

# Nutritional requirements for germination and *in vitro* development of three Orchidaceae species in the southern Brazilian Amazon<sup>(1)</sup>

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## ABSTRACT

Tissue culture is an alternative form of producing healthy, vigorous and regular plants on a large scale. The purpose of this study was to evaluate the most efficient culture medium for *in vitro* plantlet germination and development of three Orchidaceae species. Seeds disinfested of three species were dispersed in distilled water and dripped into basic Murashige and Skoog (MS) medium. The experimental design was completely randomized in a factorial 3 x 4 (three species x four culture media), with 5 replications. Four treatments were established: (1) full-strength MS medium, with the full nutrient concentration (MSØ), (2) full-strength MS medium plus 0.3% activated charcoal (MSØ ACh), (3) half-strength MS medium (½ MS) and (4) half-strength MS medium with 0.3 % activated charcoal (½ MS ACh). Germination was evaluated after 15, 20, 25, 30, and 60 days. The shoot height, leaf number and length, root number and length of plantlets of the three studied species were assessed. In *A. variegata*, 73% germinated after 60 days in ½ MS ACh medium. In the same period, 100% of *E. viviparum* and *S. gloriosa* seeds germinated in MSØ ACh medium. The plant height, leaf number and length, root number and length were significantly higher for the species *A. variegata* and *E. viviparum* in MSØ ACh medium. The culture media ½ MS and MSØ with addition of activated charcoal favored *in vitro* germination for the three orchid species of this study.

**Keywords:** culture medium, orchids, tissue culture *Aspasia variegata*, *Epidendrum viviparum*, *Schomburgkia gloriosa*.

## RESUMO

### Requerimentos nutricionais para germinação e desenvolvimento *in vitro* de três espécies de Orchidaceae do sudoeste da Amazônia brasileira

A cultura de tecidos é aplicada como alternativa de produção de mudas saudáveis, vigorosas, uniformes e em larga escala. O objetivo deste estudo foi avaliar o meio de cultura mais eficiente para a germinação e desenvolvimento *in vitro* de plântulas de três espécies de Orchidaceae. Sementes desinfestadas das três espécies foram colocadas em água destilada e gotejadas em meio de cultura básico de Murashige and Skoog (MS). O desenho experimental foi completamente ao acaso em um fatorial 3 x 4 (três espécies x quatro meios de cultura) com cinco repetições. Constituído de quatro tratamentos, sendo: (1) meio MS com concentração total de nutrientes (MSØ), (2) meio MS com concentração total de nutrientes acrescido de 0,3% de carvão ativado (MSØ C.A), (3) meio MS com metade das concentrações de nutrientes (½ MS) e (4) meio MS com metade das concentrações de nutrientes, acrescido de 0,3 % de carvão ativado (½ MS C.A). A avaliação da germinação foi realizada aos 15, 20, 25, 30 e 60 dias. Foram mensuradas altura da parte aérea, número e comprimento das folhas e número e comprimento de raízes de plântulas das três espécies estudadas. Em *A. variegata* verificou-se uma germinação de 73% aos 60 dias em meio ½ MS C.A. *E. viviparum* e *S. gloriosa* obtiveram 100 % de germinação para o mesmo período, em meio MSØ C.A. A altura da planta, número e comprimento de folhas, número e comprimento de raízes foram significativamente maiores para as espécies *A. variegata* e *E. viviparum* meio MSØ C.A. *S. gloriosa* obteve os menores índices de desenvolvimento de plântulas, para todas as características analisadas, independente do meio analisado. O meio de cultura ½ MS and MSØ com adição de carvão ativado favoreceu a germinação *in vitro* para as três espécies de orquídeas estudadas.

**Palavras-chave:** cultura de tecidos, meio de cultura, orquídeas, *Aspasia variegata*, *Epidendrum viviparum*, *Schomburgkia gloriosa*.

## 1. INTRODUCTION

Different species of orchids are among the commercially most exploited plants, and can be used for ornamental, food, cosmetic and medicinal purposes. They are herbaceous, perennial plants with extremely varied shapes and colors, and sought worldwide by orchidologists and for floriculture (ROBERTS and DIXON, 2008).

The programs of breeding and reintroduction of species in their natural habitats have aroused the interest for native orchids with ornamental potential. This is particularly true for the species *Aspasia variegata* Lindl., *Epidendrum viviparum* Lindl. and *Schomburgkia gloriosa* Rchb.f, found in the state of Mato Grosso (KOCH and SILVA, 2012), due to their high ornamental value, robustness and potential for use in hybrid production, landscaping and commercial flower production.

