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Use of complex supplements and light-differential effects for micropropagation of *Hadrolaelia purpurata* (= *Laelia purpurata*) and *Encyclia randii* orchids

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ABSTRACT. In the current study, complex (coconut water and ripe-banana pulp) and specific (micronutrients, peptone and activated charcoal) supplements were added to Knudson-C medium (KC) medium to stimulate the *in vitro* propagation of ***Hadrolaelia*** *purpurata* (=*Laelia purpurata*) and *Encyclia randii* orchid species. The highest number of *H. purpurata* seedlings occurred in KC medium containing 90 g L^{-1} banana pulp maintained with 24-hour illumination. Positive effects of the banana pulp addition in KC medium were reported for the formation of seedlings and leaves from *E. randii* seeds. The seedling number from seeds of *E. randii* maintained with continuous illumination was also high in KC medium containing coconut water. Positive effects due to the addition of peptone were reported only in the case of seedling formation from *E. randii* seeds maintained under continuous illumination. The highest number of *H. purpurata* seedlings and leaves of *E. randii* occurred in cultures maintained in continuous illumination. The successful asymbiotic germination of the three-year-old seeds of two Brazilian native species from different regions at reduced costs offers an opportunity for the use of the techniques in commercial nurseries and for *ex situ* conservation of the native and explored orchid species.

Keywords: asymbiotic seed germination, banana, coconut water, peptone, micronutrients, Orchidaceae.

Uso de suplementos complexos e efeito diferencial de iluminação para a micropropagação de orquídeas *Hadrolaelia purpurata* (= *Laelia purpurata*) e *Encyclia randii*

RESUMO. No presente estudo foram adicionados ao meio de cultura Knudson-C (KC), suplementos específicos (micronutriente, peptona, carvão ativado) e complexos (água de coco e polpa de banana) para estimular a propagação *in vitro* das espécies de orquídeas *Hadrolaelia purpurata* (=*Laelia purpurata*) e *Encyclia randii*. O número maior de plântulas de *H. purpurata* foi observado no meio KC contendo 90 g L^{-1} de polpa de banana com iluminação contínua (24h). O efeito positivo da adição de polpa de banana foi descrito para a formação de plântulas e folhas de *E. randii*. O número de plântulas de *E. randii* mantidas com iluminação contínua também foi alto em meio contendo água de coco. Um efeito positivo da adição de peptona foi descrito somente na formação de plântulas de *E. randii* mantidas sob iluminação contínua. O maior número de plântulas de *H. purpurata* e folhas de *E. randii* ocorreu com iluminação contínua. O sucesso na germinação assimbiótica de sementes com três anos de idade, das duas espécies de orquídeas nativas do Brasil, de regiões diferentes, com custo reduzido, é uma técnica oportuna para ser usada em viveiros comerciais, e para a conservação *ex-situ* destas espécies nativas e exploradas de orquídeas.

Palavras-chave: germinação assimbiótica de sementes, banana, água de coco, micronutrientes, peptona, Orchidaceae.

Introduction

Asymbiotic seed germination techniques are employed to preserve and protect orchid species from extinction caused by the continuous loss of their natural habits, which has been occurring since the seventies (ARDITII et al., 1982). Due to the damage provoked in their natural habitats by forest destruction and indiscriminate collection by orchid lovers, many orchid species have become endangered (BHADRA; HOSSAIN, 2003; LEE et al., 2006; YAMAZAKI; MIYOSHI, 2006). The primary goal of some research concerning this problem is to germinate orchid species and allow them to grow to maturity, with the intent of later returning them to protected natural environments.

