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Seed storage and asymbiotic germination of *Hadrolaelia grandis* (Orchidaceae)

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ABSTRACT

Hadrolaelia grandis is an endangered species of orchid that is experiencing population declines due to habitat destruction and collection for ornamental purposes. Seed storage, *in vitro* germination and development of seedlings for propagation were investigated. Tetrazolium test (TZ) and asymbiotic germination were used to evaluate seeds stored at −20 °C and −80 °C for 24, 30, and 36 months. Seeds were germinated on Woody Plant Medium (WPM), Murashige & Skoog (MS), Vacin & Went (VW) and Knudson C (KC) media. The temperature of −80 °C was the most effective for seed storage since the percentage of viable seeds (81.86%), as determined by TZ, was higher than those stored at −20 °C (64.24%) for 36 months. WPM proved to be the most effective medium for seed germination and seedling development (94.71%, and a germination speed index of 25.33) after 36 months of storage at −80 °C. Protocorm elongation and root development were achieved on WPM medium containing activated charcoal (1 g L^{-1}), and after 16 weeks seedlings were successfully acclimatized after 12 weeks (91.07% survival rate) using vermiculite as a substrate.

1. Introduction

Hadrolaelia grandis (Lindl & Paxton) Chiron & VP Castro is a popular ornamental epiphytic species of the family Orchidaceae, endemic to the Atlantic Forest in the states of Bahia and Espírito Santo (Barros et al., 2015). It has large flowers with yellow-bronze petals and sepals, a pink central lobe and white lateral lobes (Fig. 1). It was listed as "Vulnerable" in the Red Data Book, Flora of Brazil due to population declines caused by collection for ornamental purposes and habitat destruction (Martinelli and Moraes, 2013).

Orchid seeds are very small, extremely light, do not contain endosperm and typically have lower germination rates (5%) under natural conditions making them difficult to propagate with conventional methods (Arditti and Ernst, 1993). In order to germinate in their natural environment, orchid seeds need to encounter a particular mycorrhizal fungus and may be reliant upon a different fungus at a subsequent life stage (Seaton et al., 2013). However, one characteristic of orchids that facilitates their conservation is their dust-like seed. A single seed capsule is capable of producing tens of thousands to millions of seeds, and so is all that is needed to seed bank a species (Swarts and Dixon, 2009). However, there are few studies on the longevity of orchids seeds (Suzuki et al., 2012). Orchid seeds are known to display orthodox behavior and extended longevity when subjected to drying (<5% moisture content) and freezing (−20 °C), under the conditions commonly utilized in many seed banks (Seaton et al., 2013). Thornhill and Koopowitz (1992) recommended that orchid seeds stored for conservation purposes be kept at, or below, temperatures of −70 °C.

Seed viability is commonly assessed using the tetrazolium test (TZ) and *in vitro* germination. The TZ test is one of the most reliable ways to evaluate seed quality and vigor. Seed set without embryos or with low viability seed is common, thus the ability to test their physiological quality before and after conservation is of great importance (Hosomi et al., 2011). *In vitro* germination techniques have resulted in more reliable germination and propagation of many orchid taxa (Kauth et al., 2008). Asymbiotic germination represents an ideal system for studying the growth and development of orchid seeds and seedlings (Kauth et al., 2008), and many culture media have been successfully used for the germination of various species of orchids (Arditti and Ernst, 1993), such as MS (Murashige and Skoog, 1962), KC (Knudson, 1946) and VW (Vacin and Went, 1949). However, recent studies have indicated that the ideal medium for initial germination is not always the best for seedling development (Kauth et al., 2008). All of this work notwithstanding, there remains no studies of asymbiotic germination of *Hadrolaelia grandis*.

Acclimatization continues to be a major bottleneck in the micropropagation of many orchids. A substantial number of micropropagated plants do not survive transfer from *in vitro* conditions

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Fig. 1. *Hadrolaelia grandis* flower. Bar = 80 mm.

to the environment of the greenhouse or field. The greenhouse and field have substantially lower relative humidity, higher light levels, and represent septic environments that are stressful to micropropagated plants in comparison to *in vitro* conditions (Chugh et al., 2009). Meanwhile, several alternative substrates to tree fern, which is in danger of extinction, are being tested, such as coconut powder, coconut fiber and vermiculite (Santos et al., 2016).

Therefore, the objectives of this study were to assess the viability of seeds stored at different temperatures and for different periods of time and determine the best medium for *in vitro* germination and seedling development of *Hadrolaelia grandis*. Furthermore, this study aimed to assess *ex vitro* development of seedlings in different substrates in order to establish a protocol for use in conservation and *in vitro* propagation of this vulnerable species.

