FOLIA POMERANAE UNIVERSITATIS TECHNOLOGIAE STETINENSIS
Folia Pomer. Univ. Technol. Stetin. 2012, Agric., Aliment., Pisc., Zootech. 293 (21), 51–60

Danuta KULPA, Joanna KATROŃ

SEED GERMINATION AND PLANT DEVELOPMENT OF *BLETILLA STRIATA IN VITRO*

KIEŁKOWANIE NASION I ROZWÓJ ROŚLIN *BLETILLA STRIATA* W KULTURACH *IN VITRO*

Department of Plant Genetic, Breeding and Biotechnology, West-Pomeranian University of Technology, ul. Janosika 8, 71-424 Szczecin, Poland, e-mail: danuta.kulpa@zut.edu.pl

Streszczenie. Celem badań było opracowanie metody kiełkowania nasion *in vitro* i regeneracji chińskiej rośliny leczniczej – bletilii pasiastej (*Bletilla striata)*. Stwierdzono, że do kiełkowania nasion tego storczyka w kulturach *in vitro* najbardziej odpowiednia jest pożywka Knudson "C" (Knudson 1946) bez dodatku roślinnych regulatorów wzrostu. Kiełkujące w kulturach *in vitro* siewki winny być regenerowane na podłożu według Knudsona (1946) z dodatkiem 0,2 mg · dm^{-3} NAA. Zregenerowane rośliny, wysadzone w podłoże firmy Hollas, są zdolne do wzrostu w warunkach szklarniowych.

Słowa kluczowe: mikrorozmnażanie, roślinne regulatory wzrostu, rośliny lecznicze, storczyki.
Kay words: micropropagation, medicinal plant, plant growth regulators, orchids.

INTRODUCTION

Progressive degradation of natural environment and dynamic changes in habitat conditions cause dying out of many orchid species, threatening them with extinction. Legal protection of orchids is not satisfactory and conventional method of reproduction has also proved to be unsatisfactory. One of distinguished representatives of orchid family is *Bletilla striata* (Thunb.) Reichb. f., originated from the temparate zone of east Asia occurring on the area from Tibet, China to Japan (Brown 2005, Chung and Chung 2005). They are perennials with attractive flowers and delicate honey scent which adapt easily to Polish climatic conditions. Protected with the mulch of dried leaves or polyethylene may overwinter in the ground (Kukułczanka and Gracz-Nalepka 1984).

This species is traditionally used in Chinese medicine because of its medicinal properties. The compounds isolated from underground corms are applied to treat the damage of mucuous membrane of alimentary canal, ulcers, bruises and burns (Li et al. 2005, Wang et al. 2006, Gutiérrez 2010). Currently there is a growing interest in this species connected with promising studies on the usefulness of this plant extract for the treatment of breast or liver cancer (Qian et al. 2003, Lee et al. 2009).

Seed germination and protocorm development in orchids under natural conditions is a complicated process since they belong to slow-growing plants and the process depends on the presence of mycorrhizal fungi (Stoutamire 1974, Godo et al. 2010). The method which seems to be helpful both for mass propagation of plant tissues and their long-term storage is *in vitro* culture. It allows to obtain even several million plantlets, genetically identical with mother plant and the material obtained by this method is characterised by perfect health. In literature there are many publications on orchid seed germination and their shoot proliferation *in vitro*, however, seed germination *in vitro* of terrestrial orchid species has been considered to be more difficult than that of epiphytic species (Arditti and Ernst 1993). So far there has been no accord among authors as to the optimum composition of the medium for the germination and regeneration of mature seeds of *Bletillia striata*. The only reports concern *in vitro* germination of immature seeds *with* different time passed after pollination for storage purposes in liquid nitrogen. Therefore, the aim of this paper was to determine the effects of medium mineral composition and plant growth regulators on germination and development of *Bletilla striata* Rchb. f. in cultures *in vitro*.

