Brazilian Journal of Botany (2021) 44:811–819
https://doi.org/10.1007/s40415-021-00753-5

BIOCHEMISTRY & PHYSIOLOGY - ORIGINAL ARTICLE

The cytokinin 6-Benzylaminopurine improves the formation and development of *Dryadella zebrina* (Orchidaceae) in vitro shoots

Jackeline dos Santos Anjos[1] · Clarissa Alves Stefanello[1] · Leila do Nascimento Vieira[1] · Luiza Giacomolli Polesi[2] · Miguel Pedro Guerra[2,3] · Hugo Pacheco de Freitas Fraga[1]

Received: 1 June 2021 / Revised: 6 September 2021 / Accepted: 10 September 2021 / Published online: 21 September 2021
© Botanical Society of Sao Paulo 2021

Abstract

Plant micropropagation comprises biotechnological tools used for the conservation and mass propagation of orchid species. However, there are few reports of its use for orchids of the subtribe Pleurothallidinae. The present study evaluated the effects of 6-benzylaminopurine (BAP) on shoots formation and development of *Dryadella zebrina* (Porsch) Luer. For this, seeds were in vitro germinated, and the plantlets were submitted to different concentrations of BAP (0, 3, 6, 9, 12, and 15 µM). The plantlets derived from the treatments 0, 6, and 15 µM of BAP were collected after 60 days in culture and subjected to light microscopy analysis. Our results indicated that BAP increased the formation of the new shoots of *D. zebrina*, especially in treatment with 6 µM, and its use is indicated for the in vitro multiplication phase. The anatomical analyses of the roots showed a deleterious effect of 15 µM BAP on the meristematic region, with the presence of more vacuolated cells in this zone. Our results represent the first successful report of in vitro propagation for the genus *Dryadella* and may serve as a basis for further studies of in vitro propagation of phylogenetically related species.

Keywords In vitro culture · Micropropagation · Morphoanatomy · Plant biotechnology · Pleurothallidinae

1 Introduction

The family Orchidaceae is one of the largest families of flowering plants, and orchids have a high economic, ecological, and medicinal importance (Lo et al. 2004; Dressler 2005). Pleurothallidinae is a Neotropical subtribe of the family Orchidaceae, which holds about 5,100 species, distributed in 44 genera (Karremans and Davin 2017).

Dryadella zebrina (Porsch) Luer is one of the species belonging to the subtribe Pleurothallidinae and is characterized by presenting compact plants, with short rhizome, roots covered by velamen, and a uniflora inflorescence (Luer 1999). Its flowers stand out for presenting yellow sepals and petals with reddish-brown color spots (Luer 2006).

Orchids, including *D. zebrina*, produce a large number of seeds. However, the germination rate in nature is considered low, due to the absence of nutritional storage and the need for interaction with mycorrhizal fungi (Arditti and Ghani 2000). Also, orchids are highly susceptible to losses in biodiversity, which can be caused by deforestation, pollinator decline, and predatory collection, thus leading to population reduction in natural environments (Hossain et al. 2009; Swarts and Dixon 2009).

Micropropagation techniques are widely used for several orchid species and consist of growing plant tissues in an aseptic environment, under adequate and controlled physiological conditions. These techniques allow obtaining a large number of plants in a small space and in a short time, as well as maintaining the genetic identity of individuals (Li et al. 2018).

Plant growth regulators (PGRs) supplementation to the culture medium is used in orchid micropropagation to improve the plantlet development (Miyoshi and Mii 1995; Bhattacharyya et al. 2016). This PGR supplementation is one of the main strategies to control and regulate the in vitro

✉ Hugo Pacheco de Freitas Fraga
hugofraga@ufpr.br

[1] Department of Botany, Federal University of Paraná, Curitiba, PR 81531-980, Brazil

[2] Graduate Program in Plant Genetic Resources, Federal University of Santa Catarina, Florianópolis, SC 88034-001, Brazil

[3] Graduate Program in Agricultural and Natural Ecosystems, Federal University of Santa Catarina, Curitibanos Campus, Curitibanos, SC 89520-000, Brazil

morphogenesis, especially with the use of cytokinins and auxins (Aremu et al. 2016).

The balance of auxin and cytokinin appears to play a major role in in vitro morphogenesis, with higher cytokinin levels favoring shoot formation, and higher auxin stimulating root formation (Motyka et al. 1996). Benzylaminopurine (BAP) is one of the most common cytokinins used for in vitro propagation and promotes shoot and axillary bud proliferation (Martins et al. 2018). Auxins, in general, can induce cell elongation, tissue expansion, and cell division, and the formation of calluses and roots (Su et al. 2011).

The use of BAP in the orchids micropropagation is efficient in the induction of multiple buds formation and in the regulation of organogenesis (Adhikari et al. 2019; Castillo-Pérez et al. 2020). However, there are no reports on the effects of BAP in the in vitro organogenesis of Pleurothallidinae species.

To investigate the in vitro morphogenesis of *D. zebrina* and to establish an optimal concentration of BAP for organogenesis, we evaluated the shoots formation and development by means of morphoanatomical analyses. The results obtained here represent the first successful report of in vitro culture for species of the genus *Dryadella* and can contribute to increasing knowledge about the micropropagation of Pleurothallidinae species.

2 Material and methods

Plant material – *Dryadella zebrina* plants were collected in Rio do Corvo, Piraquara, Parana (25°19′42.9″ S; 48°54′50.2″ W) and maintained in the greenhouse of the Department of Botany of the Federal University of Paraná (Curitiba, Brazil). Specimen was deposited at the Herbarium UPCB (UFPR), voucher number DC Imig, 405. The seeds were obtained from mature fruits of plants manually cross-pollinated.

In vitro germination – The *D. zebrina* fruits were washed in running water and surface sterilized with 70% ethanol for 10 min, followed by immersion in 2% (v/v) sodium hypochlorite solution with 0.1% Tween-20™ for 30 min, and subsequently washed six times with sterile distilled water.