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Under natural conditions, the germination of Orchidaceae seeds depends on a symbiosis with specific fungi for each species, resulting in low germination rates. Thus, the use of some biotechnological tools, such as tissue culture, makes the establishment of a large number of plants within a short cultivation period possible (SUZUKI and FERREIRA, 2007). The response of the orchid family to *in vitro* culture is mostly satisfactory, and the advantages over traditional propagation methods are numerous. For example, multiplication by mass cloning is fast and produces propagules with high levels of plant health, resulting in plants with appropriate characteristics for propagation (FIGUEIREDO et al., 2008). In this way, the tissue culture technique contributes to the preservation of the species and has a series of advantages, including the handling of a large number of plants within a short period under aseptic conditions (UNEMOTO et al., 2007).

The culture medium can optimize micropropagation protocols and is a key factor for the success of *in vitro* culture. This medium is composed of essential substances for tissue growth, according to the nutritional requirements of each species, and is largely responsible for the pattern of *in vitro* development (PACEK-BIENIEK et al., 2010). Thus, the culture medium should be chosen

according to the study objective and the study species (PASQUAL, 2008). The most commonly used culture medium for micropropagation is MS, consisting of inorganic salts, carbohydrates, vitamins with sufficiently high nutrient concentrations for plant development, preventing nutrient excess or lack in the culture medium (MURASHIGE and SKOOG, 1962). Adding activated charcoal to the culture medium is one of the most widely used measures for *in vitro* plantlet propagation, growth and development, since charcoal is believed to have beneficial effects on some orchid species, e.g., increases in plant height and rooting (THOMAS, 2008; PACEK-BIENIEK et al., 2010).

Doses of activated charcoal between 0.1 and 0.2 % added to the culture media promoted the development of the aerial part of *Miltonia flavescens* (Lindl.) Lindl. (CHAPLA et al., 2009). While for *Catasetum fimbriatum* (C. Morren) Lindl., higher doses 0.5 % were required to promote shoot development (MORALES et al., 2006).

The objective of this study was to evaluate the effect of concentration of MS culture medium and the use of activated charcoal on germination and development of *in vitro* plantlets of three native orchid species in the southern Brazilian Amazon.



Figure 1. Flowers of *Aspasia variegata* (A), *Epidendrum viviparum* (B) and *Schomburgkia gloriosa* (C).

## 2. MATERIAL AND METHODS

Ripe fruit seeds of three orchid species (Figure 1) were collected, and the mature capsules were taken to the laboratory for plant and seed improvement.

The capsules were disinfected by immersion in 70% alcohol solution for 2 min, rinsed with autoclaved distilled water and then with commercial sodium hypochlorite solution (2.5% of active chlorine) for 10 min, and shaken by hand in a laminar flow hood. Then they were washed in 70% ethanol for 1 min and rinsed four times with autoclaved distilled water. The capsules were opened and the seeds removed. To the flasks with seeds, 10 mL of autoclaved distilled water was added, and 1 ml of the solution with the seeds was syringed into the flasks containing 40 ml of culture medium according to the treatments.

For germination of the three species (*Aspasia variegata*, *Epidendrum viviparum* and *Schomburgkia gloriosa*), the culture media used for seed inoculation were prepared

with four different compositions, corresponding to the following four treatments: 1) full-strength MS medium, with full nutrient concentration (MSØ), (2) full-strength MS medium plus 0.3% activated charcoal (MSØ ACh), (3) half-strength MS medium (½ MS), and (4) half-strength MS medium plus 0.3% activated charcoal (½ MS ACh). All media were enriched with 30 g L<sup>-1</sup> sucrose, 7.0 g L<sup>-1</sup> agar and the pH adjusted to 5.7 ± 0.1, and immediately sterilized by autoclaving (121 °C at 1.05 kgf cm<sup>-2</sup>) for 20 min.

The experimental design was completely randomized in a factorial 3 x 4 (three species x four culture media), with five replications (20 plantlets of each replication). After seeding, the flasks were sealed with plastic film and placed in a growth chamber at an average temperature of 25 ± 2 °C, 16 h photoperiod and light intensity of two fluorescent lamps of approximately 50 µmol m<sup>-2</sup>s<sup>-1</sup>.

The germination percentage was assessed after 15, 20, 25, 30, and 60 days of cultivation. The germination speed index (GSI) was calculated as the sum of the number

of germinated seeds per day divided by the number of days between sowing and germination, as described by MAGUIRE (1962).

