Comparison of In-Vitro Micropropagation Growth Media Solutions for Prosthechea cochleata (L.) W.E. Higgins vars. diandra and triandra

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Research Article

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Abstract

Prosthechea cochleata or clamshell orchid is recognized as a species of both conservation and commercial importance. It has long been prized by orchid breeders and growers for its unique flower and hardy disposition. The commercial market has failed to meet the demand for this species since it is still targeted for illegal collection in the wild. This study examines the effectiveness of the banana powder on two variants (var. diandra and var. triandra) with disparate home ranges and levels of genetic diversity using two commercially available agar-based media (PhytoTechnology P668 and P748). Undifferentiated protocorms of the two variants were monitored for shoot growth and differentiation across a period of 156 days. Banana powder supplement (P748) was more effective in initiating shoot formation in both variants compared to control media (P668) ($F = 65.11$, $p < 0.001$, $df = 81$). The diandrous variant grown with banana showed the highest mean shoot count (98.17 shoots/flask) at the end of the monitoring period. Results suggest that banana supplement is an effective source of plant growth regulators and organic nutrients necessary to promote shoot formation and seedling development in this species. This research is important for the conservation of *P. cochleata* as it identifies an effective and cost-efficient method for micropropagation, which can aid in increasing the wild populations of this species. Additionally, the findings have implications for commercial orchid production, as it can potentially improve the cultivation and yield of this species for economic purposes.

Key Message

This paper compares two commercial micropropagation media for *Prosthechea cochleata* and provides an explanation for improved growth/differentiation rates and means for resource-effective propagation techniques for conservation and horticulture.

Introduction

The Orchidaceae family is one of the largest families of angiosperms, containing an estimated 800 genera and 30,000 species (A.P.G. III). While most orchids are naturally found in tropical regions, orchids are present on every continent except Antarctica, with some species found on Subantarctic islands (Swarts and Dixon 2009). Known for their vast array of colors and forms, orchids have a certain allure that captures the attention and fascination of horticulturists, plant enthusiasts, and collectors. Consequently, these plants have been commercialized and globally exploited, leading to their blanket protection under CITES Appendix II. Effective propagation methods are needed to secure these species for conservation and cultural use.

Unusual seed structure makes orchids difficult to propagate. Orchids produce amongst the smallest seeds of any plant, often less than 1mm in length. Their small size is primarily attributed to the lack of endosperm, allowing the microscopic seeds to be wind-dispersed. In most plants, the endosperm provides energy and nutrient storage for germination. Without an endosperm, an orchid seed cannot germinate alone and must obtain the energy necessary for germination in the environment. All orchids

rely on associations with mycorrhizal fungi to obtain the energy for seed development (Rasmussen et al. 2015). These mycorrhizal relationships vary by species and range from broad to narrow but are obligatory for orchid reproduction (Hadley 1970; McCormick et al. 2004). Due to this reliance on fungi, propagation using symbiotic methods is cost and time prohibitive. Asymbiotc micropropagation provides orchid\ seed directly with the nutrients and sugars necessary for germination that would otherwise be obtained through mycorrhizal relationships. Once germinated, orchid seeds form masses of undifferentiated cells or protocorm-like bodies (PLBs) from which roots, shoots, and leaves emerge as the plant matures (Fig. 1).

Continued advances in orchid culture and orchid breeding have led to effective in-vitro methods to propagate a wide variety of orchids from seed. Laboratory-based methods, such as micropropagation, have effectively produced seed-born orchids for conservation and ornamental breeding (Kulak et al 2022). As a result of the market increases, orchid growth media is now commercially available for many different species and can be tailored by incorporating plant growth regulators (PGRs), minimal salts, and a wide array of organic compounds. Organic-based supplements contain natural forms of PGRs and include media additives such as coconut water, banana powder, and potato powder in addition to vitamins, and amino acids that can act as micronutrients or growth factors (De Stefano et al., 2022). In orchid horticulture, bananas are a common organic additive, rich in auxins, that has been shown to promote differentiation and shoot growth (Ascher et al. 1981; Arditti 1993; Justine et al. 2022). Beyond auxin contributions, preliminary studies indicate the presence of additional bioactive compounds, including micronutrients and organic matter, in bananas which contribute to orchid development (Haruna & Yahaya 2021).