After this procedure, the seeds were removed and suspended in 750 µL of sterile water. We pippeted 250 µL of the suspension into each Petri dish (90 × 15 mm) containing 25 mL of culture medium. The germination culture medium was composed by WPM salts and vitamins (Lloyd and McCown 1980), supplemented with 2% sucrose (w/v), 0.1% glutamine, and gelled with 3 g L^{-1} of Phytagel™ (Sigma-Aldrich). The pH was adjusted to 5.8, and culture medium was sterilized in autoclave at 121 ºC for 20 min. The cultures were maintained at 25 ± 2 °C, under a photoperiod of 16 h of light under white LED lamps (40 µmol m^{-2} s^{-1}).

The plant material was morphologically analyzed to monitor the germination process. Germination was evaluated after 30, 60, 90, and 120 days of culture with the aid of a stereomicroscope (SMZ-171, Motic). Seeds that developed green protocorms with a well-developed stem apex were considered germinated.

Plantlets elongation – The green protocorms were transferred to the culture medium with the same components described above, supplemented with 1 µM of 1-naphthalenacetic acid (NAA). The culture medium pH was adjusted to 5.8 and autoclaved at 121 ºC for 20 min.

The plantlets were distributed homogeneously in the Petri dishes and were kept 60 days in a growth room, with a temperature of 25 ± 2 °C, under a photoperiod of 16 h of light under white LED lamps (40 µmol m^{-2} s^{-1}).

Shoot proliferation experiment – The plantlets obtained in the elongation step were used for this experiment. The culture medium was the same as described for plantlets elongation plus six BAP concentrations (0, 3, 6, 9, 12, and 15 µM). The culture medium pH was adjusted to 5.8 and autoclaved at 121 ºC for 20 min.

The explants were inoculated in glass flasks (8.5 cm high × 5.8 cm in diameter) containing 30 mL of culture medium. Seven plants per flask were inoculated, with seven flasks per treatment. The flasks were maintained in the growth room at a temperature of 25 ± 2 °C, under a photoperiod of 16 h of light under white LED lamps (40 µmol m^{-2} s^{-1}).

After 60 days of inoculation, the following variables were morphologically evaluated: (1) Percentage of oxidation and survival; (2) Number of shoots; (3) Average number and length of leaves; (4) Average number and length of roots. The experiments were conducted in a completely randomized design, and the results were subjected to regression analysis, testing the linear and nonlinear models, being applied the one that best fitted to the data according to ANOVA test. The statistical analysis was performed using Statistica® 6.0 for Windows version 8.0.

Morphoanatomy analysis – After 60 days in culture, we collected samples from the median region of leaves and root apices. We collected the samples only from the control treatment, the treatment that indicated the best number of shoots induction, and the treatment supplemented with the highest BAP concentration.

The fixation of the material was carried out in a solution of 0.1 M phosphate buffer and 2.5% paraformaldehyde at 4 °C for 24 h. The material was then washed twice for

15 min with 0.1 M phosphate buffer and dehydrated in an ethanol series (30, 50, 70, 90%, and absolute ethanol).

The material was infiltrated following the ratio (2:1; 1:1; 1:2) of absolute ethanol and methacrylate resin (Historesin, Leica®), and a 24 h immersion in pure historesin. The samples were embedded in historesin and hardener in a 15:1 ratio. After 24 h, the blocks were sectioned (5 μm) in a rotating microtome (Slee Technik®). The sections were treated with an aqueous solution of toluidine blue (0.05%) and phosphate buffer 0.1 M, pH 6.8 (O'Brien et al. 1964). The most relevant histological aspects were observed and recorded in an optical microscope (Olympus BX40) coupled with a digital camera (Olympus DP71).

3 Results

In vitro germination and development – The germination rate was 90%, indicating an adequate maturation stage and high viability of the seeds used in further experiments.

The seeds germination followed the typical stages of orchid plantlet development: (1) Viable embryo; (2) Swollen embryo; (3) Embryo enlargement and testa rupture; (4) Emergence of the protomeristem; (5) First leaf emergence; (6) Leaf elongation (Fig. 1).

The seeds of *D. zebrina* underwent changes in color and size during the development stages. When inoculated, the seeds were colorless and extremely small (Fig. 1a). At 60 days of inoculation, small rounded and chlorophyll structures were observed, forming a sharp structure at the end, as well as the presence of rhizoid in the basal portion (Fig. 1b and c). At 90 days of culture, the protocorms indicated a pair of leaves and rhizoids (Fig. 1d). After 120 days of culture, the plantlets were already well developed and suitable for subculture to the plantlet elongation culture medium.

After 30 days in the elongation culture medium, the plantlets were well developed, with a well-formed shoot and root apices, and uniform length (~ 1 cm).

BAP effects on shoots proliferation – After 60 days in culture, the plants of all treatments indicated mean survival greater than 97%. However, BAP concentrations greater than 9 μM caused a slight decrease in plant survival. Plant oxidation was higher on treatments from 9 to 15 μM of BAP, duplicating the number of oxidized plants (~ 18%), when compared to treatments supplemented with lower concentrations of BAP (0, 3, 6 μM). Those oxidized plantlets presented brownish color and growth inhibition. Thus, it was possible to observe that concentrations up to 6 μM of BAP are more suitable for the in vitro development of *D. zebrina*.