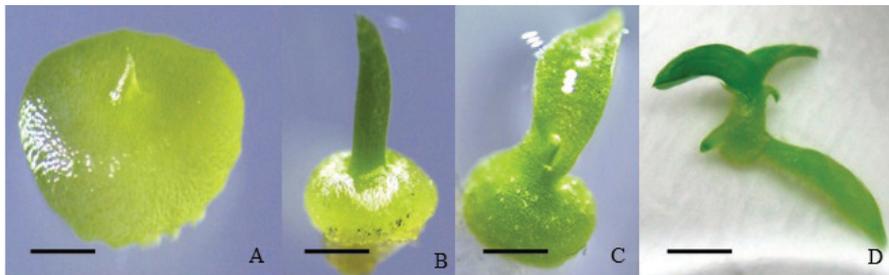
$GSI = (G1 / N1) + (G2 / N2) + (G3 / N3) + \dots + (Gn / Nn)$ , where: GSI = germination speed index, G1, G2, G3, ..., Gn = number of plantlets counted in the first, second, third, and last evaluation; N1, N2, N3, ..., Nn = number of days from sowing to the first, second, third, and last evaluation. The seeds were considered germinated after seed breakage and primary leaf growth.

After 60 days, 20 plantlets of each replication were randomly evaluated to measure the plant height and the leaf and root number and length. The data were subjected to analysis of variance and the means compared by the

Tukey test at 5%. The data of the above characteristics were transformed by . Statistical analyses were carried out with the statistical software SISVAR (FERREIRA, 2011).

### 3. RESULTS AND DISCUSSION

The beginning of germination of all studied species was characterized by seed swelling, due to water and nutrient uptake, followed by disruption of the seed integument and release of the embryo (Figure 2A). Subsequently, the development of protocorms (Figure 2B), protocorm growth (Figure 2C), and seedling development were observed (Figure 2D).



**Figure 2.** Germination stage of orchid seeds. (A) seed swelling and emergence, (B) development of protocorms, (C) formation and expansion of the protocorm leaflets, (D) emerging plantlet with leaflets and rhizoids. Figure 2 A-C Bars=1mm; Figure 2 D Bar = 1cm

According to Pridgeon et al. (1999) and Kraus et al. (2006), protocorms are a transitional structure following orchid seed germination, from which the beginning of the stem and root system is formed. This stage ends with the development of the first leaves, and from this moment onwards, the protocorm is characterized as seedling.

Structural evidence of seed germination, with seed disruption and growth of the primary leaflets, was first observed in *Schomburgkia gloriosa* (6.3%) and *Epidendrum viviparum* (5.3%) after 20 days of culture, and in *Aspasia variegata* (70%) after 60 days (Table 1).

Other studies on ornamental Orchidaceae such as *Cattleya walkeriana* and *Schomburgkia gloriosa* also reported the onset of germination after 15 days of inoculation (SOUZA and JUNGHANS, 2006).

For the three species of this study (*Schomburgkia gloriosa*, *Epidendrum viviparum* and *Aspasia variegata*), the MS media with 0.3% activated charcoal (MSØ ACh) were the most appropriate for seed germination. The onset of germination of *A. variegata* seeds began after 60 days and was 27-30% lower than that of the other species (Table 1).

**Table 1.** Seed germination percentage of *Aspasia variegata*, *Epidendrum viviparum* and *Schomburgkia gloriosa* (Orchidaceae), after 15, 20, 25, 30, and 60 days of culture, in comparison with the culture media with the compositions ½MS, MSØ, ½MS ACh, and MSØ ACh.