MATERIAL AND METHODS

Culture initiation. Study material consisted of mature closed pods, disinfected by immersion for 5 secs in 70% ethyl alcohol and run over burner flame several times. Next seeds were removed under sterile conditions and placed on sterile paper which was rolled and immersed for 5 secs in 0.2% solution of mercuric chloride ($HgCl_2$). The paper with the seeds was rinsed 4 times in sterile water. After that the seeds were put on the media: Kundson C (Knudson 1946) and MS (Murashige and Skoog 1962) with the addition of 0.5, 1.0 and 2.0 mg · dm^{-3} kinetin (KIN). Plant growth regulators were added to the media before their sterilisation and next their pH was adjusted to 5.4 by 0.1 M solutions of NaOH and HCl. The solutions were supplemented with 15 g · dm^{-3} agar and 20 g · dm^{-3} sucrose The media were poured into 50-ml Erlenmayer flasks – 20 ml per each flask and autoclaved at 121°C for 20 minutes.

Control in this experiment was the medium according to Knudson, without plant growth regulators. About 25 seeds were placed in each flask – ca 250 seeds per each medium.

During all stages of experiment glass containers with plants were placed in a growth chamber at temperature 23–24°C, light intensity 40 PAR (μE · m^{-2} · s^{-1}) and 16h photoperiod. Three months after sowing percentages of germinated plants on each medium were determined.

Plant development. Germinating seeds, obtained from the first stage of experiment were transferred onto the media with macro- and micro-elements according to Knudson (1946), supplemented with indole-3-butrytic acid (IBA), α-naphthaleneacetic acid (NAA), kinetin (KIN) and 6-benzylaminopurine (BAP) at the concentration 0.2, 0.5, 0.75 and 1.0 mg · dm^{-3} (Table 2).

Seedling cultures were placed in 50-ml Erlenmayer flasks. The media were prepared as in previous stages. Each flask contained from 3 to 5 plantlets. Three months later morphological traits of the plants were measured: height, weight, the number and length of leaves and the number and length of roots.

***Adaptation to in vivo conditions*.** Adaptation stage was conducted in a growth chamber under identical conditions as in previous stages of development. The plants regenerated on the control medium and the medium with NAA (0.2, 0.5, 0.75 $mg \cdot dm^{-3}$) were transplanted into multiplets filled with the medium for sowing and pricking out from the firm Hollas and the medium for cacti from the firm Kronen. Both the media were disinfected by autoclaving. The plants were planted, one plant per hole, 15 plants per combination.

Statistical analysis. Obtained results were subjected to variance analysis employing a completely randomized design. Mean values for the examined plant traits were compared by means of the Tukey test.

RESULTS AND DISCUSSION

According to Oszkinis (1993), only a few orchid species may germinate and initiate the growth of protocorms without the presence of a particular symbiotic fungus. Such are the species of the genera *Bletilla* and *Sobralia*, in which cotyledon grows out from a germinating seed. This is confirmed by our studies in which seeds started germination under laboratory conditions within six days after sowing. Seed disinfection by immersion for 5 seconds in 0.2% solution of mercuric chloride ($HgCl_2$) proved to be an effective treatment - infections were found only in 3% of cultures.

All authors emphasise an immense influence of media mineral composition on plant development in cultures *in vitro*. The majorities of orchids germinate well on the media with reduced content of minerals but supplemented with natural additives such as banana flesh, coconut milk or tomato juice. Dutra et al. (2009) for the germination of *Cyrtopodium punctatum* used 5 different media compositions: PhytoTechnology Orchid Seed Sowing Medium, Knudson C, Malmgren Modified Terrestrial Orchid Medium, Vacin & Went Modified Orchid Medium, and half–strength Murashige & Skoog medium. They considered Knudson C medium to be the best for germinating the seeds of that species. Vasudevan and Van Staden (2010) germinated the seeds of *Ansellia africana* Lindl used four kinds of media: MS, its modification – 1/2 MS, Phytotechnology medium (P668) and modified Knudson's C. The most effective of them were: P668 and modified Knudson C. Hirano et al. (2005) and Jitsopakul et al. (2008) germinated immature seeds of *Bletilla striata*, before their vitrification on the medium ND – New Dogashima (Tokuhara and Mii 1993). They found the beneficial impact of its mineral composition on plant development but plant observation period was limited to 6 days. In our studies to germinate fully mature seeds of *Bletilla striata* the media Knudson C (Knudson 1946) and Murashige and Skoog (1962) were used. The medium Knudson C proved to be highly useful for germinating the seeds of this orchid – irrespective of the applied kinetin concentration whereas no germination occurred on the another medium – MS (Fig. 1).