Micropropagation from shoot or root tip meristems is a direct process (known as cloning) for the multiplication of orchid plants, and is used by

orchid collectors and producers (HUSSEY, 1986). Moreover, asymbiotic seed germination seems to be one of the most appropriate procedures to replace indiscriminate collections and to preserve the genetic diversity of orchid species. Most asymbiotic seed cultures have mainly been focused on northern temperate and/or terrestrial orchids (KITSAKI et al., 2004; LEE et al., 2006; SHIAU et al., 2005; YAMAZAKI; MIYOSHI, 2006). Few studies have been devoted to the application of *in vitro* seed propagation techniques in tropical orchid species (BUYUN et al., 2004).

The *in vitro* propagation of *Hadrolaelia purpurata* and *Encyclia randii* species has not yet been reported in the literature. *Hadrolaelia purpurata* is native to Santa Catarina, a state in southern Brazil. *H. purpurata* is actually the state's flower, and it is one of the finest of all cultivated orchids due to its wide range of color forms. Moreover, it is extremely popular with local Brazilian orchid growers and is highly prized by collectors. This species has only been recently reclassified and thus divided into other genera by different authors using morphological observations. A recent study based on a molecular analysis of a ribosomal DNA fragment has confirmed the existence of two distinct groups: the Mexican species (the true *Laelia*) and the Brazilian species (*Hadrolaelia*) (CHIRON; CASTRO, 2002).

In contrast, *Encyclia randii* is native to states in northern Brazil. It is popularly described as one of the most elegant Brazilian *Encyclia* species, with a large flower epiphyte typical of the hot and humid Amazon basin and possessing an intense fragrance. There are no studies or descriptions of the *Encyclia randii* species in the specialized literature.

In the current study, complex and specific supplements were added to KC medium to stimulate *in vitro* propagation of the *H. purpurata* and *E. randii* orchid species. The differential effect of illumination periods was also tested on the seeds from *H. purpurata* and *E. randii* to determine whether they stimulate the formation of seedlings and leaves. The use of complex supplements may be more efficient than the addition of specific nutrients for asymbiotic seed germination and may have reduced costs, as expected by collectors and producers of orchids. Different illumination periods may also promote the highest production of seedlings or leaves from the seedlings of *H. purpurata* and *E. randii*.

Material and methods

Seeds of *Hadrolaelia purpurata* and *Encyclia randii* were collected from a wild natural plant culture and maintained in an experimental orchid garden of the State University of Maringá (Maringá, Paraná State, Brazil) and from an orchid producer of the state of Rondonia (northwestern Brazil), respectively. Seeds from the two plants came from natural pollinations. In the case of germination, three-year-old seeds maintained at 10ºC were superficially sterilized with 15% sodium hypochloride for 10 min. TCC (2,3,5-triphenyl tetrazolium chloride) test solution was used, as described previously, to check the seeds' viability.

After extensive washing (4-5 times) with sterile water (10 mL) under aseptic conditions, the seeds (1 mL) were distributed in the germination medium. Seeds soaked in 1 mL of sterile water were dispersed in petri dishes containing original Kundson-C medium (KNUDSON, 1946), solidified with 0.7% agar and supplemented with complex and specific nutrients. Coconut water (150 mL L^{-1}) and ripe-banana pulp of the *nanica* variety (90 g L^{-1}) were used as complex nutrients. The specific nutrients were micronutrients (1 mL L^{-1}) such as boric acid (0.056 mg L^{-1}), molibidenic acid (0.016 mg L^{-1}), copper sulfate (0.040 mg L^{-1}), zinc sulfate (0.331 mg L^{-1}) (ARDITTI et al., 1982), peptone (1 g L^{-1}), and activated charcoal (3 g L^{-1}). Each experiment was repeated four times, and cultures were incubated in a growth chamber at 25 ± 2°C under 14.9 µmol $m^{-2} \cdot s^{-1}$ and provided with light radiation from cold-white fluorescent lights (PPF) for a 14-h photoperiod or with continuous illumination (24h).

After four months of culture, the average number of seedlings and leaves per seedlings were registered for each experiment using the original KC medium and KC medium supplemented with micronutrients, peptone, activated charcoal, coconut water, and ripe banana pulp. The difference in treatments was tested by a one-way analysis of variance (ANOVA) and by the Tukey-Kramer multiple comparison test (The Assistat Software version 7.4 beta 2007; http:/www.assistat.sites.uol.com.br).

