

## ***In vitro* behaviour of *Aspasia variegata*, an epiphytic orchid from the Brazilian Cerrado**

### **Comportamento *in vitro* de *Aspasia variegata*, uma orquídea epífita do cerrado brasileiro**

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#### **ABSTRACT**

*Aspasia variegata* occurs naturally in the savanna of the Mato Grosso do Sul State, Brazil and it has been widely collected for its beautiful flowers. Additionally, its habitat has been greatly reduced and little or no investigation of its spread has been performed. Aiming to establish a protocol to obtain seedlings of the orchid *A. variegata*, different compositions of culture medium were tested to identify which one provided better *in vitro* growth and development and to assess the influence of these media in seedling acclimatisation. Thus, seeds obtained from mature capsules were inoculated in Knudson culture medium for 120 days until the protocorm stage. They were transferred to different culture media formulations, including MS and Knudson with half or full formulation, and 3.0 and 6.0g L<sup>-1</sup> activated charcoal were added to them or not. After 180 days of protocorm inoculation, seedlings were evaluated for length of roots and shoots, number of roots and leaves, and chlorophyll contents. After that, seedlings were transferred to trays containing a mixture of Plantmax<sup>®</sup> and coconut fibre (1:1) for acclimatisation. Best results for the *in vitro* growth of *A. variegata* were obtained with the use of MS medium supplemented with 6.0g L<sup>-1</sup> activated charcoal. Higher levels of chlorophyll were obtained, however, in treatments containing MS salts without activated charcoal presence, and lower levels in media containing Knudson salts with the presence of activated charcoal. The seedlings originated by higher chlorophyll levels during *in vitro* cultivation presented the highest survival rates and better development in the acclimatisation phase.

**Key words:** *orchidaceae*, activated charcoal, chlorophyll content, acclimatisation.

#### **RESUMO**

A orquídea *Aspasia variegata* ocorre naturalmente no Cerrado Sul-Matogrossense, e tem sido bastante coletada de seu habitat, devido à beleza de suas flores. Adicionalmente, sua área de ocorrência tem sido bastante reduzida e pouco ou nenhum estudo visando a sua multiplicação tem sido realizado. Objetivando

estabelecer protocolo para obtenção de mudas de *A. variegata*, diferentes composições de meio de cultivo foram testadas para observar aquela que propicia melhores condições para crescimento e desenvolvimento dos protocormos *in vitro* e ainda a influência desses meios na fase de aclimatização das mudas. Para tal, foram utilizadas cápsulas maduras para obtenção das sementes, as quais foram inoculadas em meio de cultivo básico Knudson por 120 dias até o desenvolvimento de protocormos. Estes foram então transferidos para diferentes formulações dos meios de cultivo MS e Knudson, variando a concentração de sais e de carvão ativado (0; 3,0 e 6,0g L<sup>-1</sup>). Após 180 dias da inoculação dos protocormos, foram realizadas avaliações referentes ao comprimento da maior raiz e da parte aérea, número de raízes e folhas, e quantificados os teores de clorofila. Em seguida, as mudas foram transferidas para bandejas contendo uma mistura de Plantmax<sup>®</sup> e Fibra de Coco (1:1) para aclimatização. Melhores resultados para o crescimento *in vitro* de *A. variegata* foram obtidos com a utilização do meio de cultura MS, acrescido de 6,0g L<sup>-1</sup> de carvão ativado. No entanto, os maiores níveis de clorofila foram obtidos nos tratamentos contendo sais MS desprovidos de carvão ativado, e os menores nos meios contendo sais Knudson providos com carvão ativado. As plantas oriundas dos tratamentos com maiores teores de clorofila total durante a fase *in vitro* obtiveram as maiores taxas de sobrevivência e melhor desenvolvimento na fase de aclimatização.

