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Protocorm development of *Epidendrum fulgens* (Orchidaceae) in response to different saline formulations and culture conditions

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ABSTRACT. The asymbiotic technique of orchid seeds germination is an important method of mass production of seedlings. Studies on the best culture conditions for each species are important to obtain seedlings in less time and at lower costs. Current analysis evaluates different consistencies of culture medium, saline formulations and culture conditions on the germination rate and further development of protocorms of *Epidendrum fulgens*. After 45 days in culture the protocorms were classified into three categories of development. The liquid saline formulation of Murashige and Skoog (1962) (MS) provided the highest germination rate (83.5%), and the Knudson formulation (1946) the lowest (10.9%). The different consistencies or conditions or culture conditions did not affect the germination rate percentage, except the Knudson medium, which resulted in the highest rate in response to the gelled consistency. Protocorms cultured in liquid MS medium with or without agitation showed the fastest development.

Keywords: germination, asymbiotic techique, seeds, culture medium.

Desenvolvimento de protocormos de *Epidendrum fulgens* (Orchidaceae) em reposta a diferentes formulações salinas e condições de cultivo

RESUMO. A técnica da germinação assimbiótica de sementes de orquídeas é um importante método de produção massal de mudas. Estudos que buscam condições de cultivo ideais para cada espécie são altamente desejados para obterem-se mudas em menor tempo e com menor custo. Este estudo objetivou testar diferentes consistências de meio de cultura, formulações salinas e condições de cultivo na porcentagem de germinação e velocidade de desenvolvimento de protocormos de *Epidendrum fulgens*. Após 45 dias de cultivo, os protocormos foram classificados em três categorias de desenvolvimento. A formulação salina de Murashige e Skoog (1962) (MS) líquida, sem agitação orbital, proporcionou maior taxa de germinação (83,5%), e a formulação de Knudson (1946) o menor percentual (10,9%). As diferentes consistências ou condições de cultivo não influenciaram na porcentagem de germinação, exceto para o meio Knudson, onde na consistência geleificada o percentual foi maior. Os protocormos cultivados em meio MS líquido, com ou sem agitação, apresentaram maior velocidade de desenvolvimento.

Palavras-chave: germinação, assimbiótica, sementes, meio de cultura.

Introduction

The Orchidaceae family is considered one the most developed in the plant kingdom due to the its great complexity and specialization in floral morphology and pollination types (SUTTLEWORTH et al., 1970). In fact, the orchid family shows a great diversity in size, shape and color of its flowers. From the ecological point of view, many of its members are important components in various types of vegetation (ESPEJO et al., 2002).

Although several representatives of this family have a high ornamental potential, they are not commercially exploited. *Epidendrum fulgens* Brongn. (Figure 1), an example of non-exploited ornamental orchid species, is a terrestrial orchid, native to the wet lowlands of the coastal ecosystem of Brazil, mainly in the southern and southeastern regions (DUARTE; BENCKE, 2006). The genus *Epidendrum* has approximately 1,500 species distributed between the southeastern United States to northeastern Argentina (HÁGSATER; ARENAS, 2005). *E. fulgens* has large phenotypic diversity displayed mainly in flower color, coupled to high rusticity, fast growth and long-lasting flowers that increase its potential for economic exploitation. Owing to the above characteristics, *E. fulgens* has been selected as a promising plant in the project of the Brazilian Ministry of Environment known as "Plants for the future - the South" (CORADIN et al., 2011).