2. Materials and Methods

2.1. Seed viability test

Seed viability after long-term storage (24, 30 and 36 months) in a freezer at −20 °C and −80 °C was evaluated by using a solution of 2,3,5-triphenyl tetrazolium chloride. A small sample of seeds (1 mg) for each temperature and storage period was preconditioning with 10% sucrose solution for 24 h at room temperature. The solution was then drained with a micropipette, 1% tetrazolium solution was added and the micro-tubes incubated in the dark for 24 h in a 38 °C water bath. After incubation, the tetrazolium solution was discarded and the remaining solution and seeds (0.3 mL) were placed on a glass plate with a blue background, in order to provide a better contrast between the stained seeds. The samples were analyzed under a Motic, SMZ-171 stereomicroscope and viability was evaluated by analysis of digital images taken with a Sony Cyber Shot DSC-P200 camera. Five replicates of 100 seeds per treatment were evaluated. Red seeds were considered viable and white seeds as non-viable; seeds without embryos were designated as empty. The percentage of viable seeds was calculated by dividing the number of viable embryos by the total number of embryos analyzed. The seeds used in the TZ test were discarded after evaluation.

2.2. Seed sterilization, media and culture conditions

Seeds (4 mg) were surface-sterilized for 1 min with 70% ethanol, followed by immersion in 0.75% (*v*/v) sodium hypochlorite (NaOCl) plus 0.1% Tween-20® for 5 min and rinsed six times in autoclaved distilled water. Seeds were dried on filter paper and inoculated in Petri dishes (150 mm in diameter and 20 mm in height) containing 40 ml of culture medium. Four basal media were tested: MS, KC, Woody Plant Medium (WPM, Lloyd and McCown, 1980) and Vacin & Went Himedia® (VW). MS, KC and WPM media were supplemented with 5.6 g L^{-1} Himedia® agar, 3% sucrose (*w*/*v*) and 0.1 g L^{-1} inositol, whereas the commercial VW formulation was composed of 8 g L^{-1} agar and 2% sucrose (w/v).

The pH was adjusted to 5.8 with 0.1 N NaOH or HCl before the addition of agar. The media were autoclaved for 20 min at 120 °C. The cultures were maintained in a growth room at a temperature of 26 ± 2 °C/18 ± 2 °C (day/night) and under a 16-h photoperiod provided by white fluorescent tubes at an intensity of 40 μmol m^{-2} s^{-1}.

2.3. In vitro seed germination and seedling development

Germination tests were carried out after 24, 30 and 36 months of storage with seeds stored at −20 °C and −80 °C. After surface sterilization, seeds were inoculated under a stereomicroscope and placed into Petri dishes containing culture medium. Five fields, each containing 100 seeds and five replicates per treatment in each Petri dish were marked and digital images obtained every two weeks. Experiment was repeated once. Seed germination rates were recorded eight weeks after *in vitro* sowing, considering germination as a swollen embryo bursting from the testa. The germination speed index (GSI) was calculated by the formula proposed by Maguire (1962) and simplified by Hosomi et al. (2011): $GSI = \frac{G_1}{N_1} + \frac{G_2}{N_2} + \frac{G_3}{N_3} + \frac{G_4}{N_4}$, where G_1, G_2, G_3 and G_4 are the number of seeds germinated in each period, and N_1, N_2, N_3 and N_4, are the time intervals of each counting period. After two, four, six and eight weeks, seedling development was evaluated and determined to be one of four stages: Stage I- non-germinated seed with embryo (Fig. 2C); Stage II- enlarged embryo, testa ruptured (= germination, Fig. 2D); Stage III- protocorm with pointed shoot apex and/or rhizoids (Fig. 2E and F) and Stage IV- protocorm bearing one leaf (Fig. 2G). The percentages of the different developmental stages were calculated by dividing the number of seeds/protocorms in each stage by the total number of seeds and protocorms (Stages I – IV) present in the sample.

2.4. Elongation and root development

After 10 months of culture, seedlings, about 0.5 cm long, obtained from *in vitro* germination were inoculated into flasks (6.2 cm diameter and 12.5 cm length) containing 40 ml of WPM medium supplemented with 1, 2 and 3 g L^{-1} activated charcoal. Six seedlings were cultured in each flask with 10 replicates, for a total of 60 seedlings per treatment. After 16 weeks, the average number of roots, the average length of the three largest roots and the average length of the shoot were recorded.

2.5. Transplantation and acclimatization

Seedlings with three expanded leaves (1.5 cm long) were transplanted to polystyrene trays (3 cm wide and 7 cm high) containing 30 g of substrate treatments: 1- coconut powder Vitaplan®, 2- pine bark and earthworm humus Vitaplan® 3- vermiculite Eucatex®, 4- a mixture of 1 and 2 (1:1) and 5- a mixture of 1, 2 and 3 (1:1:1). Eight plants were transplanted to each treatment substrate with seven replicates per treatment. The plants were maintained in a greenhouse at room temperature (25 ± 2 °C day/ 20 ± 2 °C night), under a photoperiod of 12 h and a light intensity of 13 μmol.m^{-2}.s^{-1}. The plants were manually irrigated every three days, and after 12 weeks the survival rate, the average shoot length (cm), the average number of roots, the average length of the three largest roots (cm), the number of leaves and the weight of the fresh mass (g) were recorded.