

Seed Germination of *Cattleyaintermedia* and *Cattleyawarneri* in Alternative culture Media

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Abstract

*In nature, germination rate of orchid seeds is very low, therefore asymbiotic germination is recommended for achieving greater number of seedlings. Composition of culture medium has great influence on performance of in vitro cultures and directly affects complexity of its preparation. The aim of this study is to evaluate germination and development of protocorms and seedlings obtained from seeds of *Cattleyaintermedia* and *Cattleyawarneri* in alternative culture media. Media tested were: Murashige and Skoog with half of macronutrients ($1/2MS$), Knudson C (KC), supplement for orchid B&G[®] (BG), supplement for orchid B&G[®] modified with two-thirds of salts (E) and commercial fertilizer Kristalon Orange[®] (KR). Seeds were sterilized, inoculated with the same volume of solution in flasks and kept in a controlled environment until assessment, which occurred 100 days after inoculation. Seed germination was assessed by the number of protocorms formed and their development through classification into two stages: protocorms and seedlings. For both species, it was found that culture media MS and E resulted in the highest amount of protocorms formed, whereas media KR and E provided the best development of protocorms thus resulting in greater conversion into seedlings. Furthermore, it was observed that in general the use of banana pulp is not beneficial for seed germination and protocorm formation of the evaluated orchid species. Additionally, results indicate that a favorable medium for germination does not deliver the same behavior for the development of seedlings.*

Keywords: orchids, asymbiotic germination, culture media, protocorms, banana pulp

1. Introduction

Orchidaceae family has a vast number of genera and species spread throughout the world, but its highest concentration is found in tropical regions. *Cattleyaintermedia* and *Cattleyawarneri* species are native to Brazil: the former is native to Brazilian South and Southeastern Coastal Atlantic Forest and the latter is endemic to Brazilian states of Espírito Santo and Minas Gerais (JORGE et al., 2011).

As they are beautiful flowers with high commercial value, these species suffer from predatory extraction. Therefore, studies aimed at improving orchid propagation techniques become more important for conservation of the species.

In vitro germination of orchid seeds allows larger numbers of seedlings and accelerates production process. Development of *in vitro* plantlets occurs faster and provides seedlings of better quality, more uniform and free from diseases.

It is well known that on *in vitro* culture medium has great influence on responses of plant tissues as it is responsible to provide necessary stimulus for triggering processes of differentiation and nutrients for formation of new cells and tissues. *In vitro* cultivation of orchids requires specific culture medium for distinct species in order to provide the best conditions for growth (VENTURA, 2007).

Traditional culture media for orchids need a series of chemical compounds in their formulation, which are often expensive and difficult to acquire. This situation makes the production of seedlings more difficult, especially by small farmers who want to begin in this activity. Research studies have indicated that one way to simplify production and reduce costs of medium is to use commercial fertilizers as nutrient base (MORAES et al., 2009a; MORAES et al., 2009b; STANCATO et al., 2001; DRONK, 2004). Furthermore, it is possible to add organic compounds to replace or supplement the levels of sugar, vitamins, amino acids and growth regulators in culture medium (GEORGE et al., 2008).

One of the most used organic compounds in culture media for orchids is banana pulp. The advantages of banana pulp for the development of plants from protocorms are already well known, however, there are few studies about the influence of this organic compound on seed germination (ARDITTI et al., 1984; ISLAM et al., 2000; FLASHSLAND et al., 1996; SHIAU et al., 2002, ZENG et al., 2012). The main goal of the present study was to analyze performance of different culture media on *in vitro* germination of seeds and on protocorms growth of *Cattleyaintermedia* and *Cattleyawarneri*. Another goal was to analyze the influence of adding banana pulp to the media used for germination of these species.

2. Material and Methods

Experiments were performed in Laboratory of Plant Cell Culture at UNIVALI university, in Itajaí, Santa Catarina. Seeds used in experiments were donated by orchid growing enterprise Orquidário Quinta do Lago, located in Itaipava, Rio de Janeiro.

For both species, experiments consisted of five culture media with and without banana pulp distributed as a completely randomized 5x2+1 factorial design and also an additional treatment.