Results and discussion

The highest number of *Hadrolaelia purpurata* seedlings occurred in KC medium containing 90 g L^{-1} banana pulp (Figure 1A, Table 1). Leaf formation from *H. purpurata* seedlings also showed significant differences (F_{NL} = 13.7221; $p < 0.001$) when compared to seedlings grown on KC medium supplemented with peptone or coconut water (Table 1).

Positive effects of the banana pulp and of coconut water addition in KC medium were also observed for seedlings and leaves formation from *Encyclia randii* seeds (F_{NS} = 15.0777, F_{NL} = 39.1674; p < 0.001) (Figure 2A, Table 1).

Micronutrients (boric acid, molibidenic acid, copper sulfate, and zinc sulfate) and the peptone addition do not seem to have a positive effect in the asymbiotic culture of *H. purpurata* seeds (Table 1). Although the germination of seeds and growth of protocorms invariably required peptone in the medium (PATHAK et al., 2001), a positive effect of peptone addition was reported only for seedling formation from *E. randii* seeds.

The effect of the activated charcoal addition in KC medium was specific because it produced an increased number of seedlings only in *E. randii* seeds maintained at a 14h photoperiod (Figure 2B).

Both the 14-h photoperiod and continuous illumination (24h) showed significant effects on a number of seedlings and leaves of *H. purpurata* and *E. randii* (F_{NS} = 45.0544 and F_{NL} = 5.5909; p < 0.001). On average, the highest numbers of *H. purpurata* seedlings and *E. randii* leaves per seedlings of occurred in cultures maintained at continuous illumination (Table 1).

The addition of banana pulp and coconut water in culture medium may be considered a liability because they are not synthetic and have different unknown compositions. Nevertheless, current experiments show that the addition of banana pulp in KC medium induces the production of the highest number of seedlings and leaves per seedling in asymbiotic culture of the *H. purpurata* and *Encyclia randii* orchid species.

Despite the fact that the growth-promoting effect of banana is still unknown, banana in combination with coconut water or activated charcoal has been a common addition since the seventies (ARDITTI et al., 1982). Complex nutrients (or unknown substances), such as banana and coconut water, are usually added in different culture media for the asymbiotic development of seedlings from different orchid-species, with or without growth regulators (DECRUSE et al., 2003; SHIAU et al., 2005). The negative effect of the banana addition in culture media has only been reported for the germination of non-orchid species (ARDITTI et al., 1982).

The use of coconut water for orchids and several other plant species for *in vitro* culture has been recommended mainly due its antioxidant characteristics (GE et al., 2004). More recently, coconut water has also been used *in situ* for animal-cell preservation (ANDRADE et al., 2002) and as a re-hydration fluid to replace fluid loss from the gastrointestinal tract (PETROIANU et al., 2004). An increasing number of investigations are searching for a better understanding of this relatively complex nutrient. Vitamins, sugar, organic acids, fatty acids, amino acids, fibers and minerals have been reported to form the nutritional composition of coconut water (RITCHER et al., 2005). Coconut water also contains a large spectrum of compounds that may be growth factors, individually or synergistically, and which show cytokinin-like activity. The identity of the two major cytokinin *O*-glucosides in coconut water was confirmed by micellar electrokinetic capillary chromatography after solid phase extraction (GE et al., 2004).