Regarding the mean number of shoots, the greatest formation of new shoots occured in the treatment with 6 μM BAP (Fig. 2). This treatment had an average of three new shoots formed per plantlet, twice the average number of shoots observed in the control treatment. In addition, the shoots

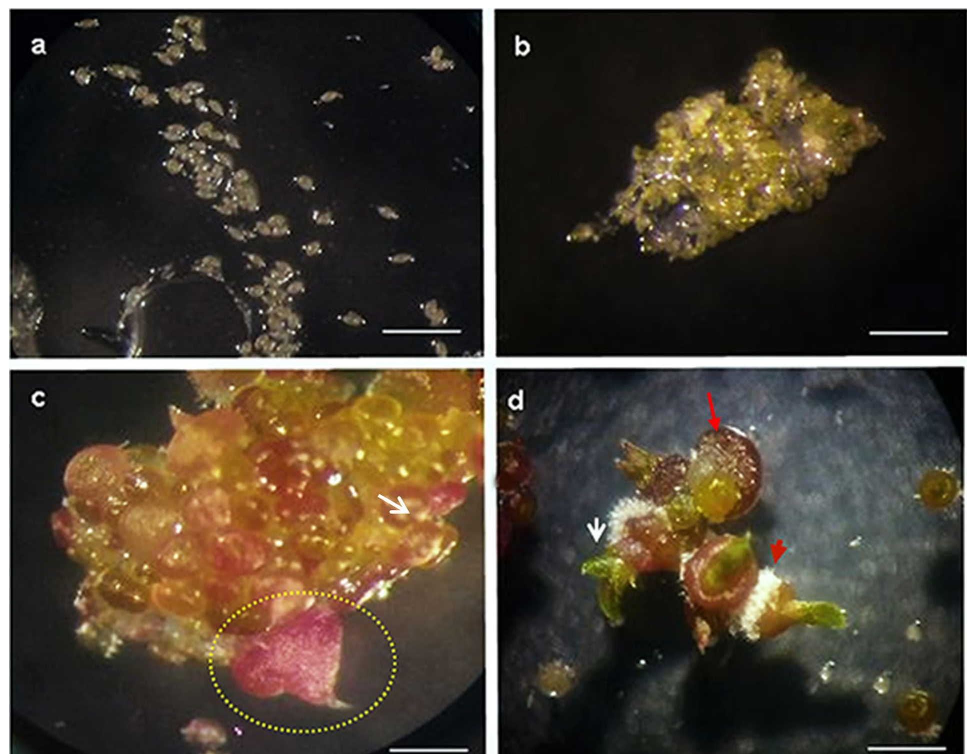

Fig. 1 Developmental stages of *Dryadella zebrina* seeds inoculated in vitro. **a** Seeds on day zero of inoculation. **b** Greenish seeds after 30 days in culture. **c** Protocorm with pink color after 60 days in culture, indicating the shoot formation (yellow circle). **d** Fully formed plantlets, with shoot (white arrow) and rhizoids (red arrow), after 90 days in culture. Bar: 1 mm

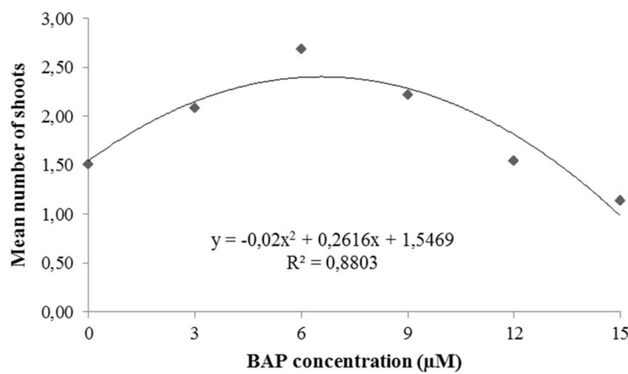

Fig. 2 Mean number of shoots formed of *D. zebrina* plantlets after 60 days in culture medium supplemented with six concentrations of 6-benzylaminopurine (BAP). *p-value* = 0.042428

obtained in this treatment showed an improved uniformity of size (Fig. 3).

The treatments supplemented with 12 and 15 µM BAP indicated a decreased shoot formation, suggesting a possible deleterious effect of BAP in these concentrations. The plantlets of these treatments were poorly developed, with few or no roots (Figs. 3d–f).

The mean number of leaves were the same between the treatments evaluated, showing 12.4 ± 3.33 leaves per plantlet for all the evaluated treatments. In the leaf length, otherwise, BAP-supplemented treatments showed a gradual decrease, from 0.84 cm in the control treatment to 0.65 in the 15 µM BAP treatment (Fig. 4).

As expected, the number and length of roots were higher in the BAP-free treatment (Fig. 5a and b). In the BAP-supplemented treatments, we observed a clear decrease in these parameters.

Morphoanatomy analysis – The root apices of *D. zebrina* analyzed in the present study indicated regions with multistratified epidermis (velamen), cortex, and vascular bundle (Fig. 6). The velamen has two layers of cells, slightly elongated, with "U" spacing, with sinuous projections (Figs. 6d and f).

The *D. zebrina* cortex has cells of circular shape, with four layers of cells, and a vascular bundle with four to seven poles of protoxylem. The vascular bundles showed phloemic cells interspersed with xylemic cells and were surrounded by the pericycle. The presence of vacuolated cells stood out in the root apices of the treatment with 15 µM of BAP when compared to the other two treatments evaluated, especially in the meristematic region (Fig. 6f).

In the leaf samples, we observed a well-differentiated mesophyll, with well-defined lacunous parenchyma cells and vascular bundles (Fig. 7). The morphoanatomical features were very similar between the leaves from the evaluated treatments.

The leaf cross-section has a semicircular shape. Its epidermis is uni-stratified, with a circular shape on the adaxial and abaxial surfaces. More developed adaxial epidermal cells were also observed, which may help in the water reserve. The presence of lacunous parenchyma was more visible in the samples from PGR-free treatment (Fig. 7a) than in the BAP-supplemented treatments (Fig. 7b and c).

The vascular system is formed by collateral vascular bundles. The number of vascular bundles is greater in the median region of the leaf blade surrounded by parenchyma cells. They are organized with different patterns of intercalation of smaller caliber bundles with larger caliber bundles (Fig. 7b and c).

4 Discussion

In vitro germination and development – In vitro development was monitored since the beginning of germination, including the formation of protocorms and the emission of the first leaves and rhizoids (Fig. 1). The present study observed the five stages of the initial germination of orchid seeds: stage 0: viable embryo, without germination; phase 1: swollen embryo, rhizoid formation; phase 2: continuous embryo enlargement, testa rupture; phase 3: the emergence of the protomeristem; phase 4: the emergence of the first leaf; and stage 5: elongation of the first leaf (Stewart and Kane 2006).