In some cases, in-vitro culture leads to over-proliferation or failure of PLBs to differentiate post-germination. Unlike commercial propagation aims, excessive multiplication of PLBs is not desirable for conservation efforts in which the goal is to preserve genetic diversity. For rare species, uncontrolled PLB proliferation can further dilute the overall genetic diversity, and waste resources, and can be problematic for overall conservation strategies. The behavior of PLBs in-vitro may be influenced by both environmental (PGR combinations) and genetic factors (seed-borne versus tissue explant). Most studies have focused on the effects of different PGRs, but fewer have addressed the potential role intraspecific genetic diversity may play on somatic embryogenesis and somaclonal variation (Chugh et al. 2009). Seeds from self-fertilized or homozygous individuals have shown differential growth habits and reduced survivorship in-vitro compared to plantlets that have been derived from outcrossing (unpublished data The Million Orchid Project, DeStefano et al. 2022).

Florida's combination of temperate and subtropical climates provides the ideal conditions for a diverse array of orchid species, many of which are rare and/or protected. There are currently 106 known native orchid species in Florida, with 58 assigned as endangered and 18 as threatened (Florida Department of Agriculture and Consumer Services, https://www.fdacs.gov/). This represents nearly half of the continental United States' orchid diversity. In addition to the threats of rapid development and habitat loss, orchids are subject to a long history of illegal poaching and human impacts, and as a result, natural

populations continue to decline (Borrero Liu et al., 2023). Since 2012, Fairchild Tropical Botanic Garden's "The Million Orchid Project" has been propagating large numbers of Florida native orchids for reintroduction efforts in urban Miami, Florida. This large-scale conservation effort includes an established micropropagation laboratory focused on native orchids which through this work has revealed new challenges in the micropropagation of several Florida orchid species.

Prosthechea cochleata (L.) W.E.Higgins, commonly known as the Clamshell or Florida Cockleshell Orchid, is an attractive tropical species that is native to Florida, Central America, Venezuela, Colombia, and the West Indies (North American Orchid Conservation Center, n.d.). In Florida, this species is restricted to the southern part of the state in growth zone 10, existing in cypress freshwater sloughs, forests, and swamps, whereas in the Caribbean this species can be found in a variety of habitat types. This species is an epiphytic orchid with yellow-green sepals and petals and a purple-brown lip that is non-resupinate and modified into a hood cupping the reproductive organs (Fig. 1). The species also includes four described variations, two of which, *P. cochleata* var. diandra and var. triandra, being the focus of this paper (Ray et al. 2019; Belfort-Oconitrillo and Pupulin 2020). Horticulturally var. triandra is derived from natural Florida populations (Ackermann 2014), although triandrous populations do exist in Puerto Rico (Ortiz-Barney and Ackermann 1999). The triandrous variation is the variant of *P. cochleata* found naturally in South Florida. Unlike var. diandra, var. triandra is not known to form specialized relationships with native pollinators in Florida, and insect visitations for var. triandra are very rare (Ray et al. 2019). Triandrous individuals have additional anthers on the column and increased pollen load that facilitates selfing, resulting in local endemism throughout the range. Higgins (2005) detailed how the two additional anthers allow the pollen tubes to bypass the rostellum, resulting in effective self-pollination. However, concerns should be raised regarding the potential for inbreeding depression due to self-pollination habits in orchids as it can affect species fitness (Juillet, 2007; Seeja et al. 2018). Ortiz-Barney and Ackermann (1999) observed low genetic variation of *P. cochleata* at the localized level throughout its broad range and discovered four distinct varieties, each represented by low local genetic diversity.

Here we assess the effectiveness of banana powder using two commercial semi-solid orchid media P668 and P748 supplied by PhytoTechnology Laboratories (Shawnee, KS) on plantlet development of *Prosthechea cochleata* var. diandra and var. triandra. This study also seeks to shed light on the effects of inbreeding by comparing the growth responses of different variants with opposing levels of genetic diversity (low vs. high), and pollination strategies (self vs. outcross).