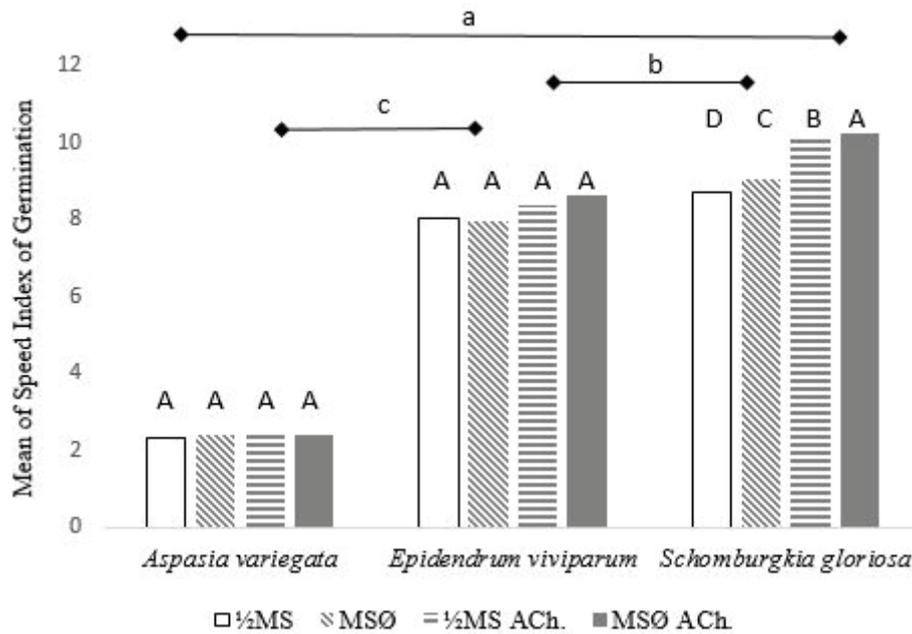
Species	Days	<i>Aspasia variegata</i>	<i>Epidendrum viviparum</i>	<i>Schomburgkia gloriosa</i>
Culture medium		Germination (%)		
½MS	15	0.00	0.00	0.00
	20	0.00	5.30	6.30
	25	0.00	22.90	21.00
	30	0.00	33.40	46.30
	60	70.00	100.00	100.00
Means		14.00	32.32	34.72
CV (%)		31.30	20.14	40.61
MSØ	15	0.00	0.00	0.00
	20	0.00	6.70	7.60
	25	0.00	22.10	22.30
	30	0.00	33.00	47.50
	60	71.80	100.00	100.00
Means		14.36	32.36	35.48
CV (%)		32.10	39.95	40.36
½MS ACh.	15	0.00	0.00	0.00
	20	0.00	7.00	11.30
	25	0.00	23.50	29.70
	30	0.00	36.90	49.00
	60	73.00	100.00	100.00
Means		14.60	33.48	38.00
CV (%)		32.64	39.86	39.34
MSØ ACh.	15	0.00	0.00	0.00
	20	0.00	6.70	11.00
	25	0.00	26.50	31.60
	30	0.00	37.90	49.10
	60	72.80	100.00	100.00
Means		14.56	34.22	38.34
CV (%)		32.55	39.77	39.30

The MS medium is widely used for the early plant development, for being rich in macro- and micronutrients and vitamins, at concentrations adjusted to the specific nutritional requirements for the development of each species (DEZAN et al., 2012). In the case of a lack of light energy and low CO<sub>2</sub> concentration, the photosynthesis of *in vitro* cultivation can be hampered, creating a demand for an external supplementation of nutrients that are essential for plantlet germination and establishment in this type of cultivation (PASQUAL, 2008).

The choice of the culture medium is extremely important for the success of orchid seed germination (SUZUKI et al., 2009). The culture medium with full salt concentrations and with different nutrient compositions was best-suited for the montane grassland orchid *Laelia cinnabarina* Bateman ex Lindl. (STANCATO and FARIA, 1996). On the other hand, in studies with *Oncidium baueri* Lindl., Sorace et al. (2008) obtained satisfactory results

with the development *in vitro* culture in MS medium with half the macro and micronutrient concentrations. The modification of the salt concentration in MS medium is an alternative for the adjustment of species-specific protocols for *in vitro* development, resulting in better plant development. Too low or even too high nutrient doses can be harmful for the plant and also raise production costs (CORDEIRO et al., 2011; CUNHA et al., 2011).

The germination speed index (GSI) of *Schomburgkia gloriosa* was higher, particularly in the medium supplemented with activated charcoal, followed by *E. viviparum* and *A. variegata*. Although the germination rates of *A. variegata* and *E. viviparum* were not significantly different in the studied media, the addition of 0.3% activated charcoal induced a 13.6 and 11.6% higher seed germination rate in *S. gloriosa* than the other MSØ and ½ MS media, respectively, without this component (Figure 3).



**Figure 3.** Speed Index of Germination (GSI) of three orchid species cultivated *in vitro* in different culture media. Means followed by the same letter in a column and capital letter in a row do not differ in the Tukey test at 5% probability.