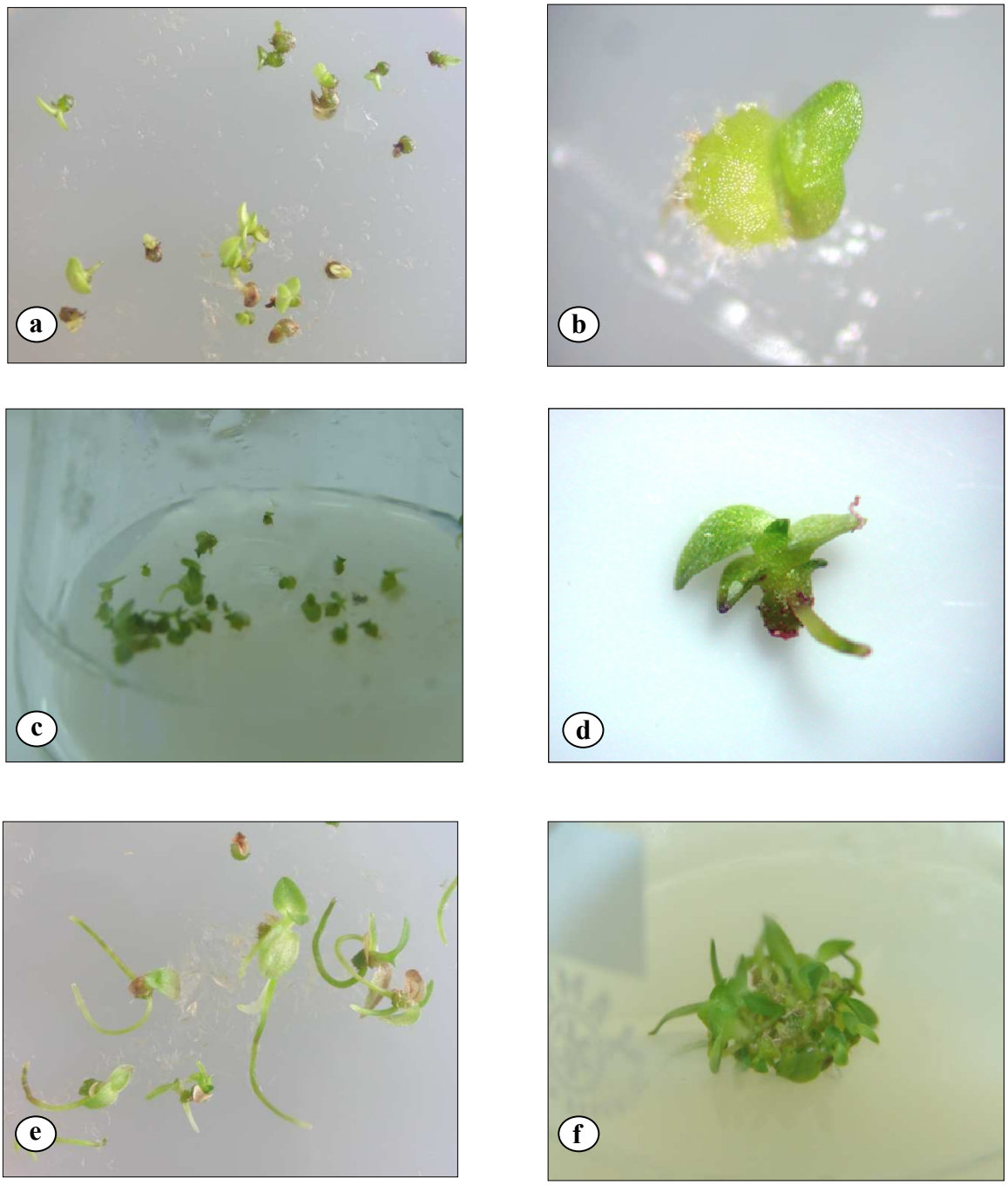

Fig. 1. Germination of mature seeds of *Bletilla striata* on Knudson C medium without plant growth regulators, photographed 2 months (a, b) and 3 months (c, d) after sowing. Seedlings developed on medium supplemented with 0.2 mg · dm^{-1} NAA (e) and 0.2 BAP (f)

Rys. 1. Kiełkowanie dojrzałych nasion *Bletillia striata* na podłożu Knudson C bez dodatku roślinnych regulatorów wzrostu po 2 miesiącach (a, b) i 3 miesiącach (c, d) od momentu wysiewu. Siewki rozwijające się na podłożu uzupełnionym 0,2 mg · dm^{-1} NAA (e) i 0,2 BAP (f)

In the majority of cases growth regulators are not added at the first stage of seed germination. However, certain species require the media to be enriched with them especially with cytokinin (Shimura and Koda 2004, Kishor et al. 2006, Stewart and Kane 2006). Orchid germination was also the object of studies of Hadley and Harvais (1968). They found that

IAA and kinetin and GA_3 applied exclusively or in combinations had a considerable morphogenic effect on development of *Dactylorhiza purpurella* protocorms. Hadley (1970), studying the same species, found that kinetin at the dose from 1.0 to 10.0 mg · dm^{-3} added with or without IAA favorably affected protocorm development at the stage of leaf formation. At the same time he noticed that symbiotic cultures of *Dactylorhiza majalis* protocorms did not require exogenic phytohormones and their development was faster and better than those from the media without fungi.