Methods

Seeds were collected from two cultivated plants of *P. cochleata* (n = 1 var. diandra, n = 1 var. triandra) in April. 17, 2021, at Fairchild Tropical Botanic Garden (FTBG), Coral Gables, Florida. The single seed capsule for var. diandra was obtained by outcross hand-pollination of two plants grown at FTBG from seed collected in October of 2017 in Haiti. The single seed capsule of var. triandra was obtained through autogamous selfing of the plant located in the FTBG greenhouse. Ripe un-dehisced capsules were collected approximately 3–4 months following pollination. The green capsules were sterilized using a

10% bleach solution with three drops of liquid dish soap and scrubbing the surface thoroughly with a toothbrush for 5 mins. Each seed capsule was then rinsed with 90% ethanol and flamed in a laminar flow hood for 2–3 seconds. Inside the laminar flow hood, Individual seeds were removed by cutting open seed capsules along the ventral ribs using a sterile scalpel. Seeds were gently shaken into glass baby jars containing P723 agar-based orchid seed germination media supplied by Phytotechnology. After approximately 30 days, a greater number of seeds had successfully germinated. Germinated seeds were placed on LED growth lights for 60 days until a mass of protocorms had fully developed and turned green.

Media Preparation: Two types of PhytoTechnology orchid maintenance media were used for this study; media p668 and P748 were prepared following the manufacture instructions: 27.31g of P668 media and 64.31 grams of P748 media, each solution contained 1.5% of agar, dissolved and boiled in one liter of deionized water. The nutrient formulas are identical except for 30g of banana powder that is pre-added to P748 media (Table 1). Approximately 20mL of media was poured into autoclavable plastic culture vessels (16 oz), which were then autoclaved at 121℃ for 20 minutes. The containers were then immediately transferred to a laminar flow hood to cool and solidify.

Table 1 List of constituents for the two PhytoTech Labs orchid growth media P668 and P748

Formula	(mg/L)
Ammonium Nitrate	825
Boric Acid	3.1
Calcium Chloride, Anhydrous	166
Cobalt Chloride • $6H_2O$	0.0125
Cupric Sulfate • $5H_2O$	0.0125
Na_2 EDTA • $6H_2O$	37.3
Ferrous Sulfate • $7H_2O$	27.85
Magnesium Sulfate, Anhydrous	90.35
Manganese Sulfate • H_2O	8.45
Molybdic Acid (Sodium Salt) • $2H_2O$	0.125
Potassium Iodite	0.415
Potassium Nitrate	950
Potassium Phosphate, Monobasic	85
Zinc Sulfate • $7H_2O$	5.3
Activated Charcoal	2000
MES (Free Acid)	1000
myo-Inositol	100
Nicotinic Acid (Free Acid)	1
Peptone from Meat	2000
Pyridoxine • HCl	1
Sucrose	20,000
Thiamine • HCl	10

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Ammonium Nitrate	825
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Cobalt Chloride • $6H_2O$	0.0125
Cupric Sulfate • $5H_2O$	0.0125
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Magnesium Sulfate, Anhydrous	90.35
Manganese Sulfate • H_2O	8.45
Molybdic Acid (Sodium Salt) • $2H_2O$	0.125
Potassium Iodite	0.415
Potassium Nitrate	950
Potassium Phosphate, Monobasic	85
Zinc Sulfate • $7H_2O$	5.3
Activated Charcoal	2000
Agar	7000
Banana Powder	30,000
MES (Free Acid)	1000
myo-Inositol	100
Nicotinic Acid (Free Acid)	1
Peptone from Meat	2000
Pyridoxine • HCl	1
Sucrose	20,000
Thiamine • HCl	10

Media Treatments

It is nearly impossible to delineate and count individual PLBs without risk of contamination, so five calluses each consisting of between 10–40 tightly bound PLBs of each variant were then transferred into the different media types (P668 and P748), sealed, and placed on growing racks. The racks were set up using a randomized complete block design (RCBD) to account for possible differences in growth conditions based on light rack location and edge effects. A total of 24 containers were made for each media and variation pairing, resulting in 48 containers per variant and 96 containers total, each containing five PLB calluses. A total of 14 culture vessels were lost to contamination and omitted from the study for var. triandra (6 cultures), and var. diandra (8 cultures). Cultures were grown at room temperature (75–78℃) under T5 LED strip lights (12 hrs light/12 hrs dark). Culture vessels were rotated on the lighting rack every month using a random block design.

