An assessment of plant growth regulators on asymbiotic development and germination of immature embryos of *Beclardia macrostachya (orchidaceae)*

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Abstract

Beclardia macrostachya is one of the rarest orchids in Mauritius. *In vitro* techniques are being used for mass propagation of this orchid for subsequent restoration programs. Successful asymbiotic germination of *Beclardia macrostachya* was obtained through embryo rescue under *in vitro* conditions. Modified half MS medium supplemented with 10% coconut milk was used as basal culture medium and the effect of plant growth regulators at different concentrations on embryo development was assessed through qualitative and quantitative parameters. Diameter of embryos, length of protocorm-like bodies (PLBs) and length of developing shoots were calculated using digital photography. Maximal growth was obtained in treatments without any plant growth regulators and with 0.5 mg/L N6-Benzylaminopurine (BAP). Higher levels of Thidiazuron/TDZ (0.3 mg/L) and BAP (1.0 mg/L) though they stimulated embryo development faster, yielded higher level of necrosis later. The results also suggest that plant growth regulator treatments that stimulate fastest embryo development from immature embryos/ovules need not be reliable for further development to PLB and plantlet regeneration.

Introduction

Beclardia macrostachya is an endangered orchid in Mauritius, where pollinator limitation has primarily lead to its population decline. No fruit set was observed since 2005 in the wild, unless manual pollination was carried out. Capsules developed from manual pollination were cultured *in vitro* as a conservation strategy for this orchid. For commercial purposes clonal propagation is preferentially used because certain characteristics, such as flower colour and longevity are requisites. However, for conservation purposes it is essential to maintain a high genetic diversity and thus, outbreeding through crosspollination and subsequent seed germination is important for maintaining stable populations in the wild.The seeds of orchids are generally exalbuminous, thus there is little energy source for embryo development and germination *in vivo*.[1] The initial developmental stage of all orchids is a non-photosynthetic protocorm that is mycoheterotrophic.[2] The mycorrhizal fungus *Rhizoctonia* which is initially pathogenic and invades embryo cells, have their hyphae later destroyed by phytoalexins and enzymes and are used as resources for embryo growth. Culturing seeds with fungal isolates from orchid roots may lead to high germination, but mycorrhizal fungi are not necessarily those that initially prompt germination. Following his earlier research works to produce virus free *Cymbidium* from infected plants from shoot apices on Knudson C agar medium that resulted in protocorm like bodies (PLB) formation,[3] Morel suggested tissue culture to be a new means to propagate orchids,[4] and became the first to have successfully cultured orchids *in vitro*.[5] Successful asymbiotic germination have been carried in orchids like *Cephalanthera falcata*;[6] *Calopogon tuberosus*;[7] *Paphiopedilum hybrid*;[8] *Bletilla striata*. However,[9] it was also demonstrated that artificially encapsulated seeds of *Spathoglotis plicata* could also be infected by *Rhizoctonia* mycelia and enhance germination under *in vitro* conditions.

The main aim of this experiment was to develop an efficient protocol for the mass propagation of *Beclardia macrostachya* for conservation purposes. Therefore, low doses of plant growth regulators Benzylaminopurine (BAP) and Thidiazuron (TDZ) were assessed for the development and germination of immature embryos of *Beclardia macrostachya*. Qualitative observations are usually not enough reliable to assess growth. Removing the tissue from the culture media for quantitative measurements increases the risk of contamination, and often requires tissue sacrifice (ex: dry weight). Therefore, a simple method using digital photography was used to assess the growth and germination under different PGR and PGR concentrations.

