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Influence of activated charcoal on seed germination and seedling development by the asymbiotic method in *Zygostates grandiflora* (Lindl.) Mansf. (*Orchidaceae*)

Agata Pacek-Bieniek[1], Magdalena Dyduch-Siemińska[2], Michał Rudaś[3]

[1] Department of Botany
[2] Department of Genetics and Horticultural Plant Breeding
University of Life Sciences in Lublin
Akademicka 15, 20-950 Lublin, Poland
[3] Central Apparatus Laboratory
University of Life Sciences in Lublin
Dobrzańskiego 3, 20-262 Lublin, Poland
e-mail: agatapacek@wp.pl

ABSTRACT

The aim of this study is to examine the influence of activated charcoal on the seed germinating ability and seedling development of *Zygostates grandiflora* (Lindl.) Mansf. in *in vitro* culture. The seeds were obtained from a sterilised orchid capsule. They were sown on a Murashige and Skoog medium + indoleacetic acid (1.5 mg dm^{-3}) and benzylaminopurine (2.0 mg dm^{-3}) without the addition of activated charcoal and on a medium that contained 1.0 and 3.0 g dm^{-3} activated charcoal, respectively, for 18 months. Initial differences in seed germination were observed after nine months since the beginning of the *in vitro* culture. The addition of activated charcoal had a positive influence on protocorm size and development. The results of this research were confirmed after 12 and 18 months of *in vitro* culture, where an intensive development of leaf and aerial roots occurred on a medium that contained the highest concentration of activated charcoal, i.e. 3.0 g dm^{-3} as a supplement. The statistical analysis showed that the asymbiotic method of orchid propagation in *in vitro* culture could be used for *Z. grandiflora*, and that the addition of activated charcoal into the medium improved this method.

Key words: *in vitro* culture, medium, orchid, propagation, protocorm

INTRODUCTION

A single orchid capsule contains millions of seeds, but they are as microscopic and weightless as pollen. Orchid seeds can measure from 0.25 to 1.2 mm in length (Knudson 1922, Hoehne 1940, Rasmussen 1985) and weigh from 0.3 to 14 µg (Burgeff 1936, Harley 1950). In spite of the very large number of seeds produced, only a few seeds germinate in nature and plant development takes as much as several years. The seeds do not possess fully developed embryos and do not contain storage tissue. Orchids obtain their required nutrients via fungi from the *Rhizoctonia* genus that lives in symbiosis with those plants. The fungi grow into the seed and their hyphae get into the deeper layers of the cells. Then the hyphae are decomposed and become a source of nutrients for the growing protocorm or undifferentiated parenchymatic cells arising during germination (Arditti 1967, Hayes 1969, Oszkinis 2004). For that reason, the first attempts of orchid propagation from seeds were unsuccessful. Moreover, to this day orchid

propagation from seeds is difficult and ornamental orchids, which can be found in floristries, are mostly *in vitro* propagated through the stem or leaf explants without the use of seeds or by the asymbiotic method using seeds but without the participation of fungi. The rising popularity of orchids has created a demand for high quality plants; therefore, *in vitro* propagation methods are more effective and faster than conventional orchid cultivation methods **(**Chen and Chang 2004, Chen et al. 2004, Tuong and Tanaka 2004). Due to the spread of effective *in vitro* orchid propagation, they have become more available on the market and less expensive. Nevertheless, most native orchid species are protected by law, and the number of endangered species will continue to increase because of the degradation of their natural environment. In order to store *in vitro* seedlings within a short span of time and preserve native orchids, the most effective micropropagation method should be utilised.

The propagation and cultivation of orchids was revolutionised after the discovery by Knudson (1922) that orchid seeds can be germinated on a simple sugar-containing medium. His work showed for the first time that the germination of orchids was possible *in vitro* without fungal association. Subsequently, he proposed a new nutrient solution for the germination of orchid seeds in 1946 (Knudson 1946). Since then, many species have been successfully raised *in vitro* from seeds.