Data Collection and Counting Methods

Data was collected over the course of 156 days by taking rounds of photos at five intervals approximately 90 days apart in the months of July, September, and December. At the end of this period, the photos were analyzed for differentiation and shoot elongation (Fig. 2a & b). At each time interval, photos were taken of the containers in a manner that ensured that all shoots or leaves were visible, with multiple photos taken at different angles if necessary (Fig. 2c & d). The number of shoots present in each container at each of the five intervals was recorded. Digital photos were used to reduce the handling and potential contamination of plantlets that could occur with the opening and resealing of sterile containers.

Statistical Analysis: The 82 containers were ordered based on a randomized complete block design (RCBD). The rate of shoot growth and the means of the total number of leaves per medium and variation grouping were compared and graphed. Flasks were assigned into four treatment groups: var. diandra/P668, var. diandra/P748, var. triandra/P668, and var. triandra/P748. Mean testing using one-way ANOVA calculations was performed to identify significant differences (95% confidence interval) in the mean number of shoots between variant and treatment type. Pair-wise group analysis was conducted using post-hoc Tukey HSD. All statistical analyses were conducted using SPSS statistical software (SPSS version 19).

Results

A total of 82 cultures vessels were tracked across four groups (P668/var. diandra n = 24, var. P668/var. triandra n = 22, P748/var. diandra n = 18, and P748/var. triandra n = 18). The total number of PLBs for each treatment grouping ranged from 414 to 552, and a total of 1,886 protocorms were included in the study. Both variants showed significantly higher shoot formation in the presence of banana powder. After 156 days, the largest increase in shoot formation was between treatments for the diandrous variant, ranging from 9 shoots for P668 media (w/o banana powder) to 1767 shoots for P748 media (w/ banana) treatment. Average shoot formation was lower for P668 treatments (0.38+/-0.22SE & 4.95+/-2.89SE) versus P748 treatments (98.17+/-16.61SE & 56.95+/-10.80SE). Time to shoot formation ranged from 45–60 days (Table 2).

Table 2 Data on the development of shoots from PLBs of two variants of *P. cochleata* when cultured on two commercial agar-based media with 15% percent banana powder (P748) and without banana powder (P668).

Growth Media	*Number of culture vessels*	*Number of culture vessels*	*Total number protocorms*	*Total number of shoots (156 days)*	*Average number of shoots/flask (156 days)*	*Standard error (SE)*	*Days until shoot formation*
P668	**var. diandra**	24	552	9	0.38	0.22	50-60
P668	**var. triandra**	22	506	109	4.95	2.89	50-60
P748	**var. diandra**	18	414	1767	98.17	16.61	50-60
P748	**var. triandra**	18	414	1025	56.94	10.80	50-60
Totals	**-**	**82**	**1,886**	**2,910**	**35.49**	**6.17**	**-**

The ANOVA analysis revealed no statistical difference in the mean number of shoots produced between var. diandra and var. triandra groupings ($F = 1.28$, $p = 0.262$, $df = 81$). There was a highly significant difference in mean shoot number between P668 and P748 groupings ($F = 65.11$, $p < 0.001$, $df = 81$). Comparisons between groups also showed that the mean number of shoots was significantly higher for the var. diandra/P748 group than all other groups (Tukey HSD p values < 0.01), and the lowest mean number of shoots produced was for the var. triandra/P668 group. For each variant, the p748 media produced significantly more shoots than P668 (Tukey HSD p values < 0.001) (Fig. 3).

The rate of shoot formation remained near zero for both variants under the P668 media treatment. For P748 media treatment, both variants began to differentiate after 50 days and rapidly increased shoot formation at approximately the 60-day mark. However, cultures of var. diandra increased in shoot count more rapidly, resulting in a higher average shoot count at the end of the 156-day period (Fig. 4).