In our studies the medium Knudson C with the addition of kinetin in the amount from 0.5 to 2.0 mg · dm^3 was used for germination stage The addition of this growth regulator was found to inhibit germination – the best germination was observed when the seeds were placed on Knudson C without plant growth regulators (Table 1). The percentage of germinated seeds from that medium was 89% whereas only 49% on the medium with 2.0 mg · dm^{-3} KIN. It was also noted that the plants from the media without growth regulators produced a larger number of longer leaves and the more numerous and longer roots (unpublished data).

Table 1. Seed germination frequency (%) of *Bletilli striata* 'Rose' depending on applied kinetin content
Tabela 1. Częstotliwość kiełkowania nasion *Bletilla striata* 'Rose' w zależności od zawartości kinetyny

Kinetin content Zawartość kinetyny (mg · dm^{-3})	Seed germination frequency Częstotliwość kiełkowania nasion (%)	Number of leaves Liczba liści	Number of roots Liczba korzeni
	Knudson C		
0.0 – control – kontrola	89.00	3.0 a	1.5 a
0.5	76.76	2.0 b	0.3 c
1.0	74.50	3.0 a	1.1 b,c
2.0	49.00	2.1 b	0.4 c
	MS		
0.0 – control – kontrola	lack of germination brak kiełkowania		
0.5			
1.0			
2.0			

[a] Values, in the same column, followed by the same letter are not significantly different at the 0.05 level according to Tukey's test.
[a] Wartości w kolumnach oznaczone tymi samymi literami nie różnią się istotnie według testu Tukeya na poziomie istotności 0,05.

