SCIENTIFIC ARTICLE

# Comparison of two types of fertilizers in the acclimatization process of in vitro seedlings of *Cyrtopodium hatschbachii* Pabst, an endangered orchid

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## Abstract

*Cyrtopodium hatschbachii* Pabst is an endangered orchid, that requires several studies to gather appropriate information for developing different conservation strategies both *in situ* and *ex situ*. This study aimed to compare the effect of a 2.5 ml.L$^{-1}$ dose of a liquid fertilizer and a 3 g.dm$^3$ dose of controlled-release fertilizer on plants from geitonogamous and xenogamous pollination during the acclimatization stage. Plants from the *in vitro* germplasm bank of the School of Forest Sciences of Eldorado, Misiones were used. Plants extracted from the flasks were first planted in plastic trays with perlite for 60 days. After this period, they were transferred to 200 cm$^3$ pots containing pine bark and perlite in a 3:1 ratio, where they remained for 30 days. Subsequently, the different fertilizers were applied, and the plants were kept under greenhouse conditions for 180 days. The results showed significant differences in leaf number, pseudobulb diameter, and plant height, but not in survival rate or plant mass. In conclusion, the tested fertilizers and doses were effective in promoting greater growth compared to the control, although survival rates during acclimatization were similar.

**Keywords:** *Acclimatization*; *Conservation*; *Micropropagation*; *Plant nutrition*; *Orchidaceae*.

## Introduction

The Orchidaceae family is one of those with the greatest number of species, surpassed only by the Asteraceae family, and it is one of the most important plant families in terms of quantity of species, which has commercial relevance in the floriculture industry. Therefore, an interest in its cultivation, propagation, and commercialization. Endless practices of illegal extraction and habitat destruction have been carried out to obtain these plants. Nevertheless, these activities have never led to their inclusion in the "endangered" category of the International Union for Conservation of Nature (IUCN) "red lists", or prohibition lists (Christenhusz & Byng, 2016; Fay, 2018; Bello-Castañeda *et al*., 2023). *Cyrtopodium hatschbachii* Pabst is one of the endangered species of the Argentine Republic, which is characterized by inflorescences with 8 to 15 pink flowers with yellow labella and a pleasant fragrance. (Surenciski *et al*., 2012; Pott *et al*., 2019; Cardoso *et al*., 2021). These are self-compatible plants pollinated by orthopterans or by rain, and hence they can produce viable fruits and seeds through self-fertilization (Cardoso *et al*., 2021).

Although *C. hatschbachii* can perpetuate its population through self-fertilization, various threats could significantly compromise the survival of these plants in their natural habitat if effective and immediate conservation measures are not implemented (Swarts and Dixon, 2017; Cardoso *et al*., 2021).

In this way, *in vitro* propagation is a technique that contributes to a great extent, making it possible to preserve endangered orchid species (De Stefano *et al*., 2022).

In comparison with conventional cultivation, this process does not require mycorrhizal fungi or long periods to obtain a massive quantity of specimens for commercialization and conservation (Frausto *et al*., 2019; Mosqueda *et al*., 2023). Nevertheless, this technique might have certain disadvantages, such as the high mortality rate of the plants during acclimatization (Deb and Imchen, 2010). The adequate use of fertilizer can be a strategy to decrease these losses, as good nutrition contributes to the survival rate of the plants when changing conditions *in vitro* to *ex vitro* (Rineksane *et al*., 2023).

The use of this technique involves a series of stages, beginning with the cultivation of seeds or explants in jars containing a growth medium under aseptic conditions with controlled light and temperature (López and Rangel, 2018; Frausto *et al*., 2019). Once the *in vitro* growth process was over, plants were extracted from the jars and subjected to an acclimatization process (De Stefano *et al*., 2022), since they must transition from *in vitro* cultivation, a controlled environment, to an uncontrolled one. The acclimatization and rustication stage has the greatest impact on the *in vitro* multiplication process, as plants must adapt to field conditions during this period.

Considering nutrition is critical since this factor noticeably affects plant survival rate during acclimatization (De Stefano *et al*., 2022; Rineksane *et al*. 2023). Thus, the type of fertilizer being used during this stage can benefit growth and adaptation for the plant (Rineksane *et al*., 2023).