Conclusion

The Clamshell Orchid, also known as the Florida Cockleshell Orchid, is a rare subspecies found in Florida, that symbolizes tropical America. Although this species is widely distributed throughout Florida, Caribbean, Central and South America, it remains popular among orchid collectors and hobbyists and is uncommon in the commercial orchid market. Research-driven in-vitro protocols such as those presented here are critical to the production of hardy and vigorous plants for conservation, breeding programs, and commercial propagation. This study utilizes a commercially available agar-based media from PhytoTechnology Laboratories. Commercial media products were chosen rather than preparing media from scratch as this method proves to be the most feasible and cost-effective for large-scale commercial production and reproducibility of research. Furthermore, commercial lab products such as these would be most beneficial for conservation efforts in developing countries (as these products are lower in cost), significantly decrease the need for large amounts of specified chemicals and require less preparation time. (Kulak et al., 2022). This is especially significant to *P. cochleata* as its range covers still-developing countries, making cost and resource-effective conservation solutions integral.

The results of this study provide clear evidence that banana powder, specifically PhytoTechnology P748 media, is effective for micropropagation of the rare clamshell orchid. Specifically, the addition of organic nutrients and PGRs present in banana powder is highly beneficial to post-germination development and shoot formation for both var. diandra and var. triandra. Furthermore, significant PLB multiplication and suppression of shoot formation was observed under the P668 medium revealing different yet equally beneficial purposes, with P668 being used for the purpose of generating large quantities of somatic embryos and P748 (banana powder) being used for the efficient formation of shoots. Shoot formation is a critical characteristic of healthy plant growth and thus is an indicator of survivorship post-in vitro (Barkoulas et al, 2007). It is important to note that banana powder is not the inducer of shoot formation, but rather a factor affecting development. Extending an understanding of organic sources of PGRs such as banana powder holds promise as a component in growth media for orchid micropropagation. This work will further our knowledge of orchid production, and the findings of this study will be utilized to influence in-vitro micropropagation methods for this orchid species and potentially other closely related species to increase propagation and reintroduction outputs.

In this study, we also have the unique opportunity to compare the embryogenesis and seedling development of variants/subspecies with different levels of genetic diversity. As expected, reduced plant vigor was exhibited by lower and slower rates of shoot formation in the genetically isolated var. triandra suggesting some degree of inbreeding depression, at least in early life stages. However, without genotyping and a better understanding of the functional genes controlling embryonic development, these findings are limited to a strong correlation.

While our findings shed light on the successful in-vitro propagation of *P. cochleata*, future research endeavors should prioritize elucidating the survivorship of these micropropagated orchids outside the in-vitro environment. Greenhouse acclimatization studies should be undertaken to fully understand the role

of organic PGR supplements on plant survivorship. To further conservation efforts, the creation of artificial outcrossing strategies specifically for the triandrous variant, considering the regular selfing, holds great promise in expanding the genetic diversity of orchid populations. Additionally, a critical avenue for future investigation lies in delving deeper into the symbiotic relationship between orchids and fungi. As the species of mycorrhizal fungus associated with *P. cochleata* across its range is currently unknown, identifying this specific fungal species involved in this intricate association and their functionality will not only enhance our understanding of orchid biology but also contribute to the development of more effective micropropagation protocols. These directions for future research underscore the importance of continuing efforts to refine and expand our knowledge in the field, ultimately paving the way for more sustainable orchid conservation and cultivation practices.

Declarations

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Contributions

JD: Conceptualization, research administration, manuscript review and editing

LGB: Investigation, experimental design, data/plate visualization, analysis, original draft preparation.

All authors have read and approved this final manuscript.

Corresponding Author

Jason Downing

Conflict of interest

The authors declare that they have no conflicts of interest.

Data availability

The experimental data and statistical analysis outputs were recorded in Excel spread sheets, deposited on computer hard drive at Fairchild Garden and Google Drive. Data can be made available upon request.