In the first 30 days in culture, it was possible to visualize the swelling of the seeds (phase 1), which had a greenish color (Fig. 1b). Similarly, Barbero et al. (2011) observed that at 45 days of culture the seeds also had swollen, rounded, or conical structures, during the germination and initial development of three species of Pleurothallidinae.

Orchid seeds follow a very uniform germination pattern, where the seeds first swell, leading to the rupture of the integument and the release of the embryo (Yam and Arditti 2009). Subsequently, a green tuberiform structure (protocorm) develops.

At day 90 in culture, we observed protocorms with a pair of leaves and several rhizoids, in stage 4 of development (Fig. 1d). Barbero et al. (2011) reported similar characteristics only after 120 days of in vitro germination of three species of Pleurothallidinae. These authors investigated the in vitro development of *Acianthera teres* (Lindl.) Borba, *Octomeria gracilis* Lodd. ex Lindl., and *Octomeria crassifolia* Lindl., and observed that two of these species had well-developed seedlings at 180 days of culture. *Octomeria crassifolia* indicated a slower development, without roots formation until 180 days in culture (Barbero et al. 2011).

For *Dryadella liliputiana* (Cogn.) Luer, Koene et al. (2020) reported in vitro germination rates below 40% and no

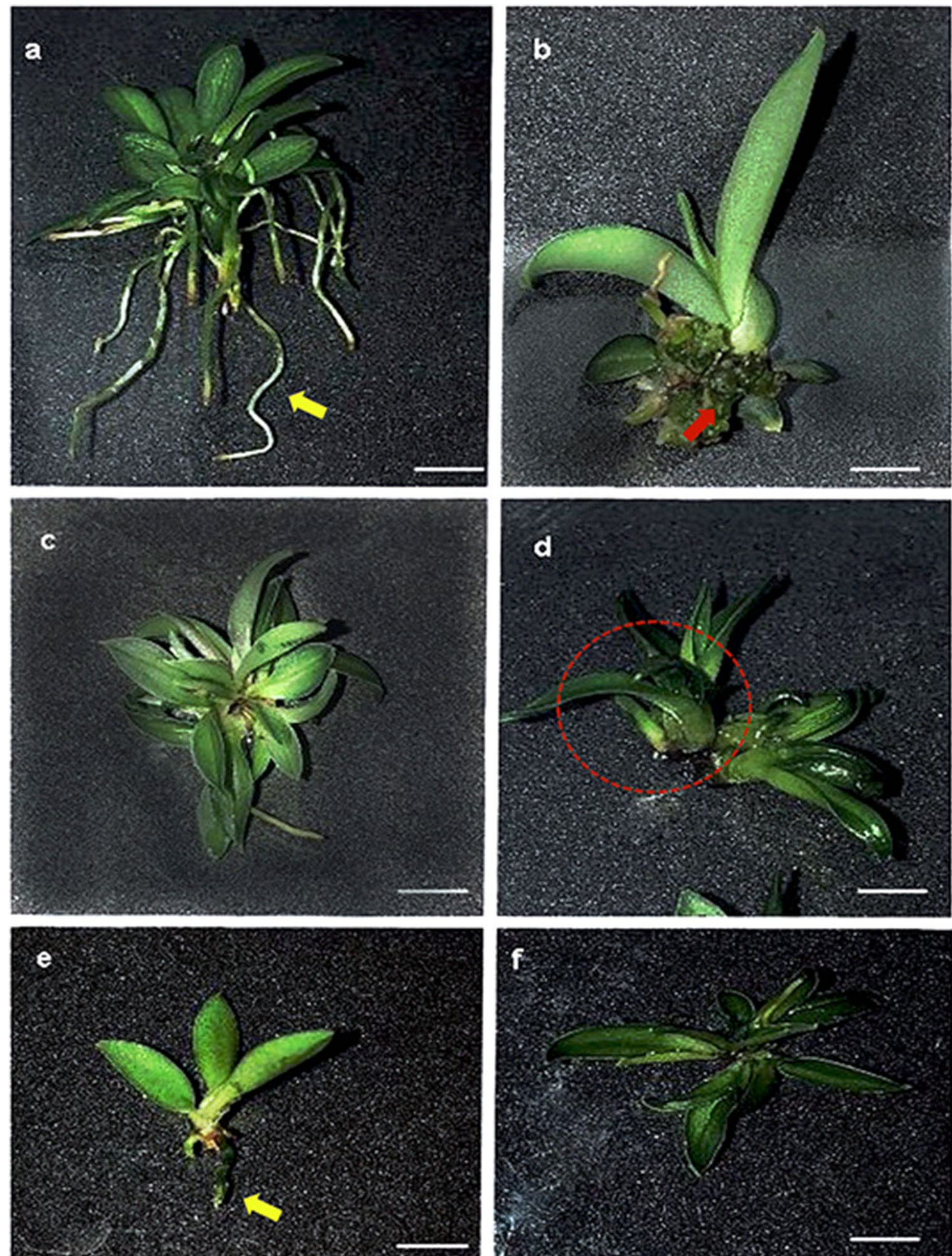

Fig. 3 Morphological features of plantlets after 60 days in culture medium supplemented with six concentrations of 6-benzylaminopurine (BAP). **a** Control treatment; **b** Treatment supplemented with 3 µM BAP; **c** Treatment supplemented with 6 µM BAP; **d** Treatment supplemented with 9 µM BAP; **e** Treatment supplemented with 12 µM BAP; **f** Treatment supplemented with 15 µM BAP. Yellow arrows indicates root, and red arrow indicates shoots formation. The red circle indicates a newly formed shoot. Bar: 1 cm

leaves emergence in the germinated protocorms. Thus, here we report the first micropropagation protocol for *Dryadella*.

In the present work, after 120 days in the germination culture medium, the obtained plantlets were subjected to rooting and elongation in an NAA-supplemented treatment. Previous bioassays in our lab with *D. zebrina* using a culture medium NAA-free indicated lower rooting rates and slower development (data not shown).