Nutritional requirements, thus media composition should change with the development of orchid seedlings. In the studies of Pedroza-Manrique et al. (2005) on successive stages of orchid cultures i.e. protocorm and seedling stage of the genus *Comparettia* falcate reaction to applied growth regulators varied. Ochowicz (1998) claims, that the stronger effect of applied regulators in the case of protocorms may be probably attributed to gradual changes in metabolism along with progressive organogenesis. Tay and Takeno (1988), examining germination and further development in *Paphiopedilum*, also stressed the need for separate studies on particular developmental stages taking into consideration varying requirements of germinating protocorm seeds and seedlings. According to Ochowicz (1998) in the case of the

species *Liparis loeselii* BAP, with or without NAA, stunted root development but its higher doses stimulated the formation of adventitious shoots. The research on *Dactylorhiza fuschii* shows an inhibiting effect of cytokinins on seedling rooting and promoting effect of higher doses on the initiation of adventitious buds on protocorms and seedlings (Arczewska 1993). Plant reaction to cytokinins to a great extent depends on applied dose.

We have also observed that the plants from the media enriched with cytokinins produced a small number of short roots whereas low concentration of BAP had a positive influence on other morphological traits (Table 2). *Bletilla* plants growing on the media supplemented with 0.2 mg · dm^{-3} BAP were tall, produced numerous and quite large leaves and had a high weight. On that medium adventitious shoot formation was observed in the case of certain part of seedlings. Kinetin (KIN) application did not result in such satisfactory results. The plants regenerated on the media with growth regulatorsat the highest dose, died.

Table 2. Values of morphological treats of *Bletilla* seedlings regenerated on media with various PGRs content

Tabela 2. Wartości cech morfologicznych siewek *Bletilla* regenerowanych na pożywkach o zróżnicowanej zawartości roślinnych regulatorów wzrostu

Plant growth regulators Roślinne regulatory wzrostu (mg · dm^{-3})		Mass Masa(g)	Plant height Wysokość roślin (mm)	Length of root Długość korzenia (mm)	Number of roots Liczba korzeni	Number of leaves Liczba liści
Control – 0.00 – Kontrola		0.040 a	1.50 b	0.56 b	1.00 b	1.56 b
IBA	0.20	0.005 b	2.50 b	0.00 b	0.66 a	3.00 a
	0.50	0.008 b	4.66 b	3.66 a	1.66 a	3.00 a
	0.75	plants got brown and died rośliny zbrązowiałe i zamarłe				
	1.00					
NAA	0.20	0.074 a	12.33 a	7.00 a	3.00 a	5.66 a
	0.50	0.065 a	8.00 a	3.66 a	1.33 a	4.00 a
	0.75	0.008 a	5.00 b	3.33 a	1.33 a	2.30 a
	1.00	plants got brown and died – rośliny zbrązowiałe i zamarłe				
BAP	0.20	0.086 a	9.33 a	6.00 a	2.66 a	4.30 a
	0.50	0.068 a	4.33 b	3.00 a	2.00 a	4.33 a
	0.75	0.041 a	3.66 b	2.00 a	0.66 a	3.00 ab
	1.00	plants got brown and died – rośliny zbrązowiałe i zamarłe				
KIN	0.20	0.005 b	2.66 b	3.66 a	1.66 a	1.33 b
	0.50	0.008 b	3.33 b	3.00 a	1.00 a	2.33 b
	0.75	plants got brown and died rośliny zbrązowiałe i zamarłe				
	1.00					

[a] Values, in the same column, followed by the same letter are not significantly different at the 0.05 level according to Tukey's test.

[a] Wartości w kolumnach oznaczone tymi samymi literami nie różnią się istotnie według testu Tukeya na poziomie istotności 0,05.