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Figures

Figure 1

Photos of *P. cochleata* var. diandra (left) and var. triandra (right) flowers. Photos taken by Jason L. Downing

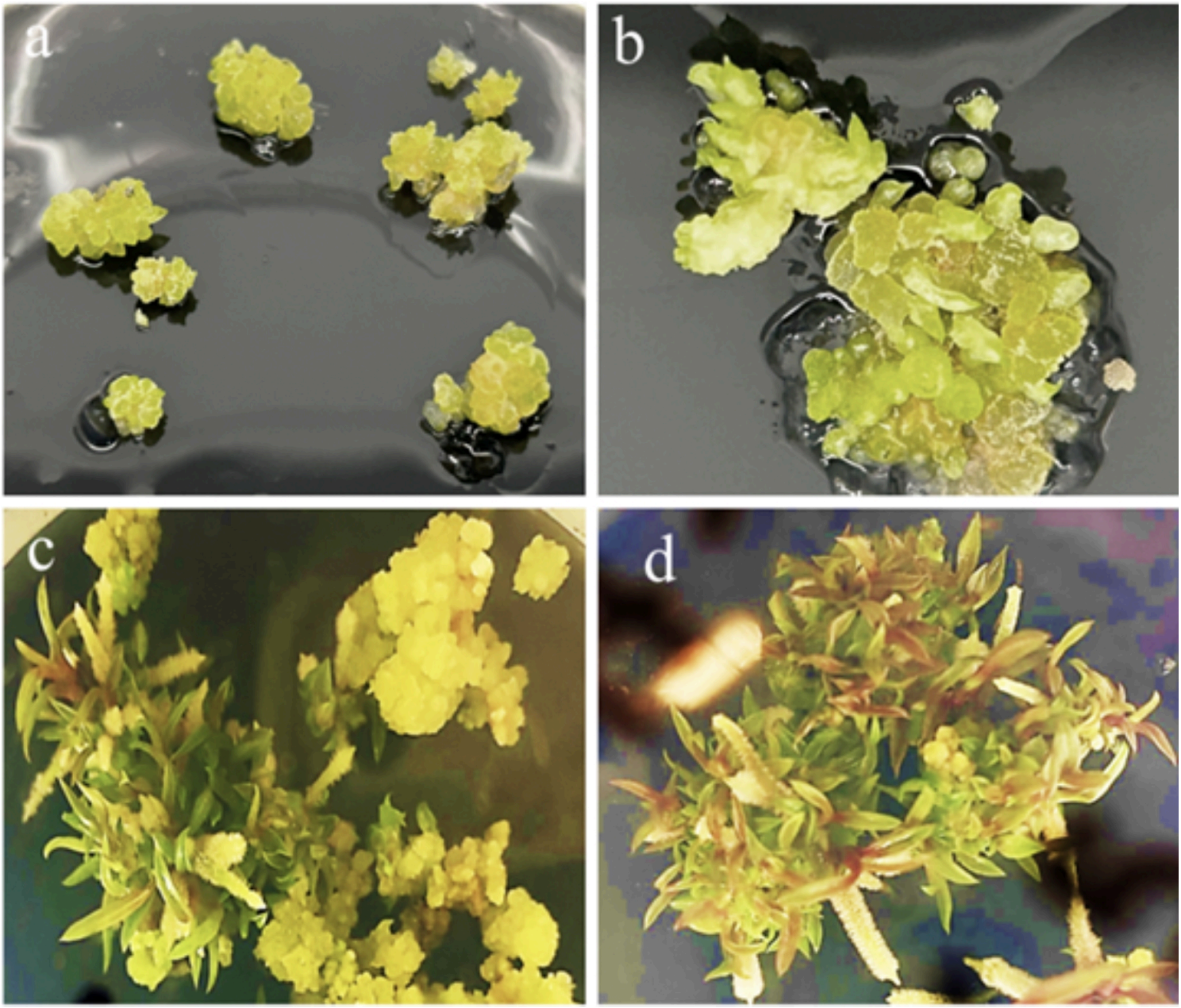

Figure 2

Micropropagation of *P. cochleata*, (a) Multiplication of PLBs 30 days post germination, (b) elongation of individual multiple shoot buds, (c) var. triandra formation of elongated multiple shoot buds and leaves cultured on p748 for 90 days, (d) var. diandra formation of elongated multiple shoot buds and leaves cultured on p748 for 90 days.