Several studies have proved that controlled-release fertilizers, as well as liquid formulations, effectively contribute to the growth and survival of orchids during optimization (Ha *et al*., 2018; Hendriyani *et al*., 2019). However, existing information about fertilizers and doses to be used is scarce and, therefore, there is a need to carry out studies in order to determine the correct nutrition of orchids during the different stages of *in vitro* cultivation, since each species has specific and particular requirements regarding the appropriate environment to achieve an optimal growth and flowering (Pardo *et al*., 2015; Hoshino *et al*., 2016).

The aim of this study was to evaluate the growth and survival of *C. hatschbachii* seedlings of geitonogamous and xenogamous origin, which were germinated *in vitro*, using two types of fertilizers: liquid formulation and controlled-release fertilizer.

## Materials and methods

The plants used in this study were obtained through *in vitro* cultivation of seeds resulting from manual pollination between flowers on the same inflorescence (geitonogamous pollination) and flowers from different plants (xenogamous pollination). These plants were kept for 2 years in the germplasm bank of the School of Forest Sciences at the National University of Misiones, located in Eldorado City.

The seedlings were extracted from and thoroughly washed with fresh water before being placed in rounded plastic trays (17 × 22 cm and 7 cm high) containing moist perlite. After that, they were covered with trays of the same type and kept under greenhouse conditions for 60 days, where the average humidity and temperature ranged from 60 % to 80 % and 23 °C to 27 °C, respectively, and the light level ranged from 27 to 73 μMol.m$^2$.sec$^{-1}$ depending on the month and cloud conditions. The substrate was watered daily to maintain a humid environment. After that period, seedlings were placed in containers of 200 cm$^3$ containing pine bark and perlite in a 3: 1 ratio. After 30 days, the plants were fertilized with two types of fertilizers. On the one hand, a controlled-release fertilizer, Plantacote 6M 14-9-15+mg+micronutrients, was applied at a dose of 3 g.dm$^3$ of substrate; on the other hand, a liquid formulation, Fertifox Potenciado 7-3-7.5 with naphthalene acetic acid, was applied at a dose of 2.5 ml.L$^{-1}$ of water, using 50 ml to water the plants every 20 days. 6 treatments constituted by 4 repetitions of 5 plants each were carried out. Each treatment is presented in Table 1.

**Table 1.** Composition of the different treatments

| Treatment | Pollination | Fertilizer |
|---|---|---|
| 1 | Geitonogamous | No fertilizer |
| 2 | Geitonogamous | Fertifox |
| 3 | Geitonogamous | Plantacote 6M |
| 4 | Xenogamous | No fertilizer |
| 5 | Xenogamous | Fertifox |
| 6 | Xenogamous | Plantacote 6M |

At the end of the trial, the following variables were measured: total plant height (in this case, the size of the pseudobulb plus the longest green leaf was considered, as several plants had not developed pseudobulbs at the time of measurement), pseudobulb diameter, number of pseudobulbs, number of green and dry leaves, survival percentage, fresh weight, and dry weight in grams. Digital calipers, rulers, and high-precision scales were used to take the measurements. Dry weight was obtained through a heater at 60 °C for 5 days in order to obtain constant weights. Data was analyzed through an Analysis of Variance (ANOVA Test) while means were compared with Tukey's Honestly Significant Difference (HSD) Test using the 2020 version of Infostat software (Di Rienzo *et al*., 2020). A completely randomized design was employed for this study.

## Results and discussion section

The statistical analysis of the number of green and dry leaves between plants derived from geitonogamous and xenogamous pollination showed highly significant differences (P = 0.0001), whereas the number of pseudobulbs did not show significant differences (Figure 1). This partially matches the information reported by Arthagama *et al*. (2021), where no significant differences were observed regarding the number of pseudobulbs and the number of leaves that the plants developed in the different treatments used. Conversely, Heredia-Rendón *et al*. (2009) demonstrated that the use of nitrogen fertilizers at different doses had a significant effect on the number of leaves and pseudobulbs formed in *Laelia halbingeriana* Salazar and Soto Arenas. In the study held by Wang (1996) on *Phalaenopsis sp*., only a small significant effect on the number of leaves was observed for the different fertilizers tested. On the other hand, Jimenez-Peña *et al*. (2019) showed that watering plants with a nutrient solution containing both macronutrients and micronutrients promotes an increase in the number of leaves and the development of pseudobulbs, whereas plants receiving a solution containing only micronutrients showed lower or fewer results.