Materials and Methods

Culture media

The culture medium used was a modified half-strength Murashige and Skoog's[10] medium,[11] as suggested by Chen *et al*. The basal culture media contained ½ strength macro and micro-elements of MS supplemented with myo-inositol (100 mg/L), niacin (0.5 mg/L), pyridoxine HCl (0.5 mg/L), thiamine HCl (0.1 mg/L), glycine (2.0 mg/L), peptone (1 g/L), NaH_2PO_4 (170 mg/L), sucrose (20 g/L) and phytagel (2200 mg/L). It should be noted that 10 % coconut water was also supplemented in the culture medium and the pH was maintained at 5.7. The five different treatments consisted of the basal media with BAP at 0.5 or 1.0 mg/L, TDZ at 0.1 or 0.3 mg/L, and the control containing no plant growth regulators. The media were poured in 50 mL glass Test-tubes (Pyrex). The media were poured in test tubes (20 mL/Test-tube) and slants were prepared to give a maximum surface for the tissues to grow.

Explants

Immature capsules (2 months old) were collected from manually pollinated plants and were surfaced washed with a detergent. They were then surface sterilized in 70% ethanol for 1 minute, followed by 1% Sodium hypochlorite for 30 minutes and washed with sterile water 3 times (25 minutes, 15 minutes, 5 minutes). The capsules were carefully cut open, and small bits (~2 cm^2) of whitish mass of embryos along with the placental tissue were sliced and placed on the culture media. A minimal of 50 culture tubes for each treatment was inoculated and readings were taken on a weekly basis. Each culture tubes that showed fungal infec-

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tion were discarded and this data was used to calculate the percentage of cultures that survived. After 3 months ten test-tubes selected, that showed uniform growth of PLBs, for each treatment and were used for digital photography. Cultured explants were maintained at a temperature of 24ºC±2 and exposed for 10 h per day to an illumination of 15.7 $\mu mol^{-1}s^{-1}$ provided by daylight-type fluorescent lamps. Only 10 h illumination was provided because these plants in their natural habitat are epiphytic, and most of the times were sheltered in the canopy and shade of the host plants.

Digital photography and growth assessment

After 3 months photographs were taken using a Canon *PowerShot A470* Digital camera. Prior to taking photograph a small piece of graph paper (graduated paper) was stuck on the test-tube as a scale to estimate the growth/increase in size (mm) of the embryo. The photographs were also used to make counts of germinating embryos. Using the software Scion Image (*Alpha* 4.0.3.2) the diameter of the embryo was calculated. For PLBs the vertical length was calculated and for the plantlets the length of the longest leaf to its base was taken. It should be noted that the exact value of the diameter is not obtained but a relative value is used to assess growth under different conditions. The test-tubes should all be of the same make so as to minimize the effect of varying refractive index.

Statistical analysis

The percentage of germination was calculated as the number of green embryos/PLBS over the total number of embryos (green PLBs + necrotized embryos). SPSS 16.0 was used for statistical analysis. ANOVA was carried out following Post Hoc Analysis using Tukey's coefficients to determine significant differences in the effects of using different PGR treatments on germination (3 months growth) and subsequent plantlet regeneration (6 months growth). It should also be noted that, since the sample size for the data recorded varied for the different treatments, the Harmonic mean of group sizes was used for the analysis.

Results and Discussion

Maximum germination of *Beclardia macrostachya* immature embryos after 3 months culture on modified ½ MS medium was obtained in Control (31.5 %) while TDZ 0.3 mg/L showed maximal necrosis (Figure 1).

At the germination stage (3 months), the embryo diameter was highest in control and lowest in TDZ (Figure 2).

Table 1. Qualitative and Quantitative data were collected to assess and compare growth under different PGR treatments.

Qualitative and qualitative parameters	Observations				
	Control	BAP 0.5	BAP1.0	TDZ 0.1	TDZ 0.3
Week 1					
Tissue colour	White	White	White	White	White
Week 2					
Tissue colour	Green/ swollen	Green/ swollen	Green/ swollen	Green/ swollen	Green/ swollen
Week 5					
Tissue colour	Green	Dark green	Dark green	Dark green	Dark green
Tissue appearance	Granular	Granular	Granular	Globular embryos distinct	Globular embryos distinct
%Survival rate of cultures	25.7	22.9	51.4	28.6	22.9
Week 10					
% of Healthy cultures	55.6	57.1	30	92.9	41.7
% germination	31.5	26.7	26.7	19.19	18.11
Growth in mm	0.62±0.02	0.47±0.02	0.59±0.01	0.37±0.02	0.44±0.01
6 months					
Developmental stage	Plantlet	Plantlet	PLB	PLB/callus brown/necrosis	All tissue
Growth in mm	1.75±0.10	1.89±0.08	1.10±0.04	1.12±0.09	