Media tested were: Murashige and Skoog modified with half of macronutrients ($\frac{1}{2}$ MS), Knudson C (KC), supplement for orchid B&G[®] (BG), supplement for orchid B&G[®] modified with two-thirds of salts (E) and commercial fertilizer Kristalon Orange[®] (NPK 6-12-36) (KR). The additional treatment (S) was prepared with organic compounds (50 g.l⁻¹ of banana pulp, 50 g.l⁻¹ tomato pulp, 50 g.l⁻¹ of avocado pulp, 50 g.l⁻¹ papaya pulp, 50 ml.l⁻¹ of potato broth, 120 ml.l⁻¹ of coconut water).

Media $\frac{1}{2}$ MS, KC e KR 30 g.l⁻¹ were supplemented with 30 g.l⁻¹ of sucrose, 3 g.l⁻¹ of activated charcoal and 7 g.l⁻¹ of agar. BG based media were combined with 10 g.l⁻¹ of agar and E medium was combined with 10 grams of sucrose in order to match concentration of 30 g.l⁻¹ of all other media. For production of additional medium S, all organic compounds (papaya, avocado, tomato, banana and potato broth pulps and coconut water) were homogenized using a blender and 20 g.l⁻¹ of sucrose, 3 g.l⁻¹ of activated charcoal and 8 g.l⁻¹ of agar were added to it. Then, pulp of ripe banana (Prata variety) was added to it in concentration of 100 g.l⁻¹ and also homogenized. The pH of every culture media was adjusted to $5,7 \pm 0,05$ before sterilization in autoclave at 120° for 20 minutes.

Sterilization and inoculation of seeds were performed in a blade flow chamber weighing 0,7 g of seeds, for both species, placing them in a disposable 25 ml syringe and then adding 20 ml of 2% active sodium hypochlorite with 2 drops of Tween 20. Seeds were immersed in the solution for 15 minutes and then washed four times with autoclaved water. Subsequently, sterilized seeds were placed in a beaker with 80 ml of autoclaved water forming a solution of seeds. This solution was under constant stirring by magnetic stirrer in order to keep seeds in suspension. Using sterile micropipette and tips, 800 μ l of seeds solution was deposited in each flask of 100 ml capacity already containing 15 ml of culture medium. Flasks were sealed with transparent polypropylene lid and edges secured with transparent film of PVC (Rolopac®). These flasks containing the seeds were kept in a controlled environment: temperature of $25 \pm 3^\circ\text{C}$ and 16 hours per day of light with average irradiance from 30 to $50 \mu\text{mol m}^{-2}\text{s}^{-1}$. A fully randomized design with four replications was carried out as statistical model considering each flask as one replication.

Analysis of seed germination was carried out by counting protocorms formed 100 days after inoculation of *Cattleyaintermedia* and after 120 days for *Cattleyawarneri*. Analysis of the initial protocorm development was performed by dividing formed protocorms into two classes: protocorms - chlorophyllous germinated seeds with or without leaf primordia and without rooting; and seedlings - protocorm with foliar and root primordia or formed roots.

Data were submitted to analysis of variance (ANOVA) and means were compared with Tukey test at 5% of significance with SisVar v.5.1 software (FERREIRA, 2011).

3. Results and Discussion

Twenty days after establishment of the experiment, we observed that germination of both species had started through visual analysis of embryos swelling and production of chlorophyll by protocorms. The exception was the alternative medium (S), in which seeds did not germinate. Because of that this medium was disregarded for statistical analysis and therefore it is not recommended for germination and early development of the species in study.

The results measured in the experiments suggest similar behavior for both species. Culture media with higher germination rate, which formed the largest total number of protocorms from seeds, were $\frac{1}{2}$ MS and E without banana pulp with respectively 410 and 402,33 protocorms formed of *C. intermedia* and 389,25 and 390 protocorms formed of *C. warneri* (Tables 1 and 2). The lowest total protocorm formation was found in KC medium followed by BG.

Nitrogen is an element of great variation among different formulations of culture media and interferes directly in plant development. Dijk& Eck (1995) stated that hybrid embryos of *Cattleya* were unable to use nitrate ions in the first sixty days of development in contrast to ammonium ions. Raghavan& Torrey (1994) also showed that ammonia was critical for germination and early development of a *Cattleya* hybrid. Rasmussen (1995) observed a reduction in percentage of seed germination of *Dactylorhizamajalis* when increasing nitrate concentration in culture medium. Recently, Suzuki et al. (2010) observed that the presence of ammonia is more efficient for germination of *Cattleya bicolor* than nitrate as higher germination rates were found in the medium with greater ammonia:nitrate proportion.