The effectiveness of seed development in a culture *in vitro* depends on a number of environmental factors, such as medium components, light intensity, temperature, etc. In this report, three different germination media and the seedling regeneration of native Brazilian orchid *Zygostates grandiflora* (Lindl.) Mansf. were investigated.

MATERIAL AND METHODS

Material source

Zygostates grandiflora (Lindl.) Mansf. (*Orchidaceae*) came from the collection of the UMCS Botanical Garden in Lublin. To obtain seeds, a flower of the studied species was hand-pollinated through geitonogamy under greenhouse conditions. For pollination, the pollinarium from a fully open flower was carried over by means of fine forceps and deposited on the stigma of another flower on the same plant. The mature fruit capsule was collected 208 days after pollination and it was stored at 5°C for six months. The seeds from this capsule initiated the new *in vitro* seed culture.

Material preparation

The capsule was treated with 0.4% sodium hypochlorite ($NaOCl_2$) for 30 minutes then rinsed three times with sterile distilled water. The capsule was dried in a laminar airflow and then dissected longitudinally with a surgical blade. The seeds were sown by being spread as thinly as possible over the surface of the culture medium in petri dishes; each dish contained 10 cm^3 of medium.

Media compositions

Seeds were grown on three different kinds of Murashige and Skoog (1962) media (MS) with 3.0% sucrose. The media were supplemented by 2.0 mg dm^{-3} benzylaminopurine (BAP) and 1.5 mg dm^{-3} indoleacetic acid (IAA), solidified with 0.7% agar-agar. They contained the following quantity of activated charcoal (AC):

- medium MS1 as the control contained no addition of activated charcoal,
- medium MS2 contained the addition of 1.0 g dm^{-3} activated charcoal,
- medium MS3 contained the addition of 3.0 g dm^{-3} activated charcoal.

The media were adjusted to pH 5.7 with 1 N NaOH or HCl before autoclaving at 121°C, for 20 min, under a pressure of 0.1 MPa. The seeds were sown on 45 petri dishes, with 15 dishes of each medium. The petri dishes with the analysed material were placed into the phytotron (photoperiod: 16 h/light; 8 h/dark) with quantum irradiance of 20 mol m^{-2} s^{-1} for 18 months.

The culture was passaged in eight-week cycles. The germination of seeds and the proliferation of protocorms were observed. After 12 and 18 months, the length of the first leaf and aerial root was measured. For this purpose, 10 plants from each medium were randomly chosen and marked. The average length of these parameters was evaluated (Tab. 1). The results of this research were also documented photographically by means of a Jenaval light microscope (LM) and Olympus SZX12 stereomicroscope. The recorded results from the experiment were statistically processed by means of the variance analysis method and Tukey's 95% confidence interval.

RESULTS AND DISCUSSION

Zygostates grandiflora (Lindl.) Mansf. plants are miniatures and measure about 10 cm. Leaves are fan-shaped and there is a lack of pseudobulbs (Fig. 1). Those epiphytic plants flower in May. The flowers are white-green, and about 2.0 × 1.8

Table 1. Mean length of first leaf and aerial root

Duration of culture	Type of medium					
	MS1*		MS2▪		MS3♦	
	First leaf (cm)	First aerial root (cm)	First leaf (cm)	First aerial root (cm)	First leaf (cm)	First aerial root (cm)
12 months	0.00	0.00	0.45	0.00	1.11	0.65
18 months	0.34	0.00	1.06	0.74	2.02	2.36
$LSD_{0.05}$						
Type of medium (A)	0.136	A × B	0.021	A × B × C	0.027	
Part of plant (B)	0.256	A × C	0.074			
Duration of culture (C)	0.336	B × C	0.156			