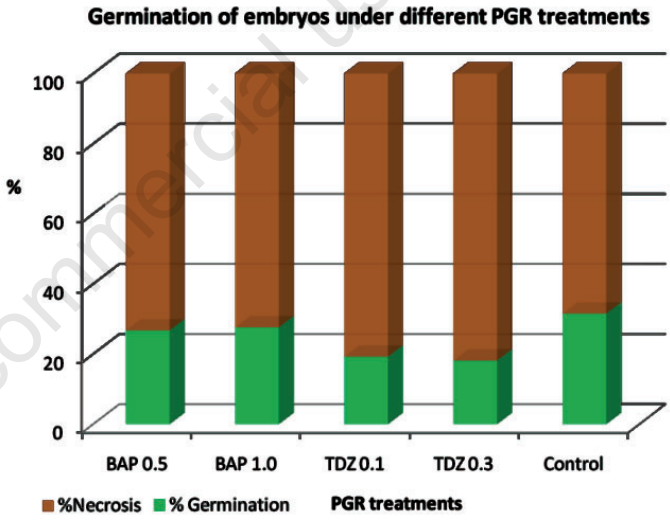

Figure 1. The chart describes the effect of different PGR on development and germination of immature embryos during 3 months under culture (n=1021 (TDZ0.1), n= 1882 (TDZ0.3), n=930 (BAP0.5), n= 1139 (BAP1.0), n = 1082 (Control).

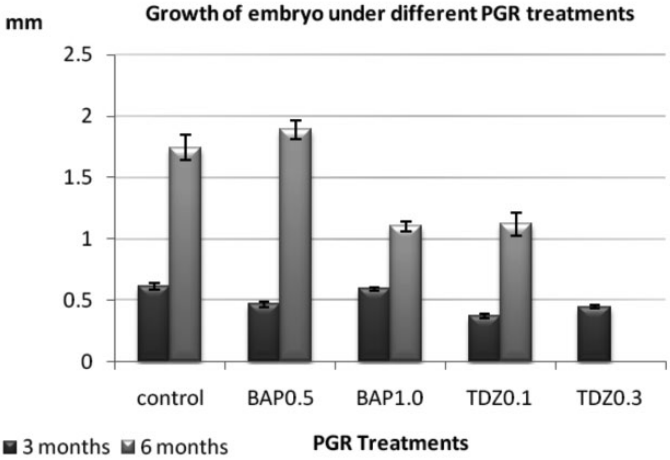

Figure 2. The chart describes the growth in diameter/length of green embryos/ PLBs under different PGR treatments [n=77 (TDZ0.1), n=78 (TDZ0.3), n=103 (BAP0.5), n=162 (BAP1.0), n=126 (Control)].

Table 2. Statistical Comparison of growth (mm) of embryos under different PGR treatments following ANOVA using Tukey's multiPle comParison method.