Furthermore, the results obtained from the different fertilizer treatments showed significant differences, indicating that plants from different pollination processes apparently exhibited very similar responses (Figure 1) to the doses of both fertilizers used with respect to the evolution of these variables. In this study, the employed doses of fertilizer had appropriate results as they manifested significant differences with the witness treatments (Figure 1).

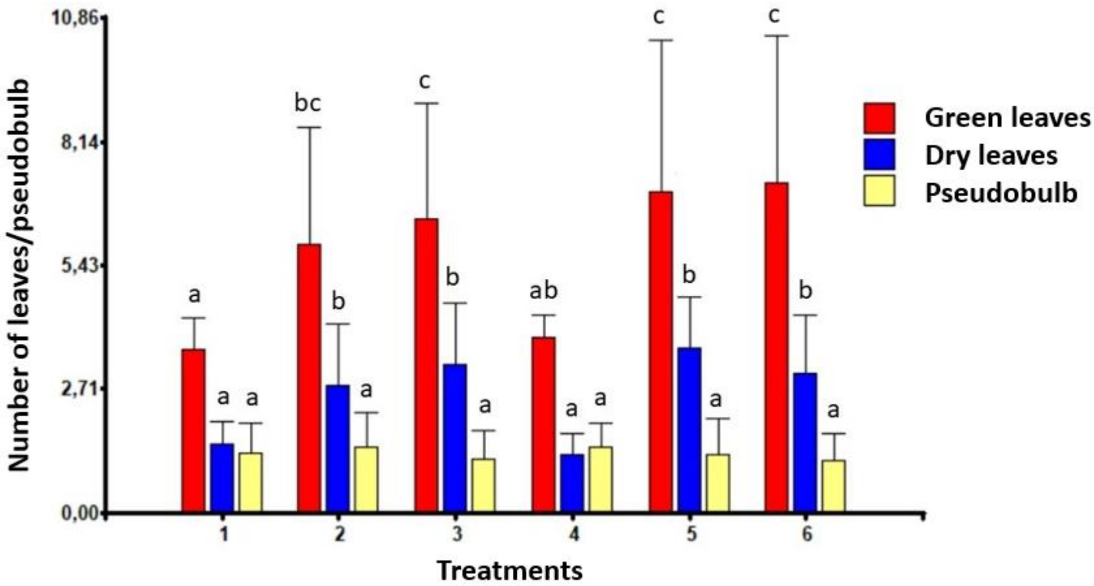


**Figure 1.** Mean values of the number of leaves and pseudobulbs developed, and the number of leaves that had died by the end of the trial for the plants with different treatments

Regarding pseudobulb diameter and total plant height (measured from the base of the pseudobulb to the tip of the largest leaf), significant differences were observed among the different treatments, with the highest values for both variables recorded in treatment 3, where a dose of controlled-release fertilizer was applied (Figure 2). In the study held by Arthagama *et al*. (2021), in contrast, no significant differences were found among the treatments when different doses of organic liquid fertilizer were employed, although differences were observed depending on the type of substrate used, indicating that substrate type is more relevant than the liquid fertilizer dose. In addition, it is also important to consider the frequency of the fertilizer, since it is a factor that can noticeably affect the growth and development of orchids (Herastuti and EK, 2020). Thereby, using foliar fertilizer every 14 days has a highly significant effect on the growth of species of the genus *Dendrobium* (Hariyanto *et al*., 2019).