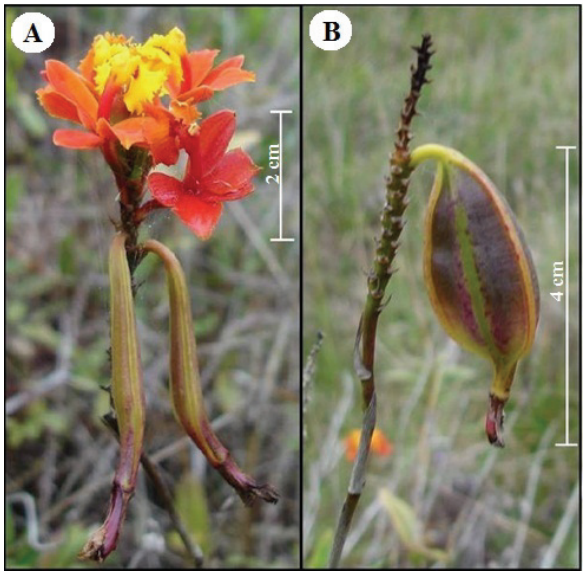

Figure 1. Flower and capsule of *E. fulgens*. A) Flower with developing capsule. B) Capsule with seeds.

Orchids have a slow vegetative growth which makes their multiplication difficult and expensive (FARIA et al., 2006). Tissue culture techniques comprise several tools to accelerate the process of germination, multiplication and development, making large scale multiplication viable, since almost all the seeds germinate *in vitro* (MORAES et al., 2002).

Orchid seeds are very small, extremely lightweight and without any endosperm. One capsule (Figure 1b) contains more than half a million seeds (ARDITTI; GHANI, 2000). Thus, a symbiotic association with mycorrhizal fungi is required for embryo germination and plant development (PETERSON et al., 2004). Knudson (1922) was the first to demonstrate the possibility of asymbiotic *in vitro* germination of orchid seeds as a viable alternative for seedling production, emphasizing the importance of sucrose for the *in vitro* growth and development of embryos. Although several formulations have been developed, due to the large number of species, the choice of the best salt formulation is essential to produce seedlings with quality and in a very short time.

Culture media may be gelled or liquid but the latter requires some kind of support or agitation to facilitate the explant´s gas exchange. The liquid media is less expensive owing to the non-utilization of agar and the agility to prepare it (CALDAS et al., 1998). In stationary liquid culture, the contact between the plant organs and the nutrient medium favors the incorporation of available nutrients, but the gaseous exchanges are impaired (LORENZO et al., 1998). Agitation enhances the uptake of nutrients due to greater surface contact with the plant whilst gas exchange is increased by the constant aeration of the medium (CIDADE et al., 2006). Furthermore, liquid media are more homogeneous since they do not form a nutrient gradient as occurs with solid ones (PASQUAL et al., 2002).

Due to their consistency, gelled media provide ideal conditions for seedling support, allowing them to stay in a favorable position in their development and favoring gas exchange due to the constant contact with air. However, nutrient assimilation is reduced since only the basal part of the explants is in direct contact with the culture medium (AITKEN-CHRISTIE et al., 1995)

This study aim to evaluates seed germination and *in vitro* development of *E. fulgens* protocorms under different culture conditions, formulations and consistencies of culture medium.

Material and methods

Seeds of *E. fulgens* from mature pre-dehiscent capsules, originating from natural pollination, were used as explants. The capsules were washed under running water with neutral detergent and submitted to an aseptic procedure with alcohol 70° GL (30 seconds) and 1% NaClO (30 min.). After, the capsules were washed in sterile distilled water and opened lengthwise. The seeds were then placed in a beaker with 100 mL sterile distilled water and kept under stirring with magnetic stirrer until inoculation. Aliquots of 350 µL from the seed suspension were inoculated into the culture medium.

Experiment 1

Three saline formulations were tested in liquid and gelled consistencies: Knudson formulation (KNUDSON, 1946), modified by FeSO.7HO (27.85 mg L^{-1}); and Na_2EDTA (37.35 mg L^{-1}) (KC); MS saline formulation (MURASHIGE; SKOOG, 1962) (MS); and MS modified with half the concentration of macro and micronutrients (MS/2). In the case of gelled medium, 7.6 g L^{-1} of agar was used (HIMEDIA®) and the culture medium was dispensed in disposables petri dishes (J. Prolab® 60 x 15 mm). In the case of liquid medium, 25 x 150 mm test tubes were used, with 10 mL of culture medium each. All the culture media were supplemented with sucrose 30 g L^{-1} and their pH was adjusted to 5.8 (±0.1) with NaOH and HCl (0.5 N) before autoclaving at 121°C (1.2 kgf cm^{-2}) per 15 min.