Unlike results of the literature, seed germination of *C. intermedia* and *C. warneri* does not seem to suffer much influence of the relationship between levels of ammonia and nitrate since greater protocorm formation occurred in MS medium, which has an intermediate (0.52) ammonia:nitrate proportion, and specially in E medium, which has a low ammonia:nitrate proportion (0.17) and the lowest concentration of ammoniacal nitrogen among all tested media. These results suggest that the choice for nitric or ammoniacal forms varies according to each species (ADAMS & ATTIWILL, 1982).

As stated by Stewart (1989), orchids that germinate *in vitro* can be divided into two groups according to their basic nutritional needs. Orchids with seeds that germinate in simple culture media, such as KC, belong to the first group. The second group comprises species that require media with higher amounts of macronutrients and micronutrients, such as MS medium. Suzuki et al. (2009) observed higher germination percentage of *Hadrolaeliatenebrosa* with KC than with $\frac{1}{2}$ MS or VW (VACIN & WENT, 1949), indicating that this species achieves better germination in poorer media culture.

Lo et al. (2004), using the same media, obtained seed germination of *Dendrobiumtosaense* only on ½ MS medium. Roy et al. (2011) stated that *Vanda coerulea* germinates at better rate in media with higher levels of nutrients such as Phytamax and MS compared with VW and KC. Hossain & Rubel (2013) achieved higher levels of germination of *Spathoglottisplacata* in MS medium, which is richer in nutrients when compared to Phytamax.

Results observed in this study indicate that both *C. intermedia* and *C. warneri* seem to belong to the second group, because better results of protocorm formation were found in media with higher levels of macronutrients, as well as better germination. Due to higher osmotic potential of the medium MS it is advisable to reduce salt concentration for germinating orchid seeds. Therefore, in this study we used MS medium with half the concentration of macronutrients. The fact that BG medium achieved inferior results than E medium for protocorm formation is due to higher salt concentration of medium BG, which consequently leads to higher osmotic potential of the medium when used with a 100% concentration of salts as well as does MS medium.

Regarding the use of banana pulp, we observed a detrimental effect on seed germination because there was less formation of protocorms in all culture media with pulp when compared to the same medium without banana pulp. These results differ from those reported by Shiau et al. (2002) and Islam et al. (200) on germination and development of *Anoectochilusformosanus* and *Cattleyasp.*, respectively. They observed that homogenized banana pulp increased germination and growth of seedlings. On the other hand, some authors state that the use of banana pulp in the culture medium for germination of orchid seeds reduces germination rate (ARDITTI et al., 1984; YAM & WEATHERHEAD, 1988; ZENG et al., 2012).

One reason for the negative effect on germination achieved with pulp addition may be the increased sucrose concentration in culture medium which consequently increases the osmotic potential of medium, reducing the amount of water available for imbibition and thus hindering initiation of the germination process (GEORGE, 1993; HOSSAIN & RUBEL, 2013). Another relevant factor is discovery of cytokinins on banana pulp (GE et al., 2008). The reduction in germination rate may be related to changes in hormonal balance caused by adding the pulp. Furthermore, the addition of pulp increases nutrient levels in the medium and this may also increase their osmotic potential, thus reducing availability of water and interfering negatively in the germination process.

Regarding the analysis of protocorms development, according to classification into two classes, the medium that allowed higher formation of protocorms was ½ MS followed by E, whereas for development of seedlings KR medium without banana pulp provided better results followed by E medium with banana pulp (Tables 1 and 2).

