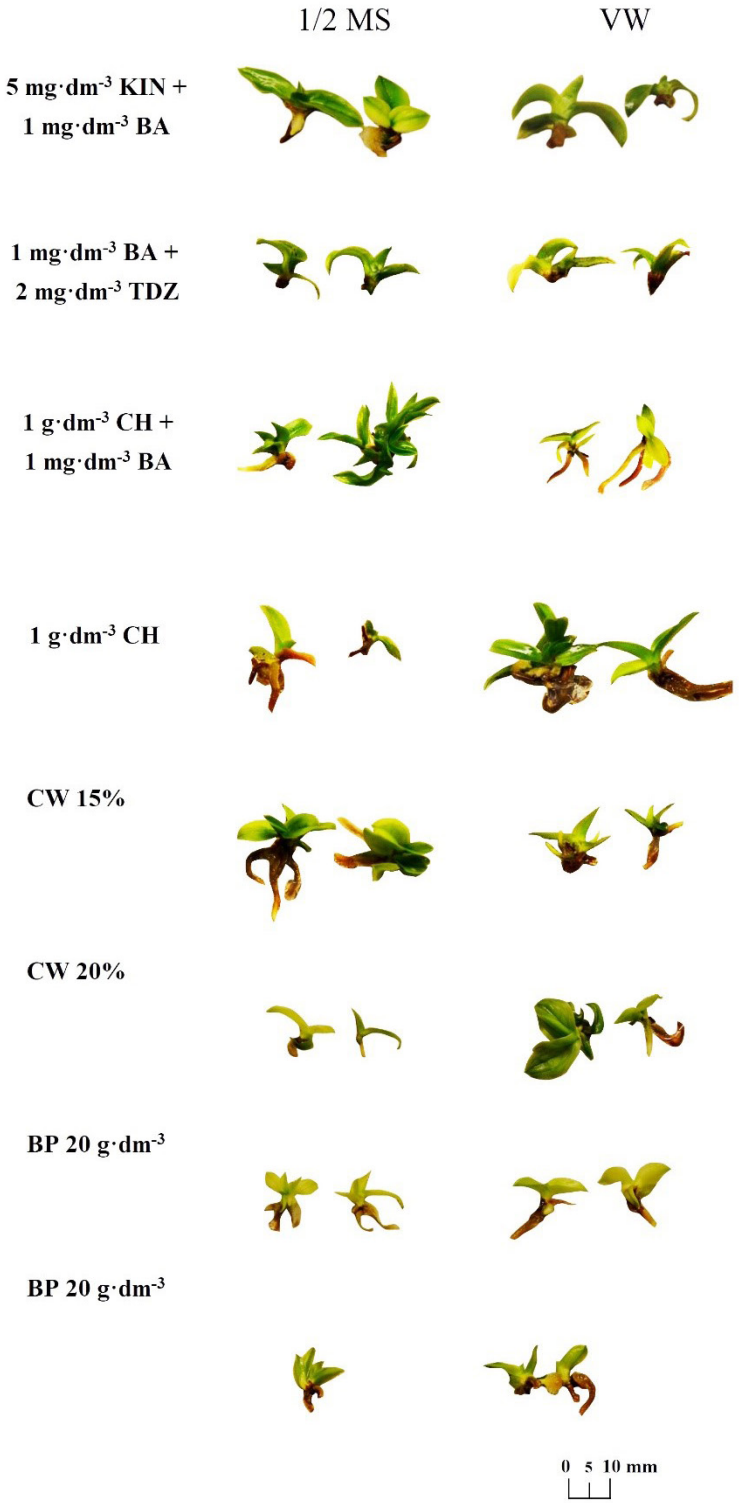

Fig. 2. Influence of the medium type, growth regulators and natural additives on growth and morphological features of *Paphiopedilum insigne in vitro*

da coerulea [Jitsepakul et al. 2013], *Paphiopedilum wardii* [Zeng et al. 2016], however the MS medium is less labor intensive and cheaper to prepare.

The use of growth regulators or natural active substances as media supplementation had an influence on morphological features of *Paphiopedilum insigne* in the presented research (Tables 3 and 4). *Paphiopedilum* plantlets with a higher number of leaves were obtained on 1/2 MS supplemented with 30 g·dm^{-3} of banana pulp (3.85 pcs.) in comparison to VW medium with the addition of 20% coconut water (2.5 pcs.). The positive effect of BP confirmed studies conducted by Zeng et al. [2012] who observed that the addition of banana homogenate added in concentration of 100 g·dm^{-3} to the VW medium increased the number of leaves on *Paphiopedilum wardii*. On the other hand, Lo et al. [2004] underlined the positive effect of MS medium supplemented with coconut water on the number of *Dendrobium tosaense* leaves. Aktar et al. [2008] stated that a positive effect of banana pulp resulted from a large content of carbohydrates.

In the presented study it was noted, that the type of media and substances used for supplementation influenced the height and width of the *Paphiopedilum insigne* rosettes (Tab. 3). The highest leaf rosettes were obtained on ½ MS media with 1 g·dm^{-3} of CH (11.95 mm) and it was also observed that 1/2 MS medium was better than VW in relation to the size of plants. The positive effect of casein hydrolysate on elongation of *Vanda teres* shoots was observed by Sinha and Roy [2004]. In the presented experiment, the BP in higher concentration positively affected the height of rosettes, what confirmed the results obtained by Zhang et al. [2013] on *Cypripedium macranthos*. On the other hand, a negative effect of 200 g·dm^{-3} of banana homogenate on leaves of *Paphiopedilum wardii* length was showed by Zeng et al. [2012].