**Palavras-chave:** *orchidaceae*, carvão ativado, clorofila, aclimatização.

#### **INTRODUCTION**

The *Orchidaceae* family has approximately 35,000 species and several hybrids, being one of the largest families in the plant kingdom (SUTTLEWORTH et al., 1994). In Brazil, around 2.400 species of orchids are known, which represents approximately 10% of all the species in this family

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(BARROS, 1996). *Aspasia* Lindl. is a plant genus first described by John Lindley in 1832 in The genera and species of orchidaceous plants. According to ULLMANN (2007) this genus has around eight epiphyte species, comprising medium-sized plants with long floral stems. *Aspasia variegata* Lindl. is found in Colombia, Venezuela, Trinidad, Guiana, Suriname and Brazil (WILLIAMS, 1974). Although the cultivation of exotic orchids has helped to make Brazil internationally famous, they have been obtained, almost without exception, through the predatory exploration of tropical forests (KÄMPF, 2000). The uncontrolled exploitation of these natural resources has resulted in the degradation of areas in almost all Brazilian territory (FERREIRA, 2000).

The orchid *A. variegata* is an epiphytic species of the Brazilian savanna which is found on tree species present in riparian forests very near the watercourse. This ambience provides good conditions for their germination and development. The species is prized by collectors because of its high ornamental value (Figure 1 A), and unfortunately its populations have decreased drastically over the last few years because of depredation and the destruction of their habitats. In addition, the specialist literature has no information about *in vitro* multiplication of *A. variegata*. A possible consequence is the future extinction of the species, and provides justification for the propagation and conservation of this valuable germplasm. MAHENDRAN et al. (2013) says that orchids in the wild are endangered as a consequence of environmental disruption, succession of natural habitats and over-exploitation for horticultural purposes. In situ conservation of dwindling populations of endangered orchid species is very difficult because of their slow growth and poor seed germination.

Consequently, *in vitro* culture can be an interesting technique for both propagation and

germplasm conservation of this species. Successful *in vitro* orchid propagation, however, is influenced by many factors such as plant genotypes, several factors involving media composition (GNASEKARAN et al., 2012) and activated charcoal (THOMAS, 2008).

The cultivation medium used in propagation is a determining factor for the success of the *in vitro* cultivation of orchids. The MS culture medium (MURASHIGE & SKOOG, 1962) is the one used most frequently in *in vitro* propagation, but several research studies on orchids use a modified basic Knudson medium (ARDITTI & ERNST, 1993). Among the additives tested in culture media, activated charcoal has been widely used. Studies have reported, however, that the concentration of salts in the culture medium directly influences the growth of *in vitro* orchids, whether by the lack or by the excess thereof (PARK et al., 2004; FIGUEIREDO et al., 2007; STANCATO et al., 2008). Furthermore, another element that requires serious attention is the acclimatisation phase of the seedlings, because the anatomical and physiological aspects of vitroplants have to be adequate for new *ex vitro* ambient conditions that differ from *in vitro* conditions in all aspects. Therefore, for the purpose of obtaining an initial efficient propagation method, we decided to research the effect of different formulations of semi-solid media with and without the addition of activated charcoal on *in vitro* development of *A. variegata* seedlings and its importance in the acclimatisation phase.

## MATERIALS AND METHODS

*Aspasia variegata* protocorms obtained from germinated seeds were transferred to glass flasks (250mL) containing 40ml of Knudson culture medium with 6.0g L<sup>-1</sup> agar and 20.0g L<sup>-1</sup> sucrose, and