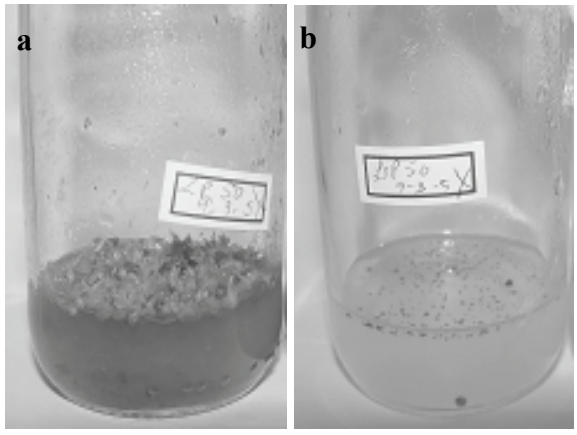

Figure 1. The highest number of *Hadrolaelia purpurata* seedlings and leaves were found in the KC medium containing 90 g L^{-1} banana pulp (A) compared to seedlings grown in KC medium supplemented with the peptone addition (1 g L^{-1}) (B).

Table 1. Comparison between the average number of seedling (NS) and leaf (NL) formation in *Hadrolaelia purpurata* and *Encyclia randii* seeds cultured for 4 months in KC medium containing different concentrations of micronutrients (M), peptone (P), activated charcoal (AC), coconut water (CW), and banana pulp (B). The cultures were maintained at a 14-h photoperiod or with continuous illumination (24h) in a randomized design with three replicates.

	H. purpurata				*Encyclia randii*			
Culture médium	NS		NL		NS		NL	
	24h	14h	24h	14h	24h	14h	24h	14h
KC medium	53.60^{aB}	48.61^{bA}	1.93^{aAB}	2.21^{aAB}	44.66^{aBC}	31.51^{aC}	1.53^{aD}	1.32^{aC}
KC + M	55.67^{aB}	48.76^{aA}	1.84^{aAB}	1.64^{aABC}	53.21^{aBC}	32.16^{aC}	1.89^{aCD}	1.39^{bC}
KC + P	0.18^{aD}	0.52^{aB}	0.50^{aB}	0.25^{aC}	76.94^{aAB}	57.35^{aBC}	2.57^{aB}	1.31^{bC}
KC + AC	11.77^{aCD}	7.67^{aB}	1.86^{aAB}	1.96^{aAB}	27.35^{bC}	72.71^{aAB}	2.25^{aBC}	2.16^{aB}
KC + CW	28.11^{aC}	0.30^{bB}	1.27^{aB}	0.76^{aBC}	93.06^{aA}	50.57^{bBC}	2.65^{aB}	1.76^{bBC}
KC + B	90.08^{aA}	35.50^{bA}	3.19^{aA}	2.99^{aA}	96.99^{aA}	100.0^{aA}	3.54^{aA}	3.04^{bA}

In columns, means followed by the same letters (in capital), and in rows, means followed by the same letters (minuscule), are not significantly different at 5% probability by the Tukey-Kramer multiple comparison test.

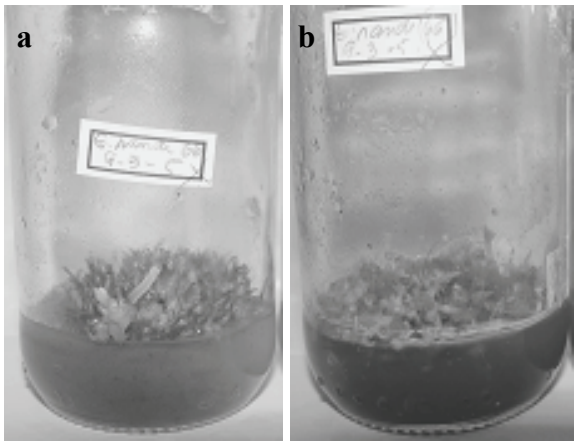

Figure 2. The highest number of *Encyclia randii* seedlings and leaves were found in the KC medium containing 90 g L^{-1} banana pulp (A) when compared to seedlings grown in KC medium supplemented with activated charcoal (3 g L^{-1}) (B).