Table 1: Number of Protocorms and Seedlings Formed from *Cattleyaintermedia* Seeds, after 100 Days of Cultivation

	Culture Media					Average
	½MS	KC	BG	E	KR	
<i>Protocorms</i>						
wiht banana	301.00 Ba	127.33 Bb	137.00 Bb	203.33 Bab	134.66 Bb	180.66
without banana	378.33 Aa	188.66 Ab	213.66 Ab	351.33 Aa	174.00 Ab	261.20
Average	339.66	158.00	175.33	277.33	154.33	cv = 19.04%
<i>Seedling</i>						
wiht banana	56.66 Ab	8.00 Bc	4.00 Bc	103.00 Aa	99.66 Aa	54.26
without banana	31.66 Bc	49.66 Abc	66.00 Ab	51.00 Bb	121.66 Aa	64.00
Average	35.00	28.83	35.00	77.00	110.66	cv = 23.61%
<i>Total of protocorms formed</i>						
wiht banana	357.66	135.33	141.00	306.33	234.33	118.07
without banana	410.00	238.33	279.66	402.33	295.66	173.73
Average	383.83	186.83	210.33	354.33	265.00	cv = 15.12%

* Uppercase letter shows difference in colum and lowercase shows difference in row

Table 2: Number of Protocorms and Seedlings Formed from *Cattleyawarneri* seeds, after 120 Days of Cultivation

	Culture Media					Average
	½MS	KC	BG	E	KR	
<i>Protocorms</i>						
wiht banana	291.00 Ba	108.50 Bc	127.75 Bbc	187.50 Bb	123.25 Bbc	167.60
without banana	365.00 Aa	204.75 Ab	214.50 Ab	347.00 Aa	174.00 Ab	261.05
Average	328.00	156.62	171.12	267.25	148.62	cv = 15.55%
<i>Seedlings</i>						
wiht banana	49.00 Ab	3.25 Bc	4.50 Bc	98.25 Aa	93.00 Ba	49.60
without banana	24.25 Bc	42.25 Abc	56.25 Ab	43.25 Bb	125.00 Aa	58.20
Average	36.62	22.75	30.37	70.75	109.00	cv = 16.78%
<i>Total of protocormos formed</i>						
wiht banana	340.00	111.75	132.25	285.75	216.25	217.20
without banana	389.25	247.00	270.75	390.25	299.00	319.25
Average	364.62	179.37	201.50	338.00	257.62	CV = 11.78%

* Uppercase letter shows difference in colum and lowercase shows difference in row

Results obtained in this study and other studies about seed germination and initial development of seedling indicate the culture medium that promotes a higher rate of germination and protocorm formation is not as effective for further development of seedlings (SUZUKI et al., 2009; SUZUKI et al., 2010; DUTRA et al., 2008; DUTRA et al., 2009, ZENG et al., 2012). Similar results were found by Jorge et al. (2010) also with *C. warneri*, obtaining high levels of germination after 20 days of culture in ½ MS in relation to KC and VW. However, after 90 days they observed greater development and root formation in KC medium.

Also in relation to the use of alternative media for germination and early development of orchid protocorms, Moraes et al. (2009a) and Moraes et al. (2009b) carried out experiments with *Cattleyatigrina* and *Cattleyaloddigessii* seed germination in ½MS and media from commercial fertilizer: Hyponex® (NPK 6,5-9-19) and Kristalon orange®. They verified that after 180 days medium based on fertilizer Kristalon orange®, containing less nitrogen compared to other means, achieved better seedling development, especially regarding number and length of roots. These studies indicate that it is feasible to replace traditional media for alternative formulations, reducing costs and simplifying the process.

The results of this work and the results of the literature state that the choice of culture medium is extremely important for the success of in vitro germination of orchids, which varies according to species in analysis. Maximizing the success of asymbiotic germination depends on identifying abiotic conditions and finding the most appropriate medium for each species.

Capsules of these species produce thousands of seeds and their germination in culture medium does not seem to place much trouble since a small amount of seeds originate large number of protocorms. Actually, in most cases, producers do not use the whole volume of sowing seeds for in vitro germination process and end up storing the rest of it. Thus, the limiting factor for them is not to generate as many protocorms as possible, but to accelerate their development, resulting in savings of material, labor and obtaining faster financial return.

4. Conclusion

Cattleyaintermedia and *Cattleyawarneri* species germinate at better rates in media containing higher amount of nutrients. MS medium with half the concentration of macronutrients and the medium BG with two-thirds of the salt concentration were found to be the most suitable for germination of seeds with the highest total protocorm formation. However, since the number of seeds is not a limiting factor for in vitro cultivation of these orchid species, using adequate media in order to accelerate production of seedlings becomes more interesting because that means savings for the producer. Thus, it is advisable to use KR and E media for germination and seedling development.

Finally, the addition of banana pulp in culture media, in general, did not result in benefits for germination of *C. intermedia* and *C. warneri*.

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