In the course of adaptation stage, plants regenerated *in vitro*, at low light intensity, constant supply of water, minerals and sucrose, must start assimilation and adapt to changeable more severe growth conditions. Oszkinis (1993) recommended hardening plants from *in vitro* cultures before transplanting into permanent medium. She gave two possible

ways of hardening plants to unfavourable atmospheric conditions. In the first one vessels with plants were exposed to daylight in a glasshouse. After a few days lids were removed and the vessels were left open. In the second one 1 dm^{-3} – 'twist' jars were filled with sterile media to 1/3, under sterile conditions, and the plants taken out of vessels were placed into the jars. When they grew almost to the top of jar, lids were removed for a few days and next the plants with earth were transplanted into normal containers. Kukułczanka and Gracz-Nalepka (1984) planted *Bletilla* in a clay soil with pH about 6.0, enriched with leaf-litter earth. Below the soil there was a pile of rubble providing the plants with good drainage to remove water excess. However, her studies focused on mature, adult plants.

In our studies in order to prepare plants for *in vivo* conditions first they were transferred from jars into disinfected multiplets filled with two kinds of sterile media – the medium for sowing from the firm Hollas and the medium for cacti from the firm Kroonen (Table 3). On the basis of observations it was found that the plants growing in peat from the firm Hollas adapted to growth with a considerably higher frequency than those rooted on the medium for cacti which was probably caused by faster drying out of cacti media and dying of poorly rooted plants.

Table 3. Frequency of plants adapted to growth in vivo two weeks after planting, depending on the content of auxin in the medium at the stage of development
Tabela 3. Częstotliwość adaptacji roślin w warunkach *in vivo* 2 tygodnie po wysadzeniu, w zależności od zawartości auksyn w pożywce na etapie regeneracji

NAA content ($mg \cdot dm^{-3}$) Zawartość NAA ($mg \cdot dm^{-3}$)	Frequency of plants adapted to growth *in vivo* (%) Częstotliwość adaptacji roślin do warunków *in vivo* (%)	
	medium Hollas – podłoże Hollas	medium Kronen – podłoże Kronen
0.0 – control – kontrola	86.7	26.7
0.20	33.3	33.3
0.50	26.7	20.0
0.75	0.0	0.0

CONCLUSIONS

The developed method is efficient and allows to obtain a large number of *Bletilla striata* plants, in a very short time. Orchids germinated in axenic cultures may be planted and introduced into natural conditions or be used as maternal material for *in vitro* plant or tissue propagation.

REFERENCES

Arczewska A. 1993. Wstępne stadia rozwojowe storczyków europejskich w kulturze zachowawczej *in vitro*. Acta Univ. Wratisl. 1515, 215–221.

Arditti J., Ernst R. 1993. Micropropagation of orchids. John Wiley and Sons, New York.

Brown P.M. 2005. Wild orchids of Florida, expanded and updated edition. Univ. Press Florida, Gainesville, Florida.

Chung M.Y., Chung M.G. 2005. Pollination biology and breeding systems in the terrestrial orchid *Bletilla striata.* Plant. Syst. Evol. 252, 1–9.

Dutra D., Kane M.E., Richardson L. 2009. Asymbiotic seed germination and *in vitro* seedling development of *Cyrtopodium punctatum*: a propagation protocol for an endangered Florida native orchid. Plant. Cell. Tiss. Organ. Cult. 96, 235–243.

Godo T, Komori M, Nakaoki E, Yukawa T, Miyoshi K. 2010. Germination of mature seeds of *Calanthe tricarinata* Lindl., an endangered terrestrial orchid, by asymbiotic culture *in vitro*. In Vitro Cell. Dev. Biol. – Plant 46, 323–328.

Gutiérrez R.M.P. 2010. Orchids: A review of uses in traditional medicine, its phytochemistry and pharmacology. J. Med. Plant. Res. 4 (8), 592–638.

Hadley G. 1970. The interaction of kinetin, auxin and other factors in the development of nort temperate orchids, New. Phytol. 68, 549–555.

Hadley G., Harvais G. 1968. The effects of certain growth substances on asymbiotic germination and development of *Orchis purpurella*. New. Phytol. 67, 441–445.