To ensure the inoculation of similar number of seeds in each treatment, the capsules were opened lengthwise and the seeds were placed in a beaker

containing 100 mL of sterile distilled water and kept under stirring with magnetic stirrer until inoculation. Aliquots of 350 µL of seed suspension were inoculated into the culture medium. An average of 34 seeds (with standard deviation of 10 seeds) was inoculated in each aliquot.

After inoculation, treatments containing liquid culture medium were kept in growth room, at mean temperature of 25°C (±2ºC) in a photoperiod of 16/8h (light/dark), with irradiance 60 μmol m^{-2} s^{-1} of photons. Cultures from treatments containing gelled medium were kept in BOD incubator at the same temperature and light conditions.

After six weeks, the protocorms' germination rate and development dynamics were analyzed by stereomicroscope. Seeds with seed-coat broken by developing protocorm were established as a pattern of germination. The developmental stages of the protocorms were: protocorms without visible apical meristem (stage 1); with apical meristem (stage 2); with leaf primordia (stage 3) (Figure 2).

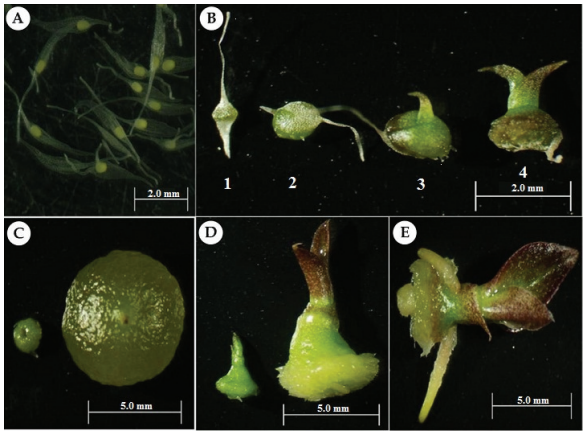

Figure 2. Seed germination of *E. fulgens* and dynamic development of protocorms. A) Seed in the initial process of germination; B) Protocorms in different developmental stages; (1) protocorm breaking through the seed tegument (stage 1), (2) protocorm with shoot meristem (stage 2), (3) protocorm with two leaf primordia (stage 3); C and D) Comparison between protocorms in the same development stages (2 and 3 respectively), germinated in liquid medium (left) and gelled (right); E) seedling with leaves and adventitious roots originated in the upper protocorm.

The experimental procedures were based on a factorial design: culture media - MS, MS/2 and Knudson (Factor A); and liquid or gelled consistency (Factor B), totaling six treatments and four replicates. The experiment had 24 experimental units. The experimental unit comprised eight samples (test tube or petri dishes). Data from germination percentage and protocorm developmental stages from each treatment were compared with contingency tables and χ^2 tests ($p = 0.05$) using Statgraphics 7.0 software.

Experiment 2

Two liquid formulations were tested: MS (MURASHIGE; SKOOG, 1962) and Y3 (EEUWENS, 1976), both modified by FeEDTA FeSO.7HO (27.85 mg L^{-1}), Na_2EDTA (37.35 mg L^{-1}) and supplemented with Morel vitamins (2 mL L^{-1}) (MOREL; WETMORE, 1951). The culture medium (30 mL) was dispensed in glass flasks (60 X 120 mm).

The cultures were maintained in a culture room (irradiance 60 μmol m^{-2} s^{-1} photons, 16 hour light period, at 25±2ºC) submitted to two culture conditions: with agitation in which flasks were submitted to constant shaking on an orbital shaker table (TECNAL TE-1401) at speed 3 (115 rpm); or without agitation, where the flasks were kept static.