The influence of the type of the medium type, cytokinins and natural biological substances used in the

Table 3. Morphological features of the *Paphiopedilum insigne* rosettes in *in vitro* cultures depending on the medium type and addition of PGRs and biologically active components

Medium type	Supplements	Rossettes features					
		number of leaves	mean	height (mm)	mean	width (mm)	mean
½ MS	5 mg·dm^{-3} KIN + 1 mg·dm^{-3} BA	2.85 ab*	3.14 A	8.30 a-c	9.44 A	12.80 a-c	14.31 A
	1 mg·dm^{-3} BA + 2 mg·dm^{-3} TDZ	3.00 ab		8.35 a-c		12.15 bc	
	1 g·dm^{-3} CH + 1 mg·dm^{-3} BA	3.45 ab		10.25 a-c		14.55 a-c	
	1 g·dm^{-3} CH	2.75 ab		11.95 a		16.35 ab	
	CW 15%	2.80 ab		10.60 a-c		18.40 a	
	CW 20%	2.75 ab		9.70 a-c		12.55 a-c	
	BP 20 g·dm^{-3}	3.65 ab		7.40 c		13.30 a-c	
	BP 30 g·dm^{-3}	3.85 a		9.00 a-c		14.40 a-c	
VW	5 mg·dm^{-3} KIN + 1 mg·dm^{-3} BA	3.15 ab	3.03 A	7.55 c	8.09 B	13.10 a-c	12.69 B
	1 mg·dm^{-3} BA + 2 mg·dm^{-3} TDZ	3.00 ab		8.35 a-c		12.15 bc	
	1 g·dm^{-3} CH + 1 mg·dm^{-3} BA	3.60 ab		7.80 bc		13.70 a-c	
	1 g·dm^{-3} CH	2.95 ab		7.90 bc		13.10 a-c	
	CW 15%	2.80 ab		7.80 bc		13.25 a-c	
	CW 20%	2.50 b		7.05 c		10.25 c	
	BP 20 g·dm^{-3}	2.75 ab		7.60 c		13.15 a-c	
	BP 30 g·dm^{-3}	3.30 ab		11.60 ab		14.85 a-c	

* means followed by the same letter do not differ significantly at p = 0.05

Table 4. Morphological features of the *Paphiopedilum insigne* rooting systems in *in vitro* cultures depending on the medium typeand addition of PGRs and biologically active components

Medium type	Supplements	Rooting features					
		Presence of roots (%)	Mean	Number of roots	Mean	Length of roots (mm)	Mean
½ MS	5 mg·dm^{-3} KIN + 1 mg·dm^{-3} BA	65 bc*	75.62 A	1.31 b	1.95 A	2.92 c	7.35 B
	1 mg·dm^{-3} BA + 2 mg·dm^{-3} TDZ	5 d		1.00 b		2.00 c	
	1 g·dm^{-3} CH + 1 mg·dm^{-3} BA	95 ab		1.53 ab		5.58 bc	
	1 g·dm^{-3} CH	85 a-c		2.41 ab		8.11 a-c	
	CW 15%	100 a		2.20 ab		11.85 a	
	CW 20%	60 c		1.25 b		6.08 a-c	
	BP 20 g·dm^{-3}	95 ab		2.16 ab		6.79 a-c	
	BP 30 g·dm^{-3}	100 a		2.40 ab		8.30 a-c	
VW	5 mg·dm^{-3} KIN + 1 mg·dm^{-3} BA	80 a-c	76.25 A	1.44 ab	1.98 A	4.25 bc	12.69 A
	1 mg·dm^{-3} BA + 2 mg·dm^{-3} TDZ	5 d		1.00 b		2.00 c	
	1 g·dm^{-3} CH + 1 mg·dm^{-3} BA	80 a-c		1.88 ab		10.13 ab	
	1 g·dm^{-3} CH	100 a		2.20 ab		8.70 a-c	
	CW 15%	90 a-c		2.00 ab		10.56 ab	
	CW 20%	65 bc		1.31 b		7.31 a-c	
	BP 20 g·dm^{-3}	95 a-b		2.47 a		7.16 a-c	
	BP 30 g·dm^{-3}	100 a		2.15 a-c		9.9 ab	

* means followed by the same letter do not differ significantly at p = 0.05

experiment on the rooting system of *Paphiopedilum insigne* plantlets is presented in Table 4. It was noted that the use of cytokinins only limited rooting of explantsand morphological parameters of roots, what was shown by Chugh et al. [2009] or Bektaş and Sökman [2016]. The number of roots was lower when the medium was supplemented with 20% of CW. In order to obtain plantlets with a good quality rooting system, it is advisable to supplement the medium with 15% CW, 20 or 30 g·dm^{-3} of BP.

CONCLUSIONS

The influence of the medium type, PGRs and natural biologically active media supplements on growth and development of *Paphiopedilum insigne* explants *in vitro* is proven. BA in concentration of 0.5 mg·dm^{-3} allowed to obtain the highest multiplication rate, however the use of KIN in concentration of 1 mg·dm^{-3} resulted in formation of plantlets of higher quality, therefore it might be advised to use the latter one before rooting or acclimatization of the plantlets. It is possible to replace cytokinins with other biologically active substance, such as banana pulp, especially before rooting of explants. It is advised to use 1/2 MS medium for *Paphiopedilum insigne* tissue culture, as there was no difference in terms of multiplication rate and the obtained plantlets were of better quality. 1/2 MS media is also superior in terms of cost and amount of work.

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