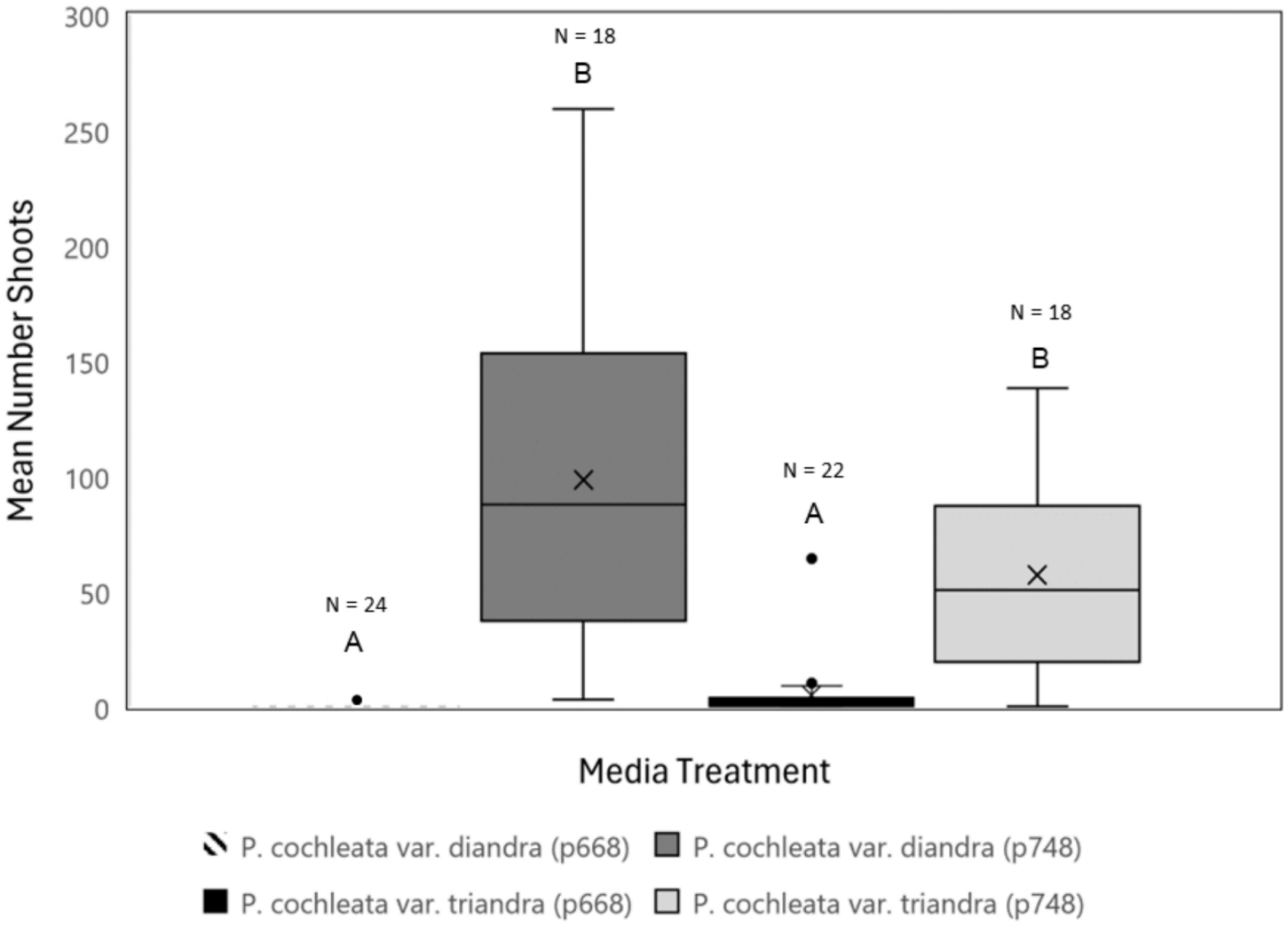

Figure 3

Box plot showing the mean number of shoots produced for each experimental group after 156 days in culture; n= number of flasks per treatment; treatments with different letters indicate a significant difference($p<0.05$) Tukey Kramer error bars indicate standard error (SE).