Hirano T., Godo T, Mii M., Ishikawa K. 2005. Cryopreservation of immature seeds of *Bletilla striata* by vitrification. Plant. Cell Rep. 23: 534–539.

Jitsopakul N., Thammasiri K., Ishikawa K. 2008. Cryopreservation of *Bletilla striata* mature seeds, 3-day germinating seeds and protocorms by droplet-vitrification. Cryo-Letters. 29 (6), 517–526.

Kishor R., Sha Valli Khan P.S., Sharma G.J. 2006. Hybridization and *in vitro* culture of an orchid hybrid Ascocenda 'Kangla'. Sci. Hortic. 108: 66–73.

Knudson L. 1946. A new nutrient solution for the germination of orchid seeds. Am. Orchid. Soc. Biull. 15, 214–217.

Kukulczanka K., Gracz-Nalepka M. 1984. Aklimatyzacja *Bletilla striata* Rchb. f. w Ogrodzie Botanicznym we Wrocławiu. Wiad. Bot. 28 (1), 83–86 .

Lee H.J., Lee H.J., Lee E.O., Lee J.H., Lee K.S., Kim K.H., Kim S.H., Lü J. 2009. *In vivo* anti-cancer activity of Korean Angelica gigas and its major pyranocoumarin decursin. Am. J. Chin. Med. 37 (1), 127–142.

Li Y., Xu C., Zhang Q., Liu J.Y., Tan R.X. 2005. *In vitro* anti-Helicobacter pylori action of 30 Chinese –herbal medicines used to treat ulcer diseases. J. Ethnopharm. 98 (3), 329–333.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15, 473–497.

Ochowicz W. 1998. Wpływ niektórych egzogennych regulatorów wzrostu na wzrost i rozwój *Liparis loeselii* (L.) Rich. Act. Univ. Wratisl. Prac. Bot. 2038 (76), 53–61.

Oszkinis K. 1993. Zagadnienia związane z procesem mikoryzy u storczyków. Hod. Rośl. Nasienn. 5, 17–21.

Pedroza-Manrique J., Fernandez-Lizarazo Ch., Suarez-Silva A. 2005. Evaluation of the effect of three growth regulators in the germination of comparettia falcata seeds under in vitro conditions. In Vitro Cell Dev. Biol. – Plant. 41, 838–843.

Qian J., Vossoughi D., Woitaschek D., Oppermann E., Bechstein W.O., Li W-Y., Feng G.S., Vogl T. 2003. Combined transarterial chemoembolization and arterial administration of Bletilla striata in treatment of liver tumor in rats. World J. Gastroenter. 9 (12), 2676–2680.

Shimura H., Koda Y. 2004. Micropropagation of *Cypripedium macranthos* var. *rebunense* through protocorm like bodies derived from mature seeds. Plant. Cell Tiss. Organ Cult. 78, 273–276.

Stewart S.L, Kane M.E. 2006. Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (*Orchidaceae*), a rare Florida terrestrial orchid. Plant. Cell Tiss. Organ Cult. 86, 147–158.

Stoutamire W. 1974. Terrestrial Orchid Seedlings, [w] Withner C.L. The Orchids Scient. Stud. 101–128.

Tay L.J., Takeno K.H. 1988. Culture conditions suitable for *in vitro* seed germination and development of seedling in *Paphiopedilum*. J. Japan Soc. Hort. Scient. 57 (2), 243–249.

Tokuhara K., Mii M. 1993. Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. Plant Cell Rep. 13, 7–11.

Vasudevan R., Van Staden J. 2010. *In vitro* asymbiotic seed germination and seedling growth of *Ansellia africana* Lindl. Sci. Hort. 123 (4), 496–504 .

Wang Ch., Sun J., Luo Y., Xue W., Diao H., Dong L., Chen J., Zhang J. 2006. A polysaccharide isolated from the medicinal herb *Bletilla striata* induces endothelial cells proliferation and vascular endothelial growth factor expression *in vitro*. Biotech. Lett. 28, 539–543.