Although the effects are species-specific, the addition of activated charcoal to the culture media commonly promotes some beneficial effects, e.g., the improvement of embryo development (CHAGAS et al., 2005) and of root growth (RIBEIRO et al., 2000). In addition, it influences the uptake of vitamins, inhibitory substances in the medium or toxic products released by explants, as well as the maintenance of pH and growth regulators (THOMAS, 2008).

In a study on *Epidendrum ibaguense* Kunth., Hossain (2008) tested the addition of activated charcoal to the medium and observed an increased number of germinated seeds, stimulation of early plantlet rooting and significant induction of protocorm formation with larger size ( $1.63 \pm 18$  mm) than the media without this component ( $1.16 \pm 11$  mm).

Orchidaceae species have different responses to supplementation of nutrient media with charcoal addition. The MS medium with activated charcoal favored the development in height, leaf number and length and number of roots of *Aspasia variegata* and *Epidendrum viviparum* plantlets. In terms of plant height and root number, the performance of *Schomburgkia gloriosa* was identical in all tested culture media. The presence of activated charcoal favored the root length of *A. variegata* compared to other species. For *E. viviparum* on the other hand, the same characteristic was increased by the absence of this component. For *S. gloriosa*, the presence or absence of activated charcoal had no influence on root length (Table 2).

**Table 2.** Mean values of plant height, leaf number, leaf length, root number and length of the orchids *Aspasia variegata*, *Epidendrum viviparum* and *Schomburgkia gloriosa*.

Species	Culture medium			
	½MS	MSØ	½MS ACh.	MSØ ACh.
	Plant height (cm)			
<i>Aspasia variegata</i>	1.90 aB	1.24aC	1.95aB	2.01aA
<i>Epidendrum viviparum</i>	0.98 bC	0.98bC	1.67bB	1.92bA
<i>Schomburgkia gloriosa</i>	0.53 cA	0.53cA	0.53cA	0.53cA
	Leaf number (un)			
<i>Aspasia variegata</i>	1.43cC	1.37bC	3.15aB	3.59aA
<i>Epidendrum viviparum</i>	1.83aA	1.99aA	1.92bA	2.00bA
<i>Schomburgkia gloriosa</i>	1.61bA	1.41bA	1.39cA	1.41bA
	Leaf length (cm)			
<i>Aspasia variegata</i>	0.46bC	0.43bD	0.65bB	0.69bA
<i>Epidendrum viviparum</i>	0.71aB	0.71aB	0.74aB	1.03aA
<i>Schomburgkia gloriosa</i>	0.45bA	0.45bA	0.43cA	0.45bA
	Root number (un)			
<i>Aspasia variegata</i>	2.00aB	1.33aC	3.01aA	3.16aA
<i>Epidendrum viviparum</i>	1.42bB	1.37aB	1.88bA	1.89bA
<i>Schomburgkia gloriosa</i>	1.50bA	1.51aA	1.59cA	1.59cA
	Root length (cm)			
<i>Aspasia variegata</i>	0.64bC	0.66bC	1.07aB	1.16aA
<i>Epidendrum viviparum</i>	1.10aA	1.11aA	0.80bB	0.83bB
<i>Schomburgkia gloriosa</i>	0.53cA	0.53cA	0.52cA	0.53cA

Means followed by the same letter in a column and capital letters in a row do not differ in the Tukey test at 5% probability.

Activated charcoal, aside from absorbing phenolic compounds, induces increased shoot formation and a greater number of roots, favoring plantlet support in the culture medium (LEDO et al., 2007).

A study on *Cattleya bicolor* Lindl. reported that apart from darkening the medium, activated charcoal is related to significant results concerning root growth of the plantlets (PRIZÃO et al., 2012). Dezan et al (2012), for example, found that the reduction in the salt concentration of MS culture medium was beneficial for the *in vitro* development of *Schomburgkia gloriosa* plantlets, as similarly found in this study with the same species.

#### 4. CONCLUSIONS

The culture media ½ MS and MSØ with addition of activated charcoal favors *in vitro* germination and the germination speed index (GSI) for the three orchid species of this study. Therefore, the development of the species *Aspasia variegata*, *Epidendrum viviparum* and

*Schomburgkia gloriosa* differed in response to activated charcoal in the culture medium.

#### AUTHORS CONTRIBUTIONS

**V.L.H.:** field analysis, data collection and analysis, manuscript preparation and review. **L.A.L.:** data collection and analysis, **E.G.P.:** data analysis and interpretation, manuscript critical review manuscript. **W.K.:** manuscript preparation and review. **C.A.S.:** manuscript preparation and review.

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