BAP effects on shoots proliferation – The elongated plants were inoculated in a culture medium containing six concentrations of BAP. According to our results, a gradual improvement in the number of shoots formation was observed until the treatment supplemented with 6 µM BAP (Fig. 2).

Similar results on the BAP positive effects have been described for the shoots induction of *Laelia crispata* (Thunb.) Garay and the hybrid *Laeliacattleya* Culminant "Tuilerie" x *Laeliacattleya* Sons Atout Rotunda x *Brassolaelia cattleya* (Soares et al. 2010). Rodrigues et al. (2015) also reported a positive influence in the number of shoots formed in *Cyrtopodium saintlegerianum* Rchb.f. Similarly, Pornpienpakdee et al. (2011) proposed that cytokinin supplementation in *Dendrobium* sp. can induce shoots and also promote

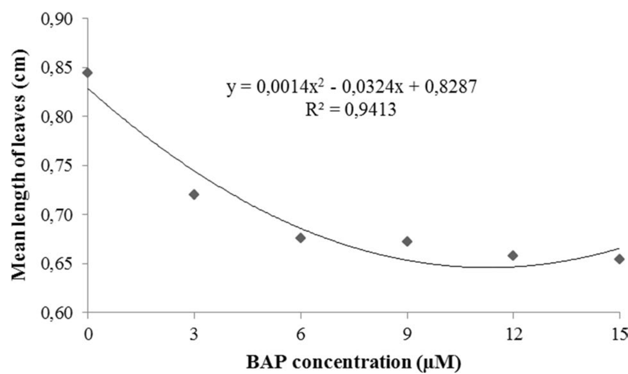

Fig. 4 Mean length of leaves of *D. zebrina* plantlets after 60 days in culture medium supplemented with six concentrations of 6-benzylaminopurine. *p-value* = 0.000005

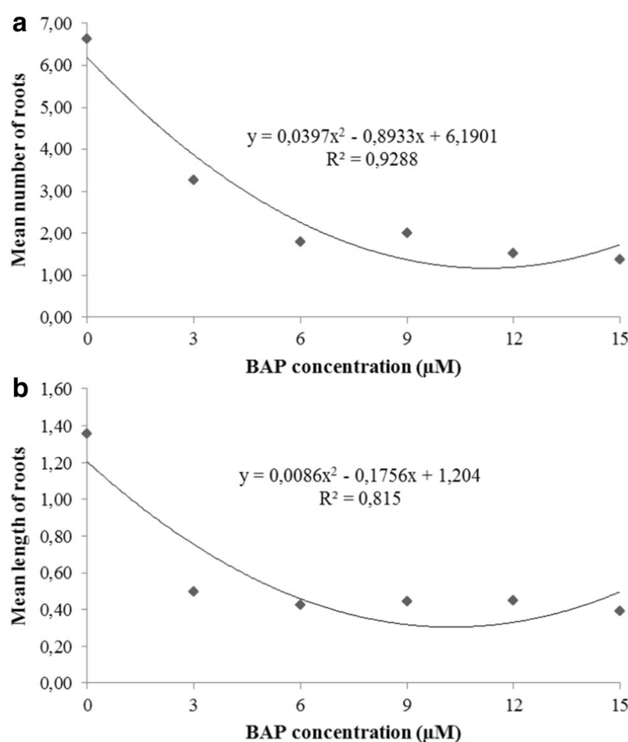

Fig. 5 Mean number **a** and length **b** of roots of *D. zebrina* plantlets after 60 days in culture medium supplemented six different concentrations of 6-benzylaminopurine. *p-value* of figure (**a**) = 0.00045; *p-value* of figure (**b**) = 0.00031

plant development. Unfortunately, there are no reports of the use of BAP for shoots induction in Pleurothallidinae species.

Cytokinins are commonly used to stimulate the development and growth of multiple shoots in vitro (Bhattacharyya et al. 2016). Cytokinins have a wide array of functions, being the regeneration and proliferation of multiple shoots closely related to the type and concentration of cytokinins used (Amoo et al. 2014). Our results indicate that the use of BAP until the concentration of 6 µM improves the formation of new shoots.

Our results also showed a decreased shoot formation and improved plantlets oxidation in treatments supplemented with BAP above 9 µM BAP. High concentrations of exogenous cytokinins can increase the cytokinin oxidase activity, decreasing the endogenous levels of cytokinins and, consequently, inhibiting cell division and preventing the induction of new shoots (Motyka et al. 1996). These higher concentrations can also reduce the size of the leaves and reduce the morphogenic responses in tissue culture, causing problems in the rooting phase (Jordan et al. 1998).

The oxidation derived from the release of phenolic compounds is caused by polyphenol enzymes, which are toxic and inhibit the growth of the explant due to changes in the absorption of metabolites (Cassells and Curry 2001). In this context, concentrations of BAP above 9 µM can cause greater tissue oxidation and negatively affect plant growth in *D. zebrina* explants.

As previously mentioned, we observed a subtle decrease in the plantlets leaf length in BAP-supplemented treatments, when compared to the control (Fig. 4). This decrease may reflect one of the physiological effects of BAP, which is the stimulus of cell division. With accelerated cell division, it is common to observe less elongated cells, which can interfere with the total leaf length (Li et al. 2020). However, during the stage of plant proliferation in the presence of BAP, this effect did not compromise the plantlet development until the concentration of 6 µM.

We observed an improved number and length of roots in the BAP-free culture medium, which was only supplemented with NAA 1 µM (Fig. 5a and b). Nayak et al. (1998) also reported that shoots of *Cymbidium aloifolium* (L.) Sw. were efficiently rooted on a culture medium supplemented with NAA (5.4 µM). In this study, the authors achieved high shoot proliferation rates on a cytokinin-supplemented culture medium and subsequently rooted the plantlets in an NAA-supplemented culture medium (Nayak et al. 1998).

Finally, it is important to stress that despite the expected inhibitory effect of BAP in *D. zebrina* root formation, this cytokinin was able to stimulate the formation of new shoots, increasing the in vitro proliferative capacity. A later stage of in vitro rooting with the obtained shoots from a BAP treatment would be sufficient to obtain a greater number of rooted plants.