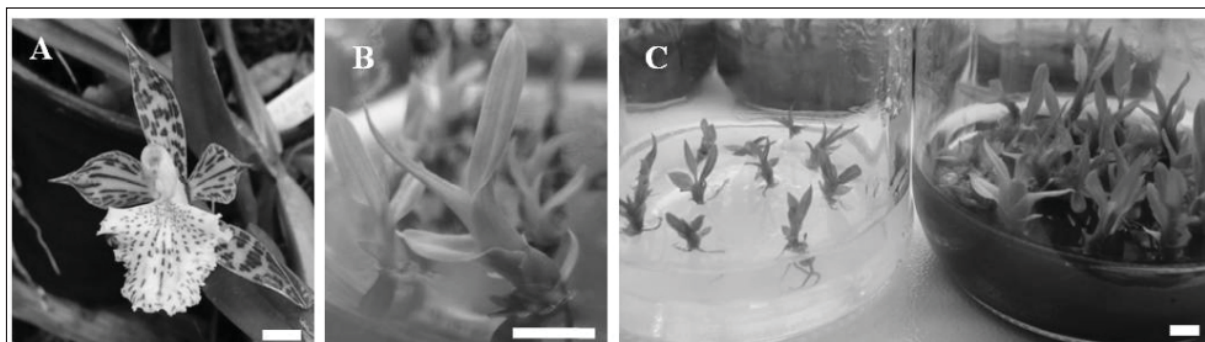


Figure 1 - Photographs showing details of the floral beauty of the *Aspasia variegata* adult plant (A); seedlings of *A. variegata* cultivated *in vitro* (B); and comparison of seedlings cultivated on Knudson medium without activated charcoal (left) and on Murashige and Skoog medium (right) with activated charcoal (6mg L<sup>-1</sup>).

MS medium (MURASHIGE & SKOOG, 1962) with 6.0g L<sup>-1</sup> agar and 30.0g L<sup>-1</sup> sucrose. All the media for the *in vitro* experiments had 0.1g L<sup>-1</sup> inositol, with and without activated charcoal, and pH 5.8±0.1 adjusted before the autoclave process (121°C and 104KPa for 20 minutes). To maximise the *in vitro* development of the *A. variegata* protocorms, different formulations of the MS and Knudson media were tested, resulting in the following treatments: T1 – Total MS, T2 – MS with half the salts, T3 – Total MS supplemented with activated charcoal (3.0g L<sup>-1</sup>), T4 – MS supplemented with activated charcoal (6.0g L<sup>-1</sup>), T5 – Total Knudson, T6 – Knudson with half the salts, T7 – Total Knudson supplemented with activated charcoal (3.0g L<sup>-1</sup>), T8 – Total Knudson supplemented with activated charcoal (6.0g L<sup>-1</sup>).

The experimental design used was entirely randomised and comprised eight treatments with ten replications each. Each replication comprised a glass flask containing 15 protocorms. All of the *in vitro* experiments were performed in a plant growth room with a 16-hour photoperiod and irradiation of 36µmol m<sup>-2</sup> s<sup>-1</sup> and temperature of 27±2°C. The protocorm conversion was evaluated at 30 and 60 days after inoculation, counting the seedlings obtained from protocorm conversion. Cultures were then co-cultivated at 90 days and analysed at 180 days after protocorm inoculation, when the *in vitro* experiment was finished. To this end, vitroplants from five replications (flasks) of each treatment were used to measure the length of the main root and aerial part, the number of leaves and roots, and the aerial part of these vitroplants was used to quantify the levels of chlorophylls a, b, and total. Three samples of 0.250g of fresh plant matter were weighed for each treatment, mashed in 8ml of acetone (P.A., Synth). The samples were centrifuged (3500rpm; 2min). The supernatant was used to obtain the absorbance (%) in the wavelength bands of 645 and 663nm with a spectrophotometer. The chlorophyll contents were determined by the Arnon method (ARNON, 1949).

The seedlings of the remaining flasks (05) of each treatment were used to evaluate the acclimatisation phase. Initially, the length of the aerial part and main root was measured with a ruler, and then the number of roots and leaves were counted. Immediately, the vitroplants were placed in expansible polystyrene trays measuring 18cm width x 23cm length x 5cm height and containing five holes on the bottom holding a mixture of Plantmax® and coconut fibre (1:1). The trays were placed on benches and kept for 90 days in a greenhouse with an intermittent irrigation system comprised of microsprinklers

activated by a timer with two daily irrigation cycles of two minutes each. The plants received leaf composting (NIPOKAN®) at weekly intervals. After the acclimatisation period, the plants were evaluated in terms of the number of roots, height of the aerial part (cm), number of leaves, size of the main root (cm) and total fresh weight (mg).

All data were subjected to statistical analysis with SISVAR software (FERREIRA, 2000), and the mean values were compared by the Tukey test at 5% probability.