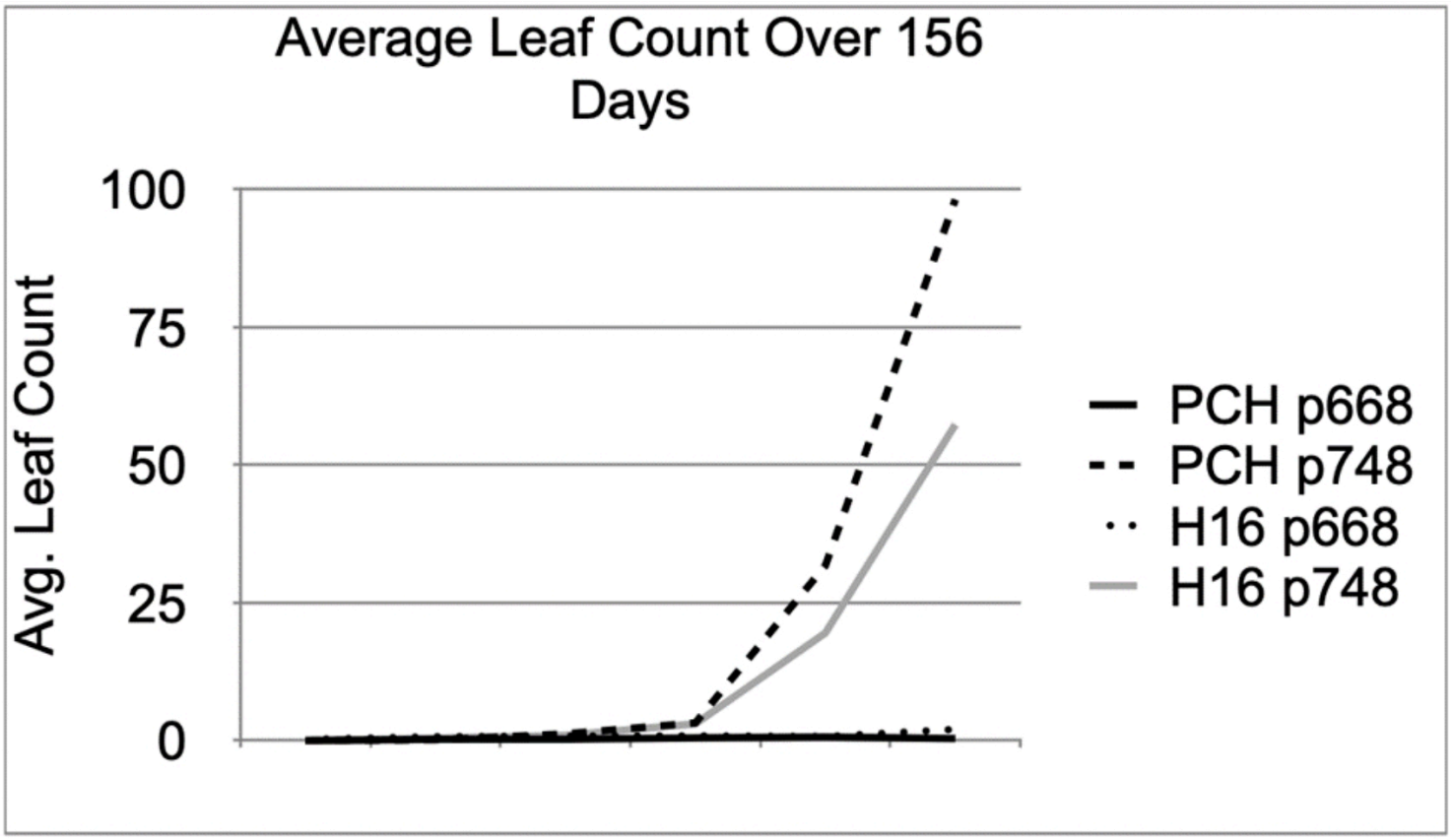

Figure 4

Graph showing the rate of shoot formation for each experimental group over 156 days.