Although the use of continuous illumination in micropropagation on a commercial scale is highly expensive for orchid producers, our results suggest that 24h light may be important for the asymbiotic seed germination and culture of some tropical orchid species. Several studies have shown that a 14-16h photoperiod is the norm for asymbiotic seed germination and culture of orchids, because seed germination of the northern temperate climate orchids is inhibited by light (PIERIK et al., 1988). The 14-16h photoperiod has also been reported as having a positive effect for some tropical orchids (BUYUN et al., 2004), but our results showed a significant positive effect in the 24-h light period. Significant effect on root induction has been observed with continuous illumination in the orchid hybrid, "BLC Pastoral Innocence" (PRIZÃO et al., 2012).

The results in current study are important because they indicate that the addition of complex and unspecific nutrients (banana and coconut water) in original KC medium is more efficient than the addition of specific and known nutrients (micronutrients, peptone, and activated charcoal) for asymbiotic seed germination and culture of the *H. purpurata* and *E. randii* species. Due to the brief period of *in vitro* culture (4 months), the number of roots was not strictly analyzed in the present study. However, root development (at least one per seedling) has only been reported in seedlings of *H. purpurata* and *E. randii* cultured in KC medium supplemented with banana pulp. Thus, it may be possible to state that banana addition in KC medium should promote adequate root development. Vyas et al. (2009) have reported significantly greater numbers of roots per shoot, as well as increased length of roots of *Dendrobium lituiflorum* Lindl., on KC medium supplemented with banana pulp. Root development is important for the continued growth of seedlings and for further transplantation for the *ex vitro* culture and thus for the conservation of both species. In spite of the lack of information on the definite nutrient composition in banana pulp, its addition to KC medium reduces costs and operational requirements for orchid producers. Besides the higher cost for the specific nutrients, such as boric acid, molibidenic acid, copper sulfate, zinc sulfate, peptone, or activated charcoal, compared to banana or coconut water, the addition of these specific nutrients also requires the use of an analytical scale (10^{-3} - 10^{-4} g) and specialized human resources. In contrast, the addition of banana pulp or coconut water in culture medium is a simple procedure for orchid producers.

There is important evidence related to efficient seedling formation from three-year-old seeds maintained at 10ºC. Mature seeds (10 months after collection) from different species of the genus *Ophrys* have also been utilized for *in vitro* development (KITSAKI et al., 2004). In the case of many orchid species, the use of green capsules is preferable because the seeds are viable or not dormant, and germination is faster using the green capsules (TOMAS; MICHAEL, 2007). The lowest frequency of seed germination in *Cephalanthera falcate* was obtained as seed maturity progressed (YAMAZAKI; MIYOSHI, 2006). The use of immature seed capsules as a seed source shortened the reproductive cycle by approximately 2 to 2.5 months for all tropical orchid species (BUYUN et al., 2004). It is thus possible that the use of immature seeds from the *H. purpurata* and *E. randii* species produces the highest number of seedling formation.

The present study may now report the successful asymbiotic germination of the three-year-old seeds of two Brazilian native species from different regions of Brazil with low costs, as expected by collectors and producers; they will benefit from these results. Seeds from dehiscent capsules may be removed, packed appropriately, and transported to laboratories in different regions. The addition of banana and coconut water as supplements is a simple strategy, with reduced costs for all orchid producers. The strategy actually offers an opportunity for using the above techniques in commercial nurseries and for ex situ conservation of this native orchid and other endangered orchid species.

Conclusion

The use of complex supplements, such as banana pulp or coconut water, were more efficient than the addition of specific nutrients (micronutrients, peptone, activated charcoal) for the asymbiotic seed germination of *Hadrolaelia purpurata* and *Encyclia randii*. Continuous illumination (24h), rather than a 14-hour photoperiod, induced the highest number of seedlings of *H. purpurata* and the highest number of leaves in the seedlings of *E. randii*. The addition of banana pulp and continuous illumination are efficient alternatives to the asymbiotic germination of orchids, but the specific effects of these strategies, such as leaf induction, is species dependent.

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