## RESULTS AND DISCUSSION

The total experiment lasted for 12 months, from seed inoculation to acclimatisation. The differentiation of protocorms of *A. variegata*, that is, the beginning of formation of leaves and roots was satisfactory in all of the tested media. However, the media containing the Knudson salts having a slight numerical advantage in relation to those with the MS salts after the first 30 days of inoculation, when a statistical difference was detected in the differentiation of protocorms in the medium with half Knudson salts (94.16%) in relation to the medium with MS salts with 3.0g L<sup>-1</sup> of activated charcoal (78.80%). Additionally no statistical difference was detected after 60 days (data not shown). This initial difference in response may be related to the low nutrient requirement in the initial germination steps in orchids. MAHENDRAN et al. (2013) affirm that the nutrient regime for orchid culture is species-specific and no single culture medium is universally applicable to all orchid species. They found great differences in *Cymbidium bicolor in vitro* seed germination when they compared four culture media, varying from 96.6% (Lindemann medium) to 62.7% (Knudson C medium) in total seed germination.

In relation to the parameters evaluated in the seedlings obtained from protocorm conversion, there was a significant effect of the culture media (Tables 1 and 2). The length of the largest root was improved when the culture medium contained MS salts, independently of its concentration (half or total salts) and activated charcoal presence. The worst performances for this parameter occurred in the media containing Knudson salts. It is worth mentioning that the Knudson medium displays considerable differences in relation to MS medium, such as lower nutritional concentration. The beneficial effect of activated charcoal on seedling development was more evident when it was added to MS medium. Other authors

Table 1 - Development analysis of *Aspasia variegata* seedlings cultivated *in vitro* for 180 days in different compositions of Murashige and Skoog (MS) and Knudson (KC) media with and without activated charcoal (AC). Chapadão do Sul, Mato Grosso do Sul, 2011.

Medium	Length (cm)		Number	
	Root	Aerial part	Roots	Leaves
MS	0.703 ab	1.592 c	4.183 bc	6.350 c
MS/2	0.737 a	1.463 c	4.617 b	6.467 c
MS + 3 g L <sup>-1</sup> AC	0.630 abc	2.351 ab	5.299 ab	6.650 bc
MS + 6 g L <sup>-1</sup> AC	0.723 ab	2.831 a	6.817 a	8.068 a
KC	0.483 cd	1.443 c	5.400 ab	6.383 c
KC/2	0.400 d	1.098 c	4.067 bc	5.650 c
KC + 3 g L <sup>-1</sup> AC	0.520 bcd	1.760 bc	5.633 ab	7.917 a
KC + 6 g L <sup>-1</sup> AC	0.428 cd	1.172 c	3.000 c	6.983 abc
CV (%)	17.34	19.31	16.16	9.63

Means followed by the same letter in the same column do not differ from each other by Tukey test at 5% significance.

also found a positive relationship between activated charcoal levels and orchid root growth; for example, *Laelia tenebrosa* showed linear root growth in relation to concentrations of charcoal (ARAUJO et al., 2006). These authors also found, however, that the media containing the highest salt concentrations needed a greater amount of activated charcoal to obtain a similar result. The presence of activated charcoal reduces light penetration to the roots, thus providing suitable conditions for root growth, and provides a favourable environment for accumulated photoprotection of auxin or co-factors (THOMAS, 2008). Some species of orchids, however, have no influence of the activated charcoal in root growth. CHAPLA et al. (2009) observed that the presence of activated charcoal in culture medium inhibited the growth of the primary root of *Miltonia flavescens* Lindl., which presented better development in charcoal-free medium. These different responses demonstrate the importance of

studying the action of additives in the development of different species of orchids.

The length of the aerial part benefited from activated charcoal presence in the MS medium, the best results being obtained with the addition of 6.0g L<sup>-1</sup> (Table 1), and consequently the lowest lengths of the aerial parts were obtained in the seedlings from the media containing MS salts without charcoal and from the media containing Knudson salts (Figure 1). Therefore, in general activated charcoal presence was beneficial for the development of the aerial part of the seedlings. PRIZÃO et al. (2012) confirmed that the presence of charcoal was particularly important in inducing roots and sprouts in the double hybrid of orchid 'BLC Pastoral Innocence' when inoculated in medium without growth regulator.