Morphoanatomy analysis – The root cortex of *D. zebrina* indicated similar features to those described by Imig et al. (2020) for several species of the genus *Dryadella*. According to Imig et al. (2020), the amount of vascular bundles in *Dryadella* roots is related to water transport functions, where a larger diameter of the bundles will promote greater flow. Ribeiro et al. (2020) also observed a similar structure with

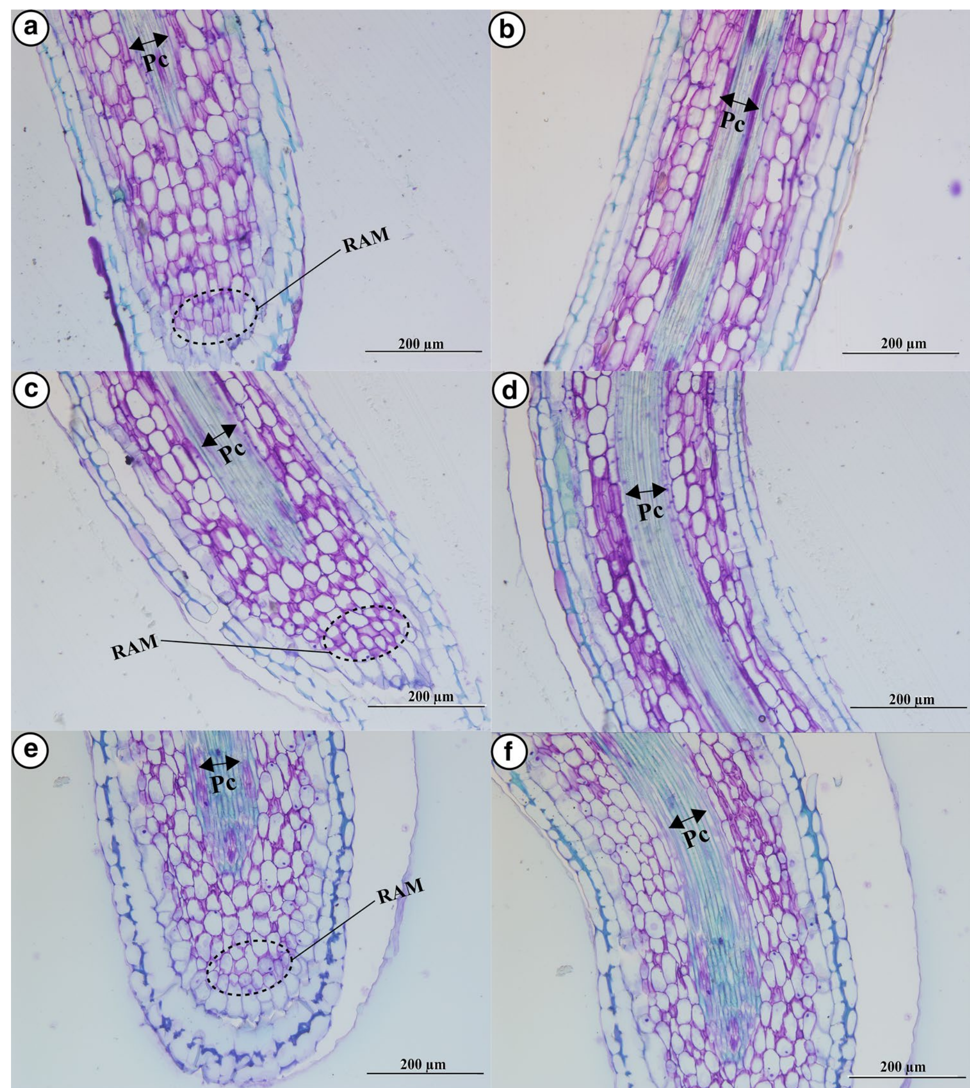

Fig. 6 Longitudinal sections *Dryadella zebrina* roots under light microscopy after 60 days in culture. BAP-free treatment (**a–b**); Treatment supplemented with 6 µM BAP (**c–d**); Treatment supplemented with 15 µM BAP (**e–f**). Pc indicates Procambium, and the black circles indicate the region of the root apical meristem (RAM)

the root morphology of *Cattleya caulescens* (Lindl.) Van den Berg and *Cattleya endsfeldzii* (Pabst) Van den Berg.

The greenish color in the root apex cells of plantlets cultured with 15 µM BAP (Fig. 6f) might be and indicative of phenolic compounds presence. Toluidine blue is a metachromatic dye, which exhibits different coloration according to the substrate it reacts to, indicating green color in the presence of non-structural phenolic compounds (Ribeiro and Leitão, 2020). The morphological analysis corroborates this indicative, where higher rates of plantlet oxidation were observed in the 15 µM BAP treatment (Fig. 3).

Light microscopy analyzes of leaf tissues showed a unistratified epidermis with a circular shape on the adaxial and abaxial surfaces (Fig. 7). Pridgeon (1982) reported similar characteristics, in which the species of the genus *Dryadella* analyzed had cells of circular shapes, elliptical, and the heterogeneous mesophyll.

In conclusion, the germination of *D. zebrina* seeds in the WPM culture medium occurred efficiently, generating well-formed protocorms. The obtained protocorms developed into healthy plantlets that could be elongated and rooted. The cytokinin BAP gradually increased the formation of new shoots of *D. zebrina* in concentrations until 6 µM, indicating its use for the in vitro shoot multiplication phase.

The anatomical analyzes of the roots and leaves allowed us to visualize the effects of BAP on the plantlets. In the roots, less organization of the meristematic region and a possible accumulation of phenolic compounds were observed in plantlets from the treatment supplemented with 15 µM BAP, especially in the cambium.