For the treatments with liquid consistency, the seeds were mixed with the culture medium and poured on a petri dish (15 x 90 mm), drained using a pipette, and then counted. The classification from the developmental stages followed the same parameters as in the previous experiment.

The experiment was designed in a factorial design: salt formulations MS and Y3 (factor A) and agitation or not of the culture medium (factor B), resulting in four treatments and four replications. The experiment had 16 experimental unit. The experimental unit consisted three flask with an average of 34 seeds (standard deviation of 10 seeds). The statistical analysis followed the same procedures as described in Experiment 1.

Results and discussion

The consistency of culture media in the first experiment did not influence the germination rate, except for the gelled Knudson medium which resulted in the highest scores (Table 1).

The germination rate was probably affected by the stirring process which caused some kind of seed damage, even at low exposure time. However, this type of inoculation method allowed the introduction of a similar number of seeds in each bottle.

Table 1. Effects of different saline formulations in liquid and solid media on germination rate (G%) and development speed of *E. fulgens* protocorm 45 days after inoculation.

Treatments		G%	Stage 1 (%)	Stage 2 (%)	Stage 3 (%)
Liquid	MS	26.3 b	30.2 b	26.3 c	43.5 a
	MS/2	34.1 a	29.3 b	37.0 b	33.7 b
	Knudson	10.9 c	46.6 a	31.8 bc	21.6 c
Gelled	MS	27.2 b	28.2 b	62.3 a	9.5 d
	MS/2	32.5 a	32.2 b	64.0 a	3.8 e
	Knudson	23.8 b	34.9 ab	63.5 a	1.6 e

*Means followed by different letters in the same column indicate significant differences according to comparisons in contingency tables and χ^2 tests ($p \leq 0.05$).

Significant interactions were found between consistency and saline formulation, where the MS/2 medium had the highest germination rate, followed by MS medium. Knudson medium resulted in the lowest germination rate in liquid medium, similar to the rate in gelled MS medium (Table 1). Similar results were reported in the germination of *Cattleya bicolor* where KC medium also resulted in the lowest germination rate (SUZUKI et al., 2010). When Martini et al. (2001) compared different saline formulations, they evaluated the *in vitro* germination of *Gongora quinquenervis*. Total necrosis of half the embryos in the Knudson medium occurred after 20 days in culture, whereas embryos cultivated in MS medium had already germinated. In a study with *Oncidium baueri* seeds, MS medium half macronutrients was more effective than the full strength MS medium in initial vegetative development (SORACE et al., 2008).

Protocorm development was evaluated among the different treatments by comparing means of protocorms percentages in each developmental stage. In the two consistencies the KC medium showed the highest amount of protocorms in stage 1 (Table 1). Suzuki et al. (2009) showed that, although it had the highest germination rate, the medium KC was not the most suitable for the development of *Hadrolaelia tenebrosa*, when compared to MS and MS/2 saline formulations. Stage 2 showed significant differences between media consistencies, the gelled medium had high proportions of protocorms in this developmental stage for all tested media (Table 1). Likewise, stage 3 showed significant differences between treatments, with liquid MS medium exhibiting the highest protocorm percentage, followed by MS/2 and KC, respectively. This fact could be attributed to the absence of micronutrients and to the low levels of nitrogen and potassium in the Knudson medium when compared to MS medium (ARAUJO et al., 2009). Mercier and Kerbauy (1991) reported that different N sources influenced the synthesis of some substances such as cytokinins and chlorophyll, and consequently the development of *E. fulgens* protocorms. Further, several authors state that the sources of nitrogen in the nutrient media positively influence orchid development *in vitro* (ARAUJO et al., 2009; PASQUAL et al., 2009, KANASHIRO et al., 2007).