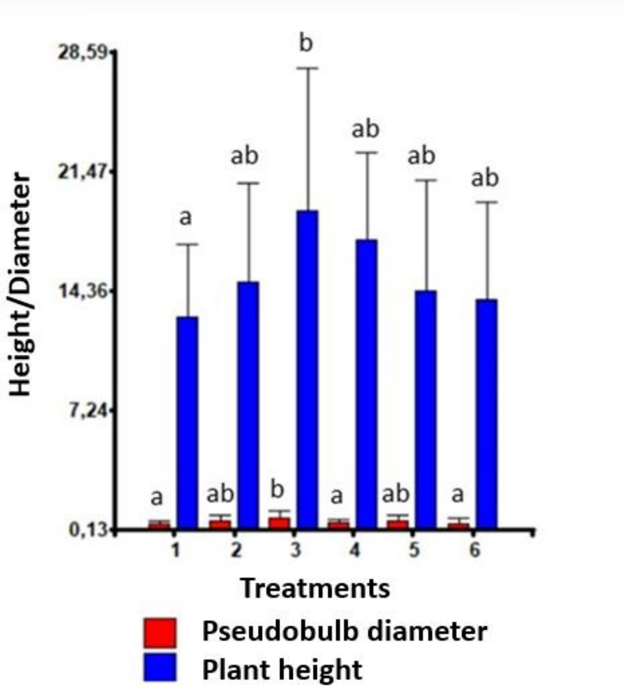


**Figure 2.** Mean values for plant height and pseudobulb diameter variables in the different treatments

As regards the survival rate, percentages ranged from 65 % to 90 % in the analyzed treatments, although no significant differences were observed. As for the fresh and dry weights, treatment 2 showed a slightly higher value with respect to the others, but with no significant differences (Table 2). According to Ha *et al*. (2018) and Hendriyani *et al*. (2019) an optimal fertilizer can promote greater growth in orchid plants, reflected in higher fresh and dry weights, thus contributing to plant survival during the acclimatization stage. Conversely, the application of inappropriate fertilizers and doses can lead to plant deterioration or even mortality.

**Table 2.** Effect of liquid and controlled-release fertilizer doses on survival, fresh weight, and dry weight variables

| Treatment | Survival (%) | Fresh weight (g) | Dry weight (g) |
|---|---|---|---|
| 1 | 65±19,14 a | 1,30±0,44 a | 0,11±0,04 a |
| 2 | 90±11,54 a | 6,79±3,89 a | 0,42±0,11 a |

Table 2 (continued)

| | | | |
|---|---|---|---|
| 4 | 75±19,14 a | 1,9±1,01 a | 0,21±0,09 a |
| 5 | 90±11,54 a | 4,54±2,39 a | 0,39±0,04 a |
| 6 | 90±11,54 a | 2,85±3,16 a | 0,34±0,04 a |

Reference. Mean values ± standard deviation are shown; different letters indicate significant differences according to Tukey's Honestly Significant Difference (HSD) Test at the 0,05 level

In this study, the fertilizers and doses tested showed significant differences among treatments in terms of the number of leaves, plant size, and pseudobulb diameter. Therefore, in *C. hatschbachii* plants derived from geitonogamous or xenogamous pollination under the conditions of this study, plant growth is influenced by the type of fertilizer used (liquid or controlled-release) at the recommended doses indicated on the label. Conversely, they have the same response for the survival, fresh weight, and dry weight variables in the acclimatization stage once they are placed in pots. Therefore, using fertilizers with different formulations and compositions can generate differences in growth during the acclimatization stage in *C. hatschbachii* plants. Nevertheless, no significant differences were observed in survival rates among the treatments or the doses of the two fertilizers applied in this study.

Previous experiments have shown that having a specific fertilizer type and dose is critical for each orchid species, as they have particular nutritional requirements which contribute to adequate growth and flowering (Ha *et al.*, 2018). For this reason, it is extremely important to have information about a fertilization system to optimally produce orchids. Studies on this species should be continued until more information is obtained regarding the most appropriate fertilization and nutrition required to produce high-quality plants for conservation purposes. Moreover, proper use of fertilizers implies fast growth processes and, consequently, timeframes and production costs for orchids are reduced (Wang and Konow, 2002).

## Conclusion

The type and dose of fertilizer used during the acclimatization of *C. hatschbachii* affected the development of the number of green and dry leaves, pseudobulb diameter, and plant height. In contrast, the quantity of pseudobulbs, as well as survival rates, fresh weight, and dry weight, showed no differences during this stage for plants derived from geitonogamous and xenogamous pollination when applying a liquid fertilizer (Fertifox) at a dose of 2.5 $ml.L^{-1}$ dose and a controlled-release fertilizer (Plantacote 6M) at a dose of 3 $g.dm^{-3}$ of substrate.

It is recommended to further investigate this type of study using other doses of these two fertilizers to determine their effects on the growth process of this species.

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