The number of leaves and roots benefited from activated charcoal presence in the MS and Knudson media, the best responses being obtained

Table 2 - Mean levels of chlorophylls *a*, *b* and *total* ( $\mu\text{g g}^{-1}$  fresh matter) in leaves of *Aspasia variegata* cultivated *in vitro* in different compositions of Murashige and Skoog (MS) and Knudson (KC) media with or without activated charcoal (AC). Chapadão do Sul, Mato Grosso do Sul, 2011.

Treatment	Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	Total chlorophyll
MS	20.609 a	14.434 a	35.044 a
MS/2	21.204 a	13.870 a	35.074 a
MS + 3g L <sup>-1</sup> AC	17.595 ab	11.388 a	28.983 a
MS + 6g L <sup>-1</sup> AC	15.878 b	12.738 a	28.617 a
KC	18.098 ab	12.073 a	30.171 a
KC/2	19.888 ab	12.645 a	32.283 a
KC + 3g L <sup>-1</sup> AC	4.672 c	3.076 b	7.748 b
KC + 6g L <sup>-1</sup> AC	6.208 c	4.038 b	10.247 b
CV (%)	12.00	19.37	12.81

Means followed by the same letters in the same column do not differ from each other by Tukey test at 5% significance.



with the addition of  $6.0\text{g L}^{-1}$  in the complete MS medium (Table 1), and consequently the lowest number of leaves was obtained in the seedlings from the media without charcoal. This shows that seedlings cultivated in the medium with higher salt availability and charcoal presence benefited in terms of growth parameters. PRIZÃO et al. (2012) confirmed that the use of activated charcoal is useful for root induction in seedlings of *Cattleya bicolor*.

With regard to the chlorophyll evaluations (Table 2), an expressive and significant reduction was evident in the contents of chlorophylls a, b and total in all seedlings cultivated in the Knudson medium with activated charcoal. In plants cultivated in the medium with MS salts, this reduction was statistically different only for the contents of chlorophyll a. This response seems to be related to the fact that activated charcoal adsorbs a considerable portion of beneficial (e.g. nitrogen) and harmful medium components. Therefore, in general the comparison of chlorophyll contents between seedlings cultivated on MS medium (half or total salts), with or without activated charcoal, shows that chlorophyll is almost the same, perhaps because MS medium is considered as one with a high concentration of salts. Consequently the nutritional components adsorbed by activated charcoal still maintained satisfactory levels of nutrients and we assume that activated charcoal reduces chlorophyll content always that it is on medium composition (Table 3). This was more determinant when seedlings were cultivated on Knudson medium because there is less nitrogen than in MS medium. It is clear, however, that activated charcoal presence further decreased nutrient availability on Knudson medium. All these factors damaged seedling growth and development, resulting in several morphological factors as minor root number and root length in seedlings came from Knudson medium added with activated charcoal, resulting in reduced nitrogen absorption affecting chlorophyll synthesis. GALDIANO JÚNIOR et al. (2012), using MS medium, did not find a great difference in the chlorophyll levels in seedlings of *Cattleya loddigesii* cultivated in different activated charcoal concentrations, thus corroborating the results we obtained with MS medium.

Sucrose level is another factor that can induce changes in chlorophyll synthesis (DIGNART et al., 2009; GALDIANO JÚNIOR et al., 2013). Several researchers have reported that, for many species, the presence of sucrose in the culture medium is the main reason for reduction in chlorophyll contents and consequently in photosynthesis; low levels of sucrose in the culture medium were correlated to an

elevated potential of carbohydrate production by the photosynthetic pathways (ADELBERG et al., 1999; KANECHI et al., 1998). GALDIANO JÚNIOR et al. (2013) compared different sucrose levels in *Cattleya loddigesii* development and observed that  $20\text{g L}^{-1}$  of sucrose support growth did not affect chlorophyll synthesis. This behaviour was not seen in the present experiment, corroborating observations made by DIGNART et al. (2009) for the orchid *Cattleya walqueriana*. They did not observe any correlation between the levels of sucrose and the levels of chlorophylls of *in vitro* plants.