MS liquid medium resulted in more protocorms in advanced developmental stages, indicating that the compounds of saline formulation positively influenced the protocorm development of *E. fulgens*. The above result agrees with that reported by Puchooa (2004) to compare MS medium with KC medium in the cultivation of *Dendrobium* sp.. This was attributed to the permanent contact of the medium with the protocorm tissues (BATYGINA et al., 2003). It has been reported that, even when classified in the same category, protocorms from liquid consistency medium were bigger than those in the solid medium (Figure 2). Moreover, a bigger size ensures a larger surface contact with the medium, which increases the nutrient absorption.

Considering the results obtained, a new experiment was established in which MS medium was employed in liquid consistency, since it showed a positive influence on protocorm development. The MS liquid medium was then tested under different culture conditions and compared with the Y3 medium which would be ideal due to the fact that this orchid species is typical of sandbanks area. According to Shylaraj et al. (2007), protocorm induction and multiplication in agitated liquid medium is suggested for the rapid multiplication of the orchid *Dendrobium* cv. Sonia. There was no significant difference ($p < 0.05$) in the germination rate in response to different culture conditions (Table 2). However, when Y3 and MS media in both conditions were compared, the latter had a higher germination percentage (Table 2).

Table 2. Effect of saline formulations of Eewens (Y3) and MS in liquid consistency, with or without orbital shaking, on the germination rate and development speed of *E. fulgens* protocorms, 45 days after inoculation. Mean of 12 repetitions.

Treatments		G %	Stage 1 (%)	Stage 2 (%)	Stage 3 (%)
Stationary	Y3	78.89 b	36.36 b	56.62 a	7.02 b
	MS	83.54 a	22.34 c	54.41 a	23.25 a
Agitation	Y3	76.28 b	53.91 a	44.87 b	1.22 c
	MS	80.82 a	30.98 b	46.64 b	22.38 a

*Means followed by different letters in the same column indicate significant differences according to comparisons in contingency tables and χ^2 tests ($p \leq 0.05$).

Significant differences were reported between treatments (Table 2) with regard to protocorms developmental stages. Treatments with a higher number of protocorms in stage 1 were the combinations Y3-static and Y3-agitation, and the agitation factor increased significantly the amount of protocorms. Mengarda et al. (2009) in their study on bromeliads concluded that the static liquid culture medium was the best in terms of proliferation rate. This result differs from that found by Pereira and Fortes (2003) who demonstrated that agitation of the medium increased the growth of *in vitro* cultivated potatoes.

Stage 2 did not show any differences between the medium. However, cultures maintained in a static medium had a higher amount of protocorms which reached the second stage of development. Cultures maintained in MS medium in two cultive conditions revealed a higher percentage of protocorms in stage 3

when compared to Y3 medium which had a higher amount of protocorms in static conditions than under agitation.

High salt concentration of Y3 medium may have caused an abiotic stress in *E. fulgens* protocorms that, albeit frequent on the coastline, is not a mangrove species and therefore cannot display such adaptation mechanism to high saline levels. Mangrove species are adapted to these conditions by accumulating salt in the cytosol until the osmotic potential level becomes lower than that in the soil solution (LARCHER, 2004).

The importance of agitation has been demonstrated in the micropropagation of cane sugar, highlighting beyond the incorporation the nutrients, the gas exchange favored by constant aeration of the medium (CIDADE et al., 2006). According to Murashige (1974), the explants submerge when in a static liquid medium, with a decrease of gas exchange. Some authors claim that the lack of agitation in liquid medium is a negative factor, attributing this fact to a reduction in the capacity of explants' sprouting (DOMINGUES et al., 1995). However, the above failed to occur in current assay since the agitation condition did not influence seed germination.

Conclusion

Results show that liquid MS culture medium, with or without agitation, enhances the asymbiotic culture of *E. fulgens* from seeds and provides a high germination rate and a fast protocorm development.

Although, the use of liquid culture medium without agitation makes the described protocol easy to apply for the *in vitro* propagation of *E. fulgens* either for its ornamental commercial purpose or for the species´s ecological conservation.

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