*Medium without the addition of activated charcoal
▪Medium with addition of 1.0 g dm^{-3} activated charcoal
♦Medium with addition of 3.0 g dm^{-3} activated charcoal

cm (Fig. 2). Some species from *Zygostates* sp. can produce flower oils that are secreted by structures called elaiophores (Vogel 1974, Buchmann 1987, pers. observ.). The *Z. grandiflora*'s elaiophore occurs on the labellar callus (Fig. 2), as in the flowers of *Ornithocephalus kruegeri*, *Oncidium loefgrenii*, *O. trulliferum*, *Ornithophora radicans*, *Rudolfiella picta* or *Gomes bifolia* (Pacek and Stpiczyńska 2007, Stpiczyńska et al. 2007, Stpiczyńska and Davies 2008, Davies and Stpiczyńska 2009, Aliscioni et al. 2009). Floral oil is usually collected by female bees, which use it mixed with pollen as a provision for larvae or nest construction (Buchmann 1987).

The capsules obtained after pollination measured 2.1 × 1.3 cm (Figs 3-5). The seeds of *Z. grandiflora* were about 250-400 μm in length and 70 μm in width. They were pale yellowish and elongated. The general morphology of the seeds was similar to those of *Cattleya* sp. (Buyun et al. 2004). The seeds usually had the largest diameter near the middle, narrowing towards the polar ends (Figs 6-7). Seed germination of the *Zygostates* species on average began after six months of culture. The seed coat split and the embryos developed into protocorms of about 0.1 cm in length six months after the sowing on all types of media. The colouration of protocorms varied from milk white at the beginning of germination to bright green some time later as in Kauth's (2005) experiment.

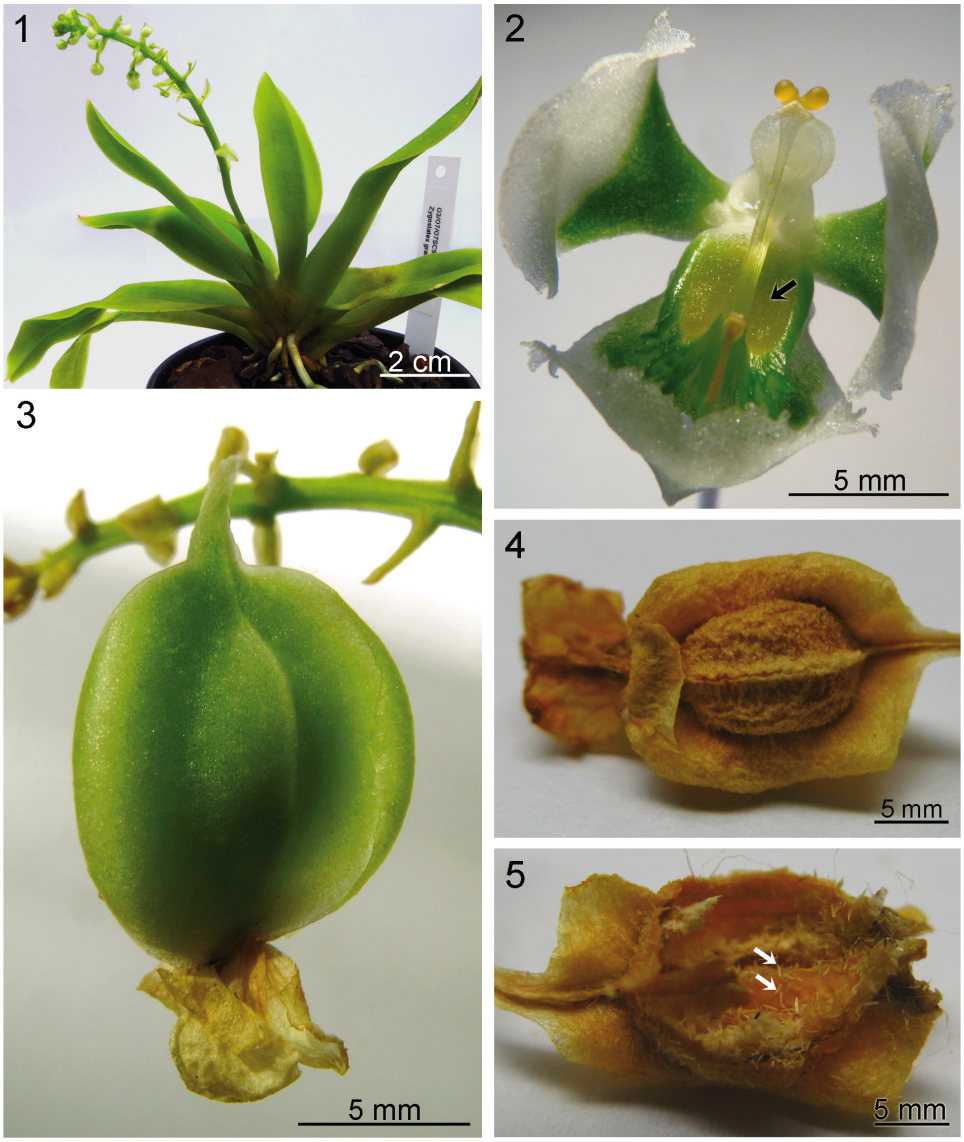
After nine months of *in vitro* culture, differences in germination were observed. In the case of the first medium (control), the size of protocorms was slightly larger than 0.1 cm and colouration did not change (Fig. 8). In the medium that contained 1.0 g dm^{-3} activated charcoal, the protocorms were 0.2 cm in diameter and rhizoids were observed (Fig. 9). The protocorms in the third medium (3.0 g dm^{-3} of activated charcoal) doubled their size (0.4 cm), and the number and length of rhizoids was the largest (Fig. 10). All protocorms on media supplemented with activated charcoal were dark green.