The capacity for survival and development of seedlings during the acclimatisation phase (Table 3) was determined by the plant length during the *in vitro* cultivation phase (Table 1). Therefore, those seedlings from the Knudson medium supplemented with  $3.0\text{g L}^{-1}$  and  $6.0\text{g L}^{-1}$  of activated charcoal resulted in only 24.0% and 36.0% of survival respectively, resulting in higher mortality rates (data not shown), similarly to those seedlings which presented the worst growth parameters (Table 1) and the lowest levels of chlorophyll *in vitro* (Table 2). The other treatments resulted in mortality rates averaging 15%. Therefore, the size of the aerial part was essential for plant survival, given that the development of algae was for a threat to the survival of the smaller seedlings. The development of algae was related to the variations in atmospheric humidity, leading to eventual humidity in the trays. DORNELES & TREVELIN (2011), during the acclimatisation of *Cattleya intermedia*, also observed an intolerance to excessive humidity, whereby some seedlings in sphagnum substrate were lost because of the decomposition of their roots. Thus, observing the growth data of the seedlings after acclimatisation (Table 3), i.e. fresh weight, presence of bud and size of roots and aerial parts, we can confirm that the best performances during this phase were obtained by the seedlings from the media that were more concentrated in salts (MS) and presented higher chlorophyll contents. Therefore, this last *in vitro* factor is apparently essential for a good performance in the acclimatisation phase. Curiously, seedlings of *A. variegata* that presented higher growth during the acclimatisation phase were not those that came from MS medium containing activated charcoal, which presented the best behaviour *in vitro*, but those that came from MS medium (half and total salts) without activated charcoal (Table 3). Nonetheless, survival average (90%) did not differ in these treatments during the acclimatisation phase. GALDIANO JÚNIOR et al. (2012) concluded that the use of activated charcoal during *in vitro* culture provided

Table 3 - Data related to the size and mean number of roots and aerial part and total fresh weight of *in vitro* seedlings of *Aspasia variegata* cultivated in different compositions of Murashige and Skoog (MS) and Knudson (KC) media with or without activated charcoal (AC), and acclimatised in an agricultural oven in coconut powder substrate. Chapadão do Sul, Mato Grosso do Sul, 2011.

Treatment	Number		Size (cm)		Fresh weight (mg)
	Roots	Buds	Root	Aerial part	
MS	7.96 ab	0.54 abc	5.00 a	4.50 a	473.92 a
MS/2	7.76 abc	0.60 ab	5.04 a	4.72 a	561.92 a
MS + 3g L <sup>-1</sup> AC	6.20 bcd	0.72 a	3.32 ab	2.52 bc	207.84 bc
MS + 6g L <sup>-1</sup> AC	5.55 ed	0.82 a	4.00 ab	2.95 b	261.23 b
KC	3.95 e	0.24 bc	1.48 d	1.67 cd	95.48 c
KC/2	8.60 a	0.68 a	2.00 cd	1.88 cd	182.44 bc
KC + 3g L <sup>-1</sup> AC	6.05 bcd	0.16 c	1.53 d	1.74 cd	117.89 bc
KC + 6g L <sup>-1</sup> AC	6.00 cd	0.13 c	1.56 d	1.50 d	83.31 c
CV (%)	30.96	86.98	45.50	36.77	63.08

Means followed by the same letters in the same column do not differ from each other by Tukey test at 5% significance.

greater development efficiency in terms of both *in vitro* and *ex vitro* survival of *Cattleya loddigesii*.

The transplantation stage continues to be a major bottleneck in the micropropagation of orchids. A substantial number of micropropagated plantlets fail to survive when transferred from *in vitro* conditions to a greenhouse or field environment, as these have substantially lower relative humidity, and higher and more intense light levels that are stressful for micropropagated plants compared with *in vitro* conditions (CHUGH et al., 2009).

Some aspects involving nutritional medium composition and activated charcoal's capacity to adsorb nutrients need to be investigated further. Additionally, the control of algae growth during the acclimatisation phase deserves special attention in future *A. variegata* propagation.

## CONCLUSION

MS salts supplemented with activated charcoal were the best medium for the *in vitro* growth of *Aspasia variegata* seedlings, which showed high chlorophyll contents during *in vitro* culture, resulting in high survival rates and better development during the acclimatisation phase. Activated charcoal in combination with Knudson salts resulted in a considerable reduction of seedlings' chlorophyll levels, producing the worst performances in the acclimatisation phase.

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