Our results represent the first successful protocol of in vitro propagation of *D. zebrina* and for the genus *Dryadella*. In this sense, the data obtained here may serve as a

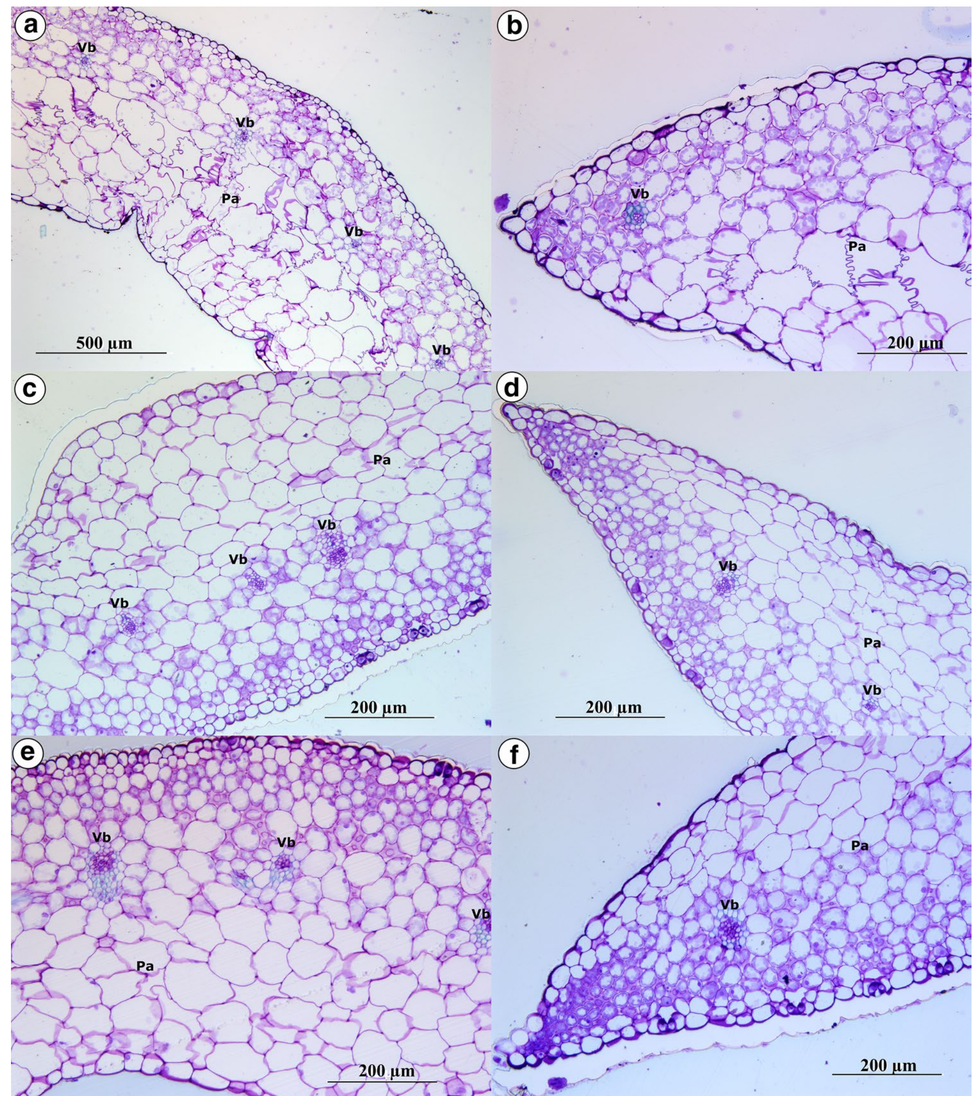

Fig. 7 Transversal sections of *Dryadella zebrina* leaves under light microscopy, after 60 days in culture. BAP-free treatment (**a–b**); Treatment supplemented with 6 µM BAP (**c–d**); Treatment supplemented with 15 µM BAP (**e–f**). Vb indicates vascular bundles (Vb), and Pa indicates parenchymal cells

basis for further studies of in vitro propagation of phylogenetically related species.

Acknowledgements We thank Dr. Eric Smidt, MSc. Daniela Imig and Rothamns Giubert Klippel de Ataide for kindly providing the seeds of *Dryadella zebrina*. We also thank Maria Clara Akiri for her contributions to the study.

Author contributions JSA and HPFF wrote the paper. JSA, CAS, LNV, and HPFF designed and performed the experiments. LGP performed the anatomical analysis. LGP, JSA, and HPFF analyzed the anatomical images. LNV, MPG, and HPFF contributed with reagents and the infrastructure used. All authors analyzed and interpreted the data and reviewed the final manuscript.

Declaration

Conflict of interest The authors declare that they have no conflict of interest.

References

Adhikari H, Pant B (2019) Effect of 6-benzylaminopurine and-naphthalene acetic acid hormonal supplements on the *in vitro* seed germination and seedling development of orchid *Otochilus albus* Lindl. Afr J Biotechnol 18:472–477

Amoo SO, Aremu AO, Moyo M, Szüčová L, Doležal K, Van Staden J (2014) Physiological effects of a novel aromatic cytokinin analogue in micropropagated *Aloe arborescens* and *Harpagophytum procumbens*. Plant Cell Tiss Org Cult 116:17–26

Arditti J, Ghani AKA (2000) Numerical and physical properties of orchid seeds and their biological implications. New Phytol 145:367–421

Aremu AO, Plačková L, Pěnčík A, Novák O, Doležal K, Van Staden J (2016) Auxin-cytokinin interaction and variations in their metabolic products in the regulation of organogenesis in two *Eucomis* species. New Biotechnol 33:883–890

Barbero APP, Barros FD, Silva EAD, Suzuki RM (2011) Influence of water stress on seed germination and early development in three species of Pleurothallidinae (Orchidaceae). Braz J Bot 34:593–601