Subsequent observations and measurements were conducted after 12 months of *in vitro* culture. On the charcoal-free medium, the protocorms were about 0.3-0.4 cm and colouration changed to green exclusively. Leaves and aerial roots did not develop (Fig. 11). On the remaining two media, protocorms transformed into seedlings. On the MS2 medium, supplemented with 1.0 g dm^{-3} activated charcoal, the seedlings possessed one or two leaves with a length of about 0.45 cm (Tab. 1). There was still a lack of aerial roots (Fig. 12). The addition of three times as much activated charcoal (3.0 g dm^{-3}) caused a more intensive growth of leaves. The first leaf length was 1.11 cm on average (Tab. 1). One-year-old *Z. grandiflora* seedlings in this medium generally consisted of 3-5 (Fig. 13) leaves and developed aerial roots that reached 0.65 cm in length on average (Tab. 1).

After 18 months in the activated charcoal-free medium, the first leaves that appeared were 0.34 cm in length on average (Tab. 1). In the case of MS2 and MS3, the leaves doubled in size, and in the MS2 medium, the development of aerial roots was observed (0.74 cm). In the third medium the aerial roots enlarged their size almost fourfold (Tab. 1).

Statistical analysis shows that the type of medium influenced initial leaf length, both after 12 and 18 months. Significant differences in the initial aerial root length after 12 months were observed only in the medium with the largest activated charcoal quantity. After 18 months, the averages for aerial roots were significantly different (Tab. 1).

In vitro plant regeneration is influenced by many factors, such as genotypes, type of explants and composition of media. Medium additives such



Figures 1-5. *Zygostates grandiflora*. Fig. 1. Plant with inflorescence. Fig. 2. Flower with elaiophore (arrow). Fig. 3. Immature capsule. Fig. 4. Mature capsule. Fig. 5. Open capsule with seeds (arrows).

as sugar, different growth regulator combinations (Faria et al. 2004, Wotavová-Novotná et al. 2007), and other organic substances (Aktar et al. 2008) are considered to be major factors for shortening the *in vitro* growth period and for more effective orchid propagation.