Bhattacharyya P, Kumaria S, Tandon P (2016) High frequency regeneration protocol for *Dendrobium nobile*: a model tissue culture

approach for propagation of medicinally important orchid species. S Afr J Bot 104:232–243
Cassells AC, Curry RF (2001) Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. Plant Cell Tiss Org Cult 64:145–157
Castillo-Pérez LJ, Maldonado-Miranda JJ, Alonso-Castro AJ, Carranza-Álvarez C (2020) Effect of 6-benzylaminopurine and potassium nitrate on the *in vitro* micropropagation of *Laelia anceps* subsp. *anceps* (Orchidaceae). Biotecnia 22:32–38
Dressler RL (2005) How many orchid species? Selbyana 26:155–158
Hossain MM, Sharma M, Pathak P (2009) Cost effective protocol for *in vitro* mass propagation of *Cymbidium aloifolium* (L.) Sw.–a medicinally important orchid. Eng Life Sc 9:444–453
Imig DC, Junior JAJ, Mauad RSA, Amano E, Smidt EC (2020) Vegetative anatomy and its systematic significance in the *Dryadella* Luer (Orchidaceae: Pleurothallidinae). Feddes Repert 131:175–187
Jordan AM, Calvo MC, Segura J (1998) Micropropagation of adult *Lavandula dentata* plants. J Hortic Sci Biotechnol 73:93–96
Karremans AP, Davin N (2017) Genera Pleurothallidinarum: The Era of Carlyle Luer. Lankesteriana 17:1–8
Koene FM, Amano E, Smidt EC, Ribas LLF (2020) Asymbiotic germination and morphological studies of seeds of Atlantic Rainforest micro-orchids (Pleurothallidinae). PLoS ONE 15:e0243297
Li YY, Chan C, Stahl C, Yeung EC (2018) Recent advances in orchid seed germination and micropropagation. In: Lee YI, Yeung ET (eds) Orchid propagation: from laboratories to greenhouses—methods and protocols: Springer Protocols Handbooks. Humana Press, New York, pp 497–520
Li SM, Zheng HX, Zhang XS, Sui N (2020) Cytokinins as central regulators during plant growth and stress response. Plant Cell Rep 40:271–282
Lloyd G, Mccown B (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Comb Proc Internat Plant Propag Soc 30:421–426
Lo S, Nalawade SM, Kuo C, Chen C, Tsay H (2004) Asymbiotic germination of imature seeds, plantlet development and *ex vitro* establishment of plants of *Dendrobium tosaense* Makino – A medicinally important orchid. In Vitro Cell Dev Biol Plant 40:528–535
Luer CA (1999) lcones Pleurothallidinarum XVIII. Systematics of *Pleurothallis* Subgen. *Pleurothallis* Sect. *Pleurothallis* Subsect. *Antenniferae*, Subsect. *Longiracemosae*, Subsect. *Macrophyllae-Racemosae*, Subsect. *Perplexae*, Subgen. *Pseudostelis*, Subgen. *Acuminatia*. Mongr. Syst. Bot. Missouri Bot. Gard. 76
Luer CA (2006) Icones Pleurothallidinarum VIII. Reconsideration of *Masdevallia*, and the systematic of *Specklinia* and vegetatively similar genera (Orchidaceae). Monogr Syst Bot 105:1–300
Martins JPR, Santos ER, Rodrigues LCA, Gontijo ABPL, Falqueto AR (2018) Effects of 6-benzylaminopurine on photosystem II functionality and leaf anatomy of *in vitro* cultivated *Aechmea blanchetiana*. Biol Plant 62:793–800
Miyoshi K, Mii M (1995) Phytohormone pré- treatment for the enhancement of seed germination and protocorm formation by the terrestrial orchid, *Calanthe discolor* (Orchidaceae), in asymbiotic culture. Sci Horticult 63:263–267
Motyka V, Faiss M, Strand M, Kamínek M, Schmulling T (1996) Changes in cytokinin content and cytokinin oxidase activity in response to derepression of ipt gene transcription in transgenic tobacco calli and plants. Plant Physiol 112:1035–1043
Nayak NR, Chand PK, Rath SP, Patnaik SN (1998) Influence of some plant growth regulators on the growth and organogenesis of Cymbidium aloifolium (L.) Sw. seed-derived rhizomes in vitro. In Vitro Cell Dev Bio Plant 34:185
O'Brien TP, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373
Pornpienpakdee P, Singhasurasak R, Chaiyasap P, Pichyangkura R, Bunjongrat R, Chadchawan S, Limpanavech P, Murashige T, Skoog F (2011) Improving the micropropagation efficiency of hybrid *Dendrobium* orchids with chitosan. A revised medium for rapid growth and bioassay with tobacco tissue culture. Sci Horticult 124:490–499
Pridgeon AM (1982) Diagnostic anatomical characters in the Pleurothallidinae (Orchidaceae). Am J Bot 69:921–938
Ribeiro OPJ, Paula-Souza J, Silva JC (2020) Morphoanatomy of vegetative organs of two species of *Cattleya* (Orchidaceae) Native to Brazil. Rodriguesia 71:e01672017
Ribeiro VC, Leitão CAE (2020) Utilisation of toluidine blue O pH 4.0 and histochemical inferences in plant sections obtained by freehand. Protoplasma 257:993–1008
Rodrigues LA, Paiva Neto VBD, Boaretto AG, Oliveira JFD, Torrezan MDA, Lima SFD, Otoni WC (2015) *In vitro* propagation of *Cyrtopodium saintlegerianum* Rchb. f. (Orchidaceae), a native orchid of the Brazilian savannah. Crop Breed App Biotechnol 15:10–17
Soares JDR, Pasqual M, Rodrigues FA, Araújo AG (2010) Etiolation and artificial light in native and hybrid orchids under *in vitro* cultivation. Cienc Rural 40:1941–1947
Stewart SL, Kane ME (2006) Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae) a rare Florida terrestrial orchid. Plant Cell Tiss Organ Cult 86:147–158
Su YH, Liu YB, Zhang XS (2011) Auxin–cytokinin interaction regulates meristem development. Mol Plant 4:616–625
Swarts ND, Dixon KW (2009) Terrestrial orchid conservation in the age of extinction. Ann Bot 104:543–556
Yam TW, Arditti J (2009) History of orchid propagation: a mirror of the history of biotechnology. Plant Biotechnol Rep 3:1

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.