The addition of activated charcoal to the medium for plant tissue cultures improves growth by adsorbing toxic metabolites (Wang and Huang 1976). Activated charcoal plays also an important role in orchid seed germination (Kull and Arditti 2002, Moraes et al. 2005). According to Moraes et al. (2005), media supplemented with activated charcoal improved the *in vitro* propagation of Brazilian native orchids. The results of the present study for *Z. grandiflora* confirmed the data obtained by Moraes et al. (2005) for other Brazilian orchids, i.e. *Laelia flava* Lindl., *Miltonia flavescens* Lindl., and *Oncidium trulliferum* Lindl.

CONCLUSIONS

The *Zygostates grandiflora* (Lindl.) Mansf. orchid can be successfully propagated by *in vitro* seed culture. This study was very important for the preservation of genetic resources and the basic researches of orchid plant breeding.

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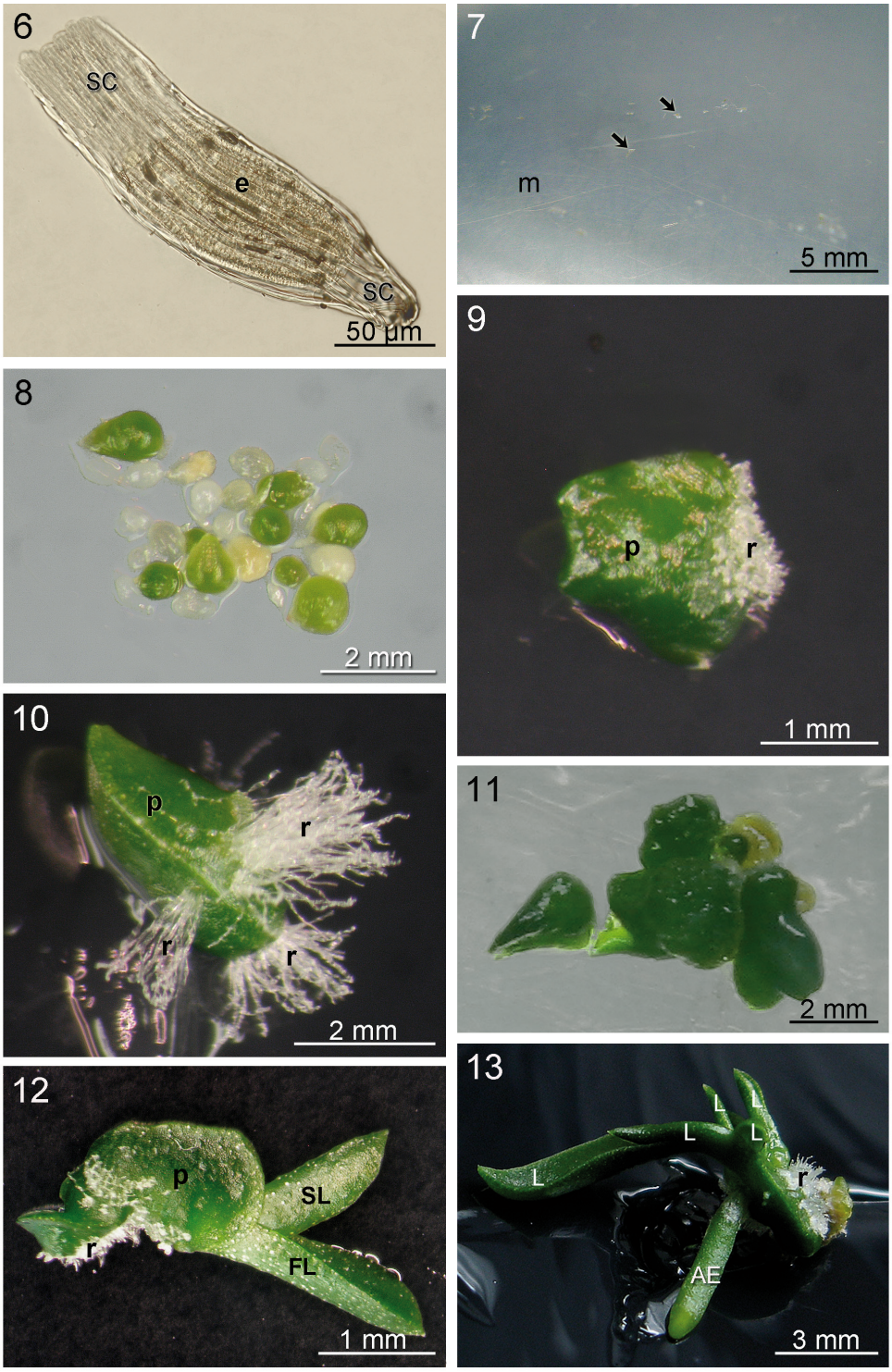

Figures 6-13. *Zygostates grandiflora.* Fig. 6. Seed details in light microscope (LM). Fig. 7. Seeds (arrows) after sowing in MS medium. Fig. 8. Protocorms in MS charcoal-free medium after nine months of culture. Fig. 9. Protocorm with short rhizoids in MS medium supplemented with 1.0 g dm^{-3} activated charcoal after nine months of culture. Fig. 10. Protocorm with well developed rhizoids in MS medium supplemented with 3.0 g dm^{-3} activated charcoal after nine months of culture. Fig. 11. Swollen protocorms in MS charcoal-free medium after 12 months of culture. Fig. 12. Seedling at the two-leaf stage in MS medium supplemented with 1.0 g dm^{-3} activated charcoal after 12 months of culture. Fig. 13. Seedling with five leaves and aerial root in MS medium supplemented with 3.0 g dm^{-3} activated charcoal after 12 months of culture

Abbreviations: AE − aerial root; e − embryo; FL − first leaf; L − leaf; m − medium; p − protocorm; r − rhizoids, SC − seed coat; SL − second leaf.

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WPŁYW WĘGLA AKTYWNEGO NA KIEŁKOWANIE I TWORZENIE SIEWEK METODĄ ASYMBIOTYCZNĄ U *ZYGOSTATES GRANDIFLORA* (LINDL.) MANSF. (*ORCHIDACEAE*)

Streszczenie: Celem pracy jest zbadanie wpływu węgla aktywnego na zdolność kiełkowania i rozwój siewek storczyka *Zygostates grandiflora* Lindl. w kulturze *in vitro*. Z wysterylizowanych owoców storczyka pozyskano nasiona, które wyłożono na pożywkę stałą o składzie podstawowym według Murashige i Skooga (1962) wzbogaconą w kwas indolilooctowy (1,5 mg dm^{-3}) i benzyloaminopurynę (2,0 mg dm^{-3}) bez dodatku węgla aktywnego i zawierającą odpowiednio 1,0 i 3,0 g dm^{-3} węgla aktywnego na okres 18 miesięcy. Pierwsze różnice w kiełkowaniu nasion zaobserwowano po 9 miesiącach prowadzenia kultury. Dodatek węgla aktywnego wpłynął korzystnie na rozwój i wielkość protokormów. Wyniki tych badań zostały potwierdzone po 12 i 18 miesiącach trwania kultury, gdzie na pożywce zawierającej najwyższe stężenie węgla tj. 3,0 g dm^{-3} jako dodatku, nastąpił intensywny rozwój liści oraz indukcja korzeni powietrznych. Analiza statystyczna otrzymanych wyników wykazała, że metoda asymbiotycznego rozmnażania storczyków w kulturach *in vitro* może być stosowana dla *Z. grandiflora*. Z zebranych danych wynika, że dodatek węgla aktywnego do pożywki doskonali tę metodę.

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