Multiple comparisons DePendent variable: growth data(mm) during first 3 months Tukey HSD (I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig.		Multiple Comparisons DePendent Variable: growth data(mm) after 6 months Tukey HSD Mean Difference (I-J)	Std. Error	Sig.	
Control	BAP0.5	.14774(*)	0.02671	0	P<0.05	-0.14429	0.10889	0.548	P>0.05
	BAP1.0	0.02183	0.02389	0.892	P>0.05	.64032(*)	0.11544	0	P<0.05
	TDZ0.1	.24284(*)	0.02909	0	P<0.05	.62597(*)	0.13575	0	P<0.05
	TDZ0.3	.17082(*)	0.02897	0	P<0.05				
BAP0.5	Control	-.14774(*)	0.02671	0	P<0.05	0.14429	0.10889	0.548	P>0.05
	BAP1.0	-.12590(*)	0.02534	0	P<0.05	.78461(*)	0.10823	0	P<0.05
	TDZ0.1	.09511(*)	0.0303	0.015	P<0.05	.77026(*)	0.12966	0	P<0.05
	TDZ0.3	0.02308	0.03019	0.941	P>0.05				
BAP1.0	Control	-0.02183	0.02389	0.892	P>0.05	-.64032(*)	0.11544	0	P<0.05
	BAP0.5	.12590(*)	0.02534	0	P<0.05	-.78461(*)	0.10823	0	P<0.05
	TDZ0.1	.22101(*)	0.02784	0	P<0.05	-0.01435	0.13522	1	P>0.05
	TDZ0.3	.14898(*)	0.02772	0	P<0.05				
TDZ0.1	Control	-.24284(*)	0.02909	0	P<0.05	-.62597(*)	0.13575	0	P<0.05
	BAP0.5	-.09511(*)	0.0303	0.015	P<0.05	-.77026(*)	0.12966	0	P<0.05
	BAP1.0	-.22101(*)	0.02784	0	P<0.05	0.01435	0.13522	1	P>0.05
	TDZ0.3	-0.07202	0.03231	0.17	P>0.05				
TDZ0.3	Control	-.17082(*)	0.02897	0	P<0.05				
	BAP0.5	-0.02308	0.03019	0.941	P>0.05				
	BAP1.0	-.14898(*)	0.02772	0	P<0.05				
	TDZ0.1	0.07202	0.03231	0.17	P>0.05				

*The mean difference is significant at the .05 level.

However, after 6 months fastest growth was observed in the treatment containing 0.5 mg/L BAP and in the control (Figures 2, 3a,b). All embryos kept under 0.3mg/L TDZ died within 6 months. Significant differences in growth were observed among the different treatments on plantlet formation (Table 1). The initial survival rate of the cultures was more dependent upon handling of the explants and infections within the cultured tissues. By the 5th week of culture more than 70% of cultures had died due to fungal infections. Infected tubes were discarded and the healthy cultures kept for further observations. By the 10th week, most of the cultures were stable and granular embryos were obvious. However, when the embryos were carefully observed, it was found that the germination rate (production of PLB/Green embryos) was low. Maximal germination (31.5%) was observed in Control (Figure 6a) and minimal germination (18.11 %) observed in TDZ 0.3 mg/L cultures (Figure 5b).

Following Tukey's test, it was found that the effect of culture under both BAP 0.5 mg/L and control were fastest and similar, while TDZ treatments and BAP 1.0 mg/L were detrimental to plantlet development (Table 2). Among cytokinins,[12,13] TDZ is known to have a higher activity than BAP and other

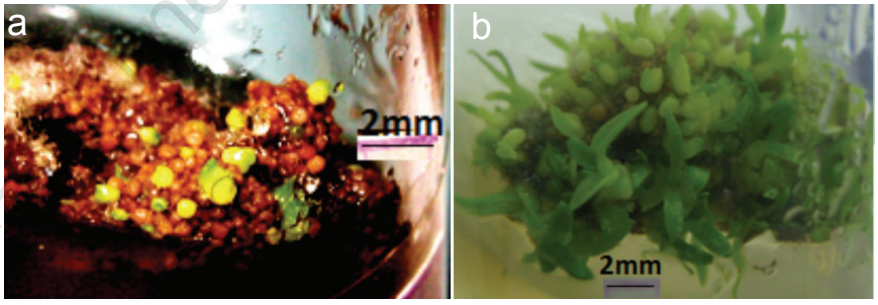

Figure 3. (a) PLB formation on media containing BAP (0.5mg/L) and (b) subsequent plantlet formation.

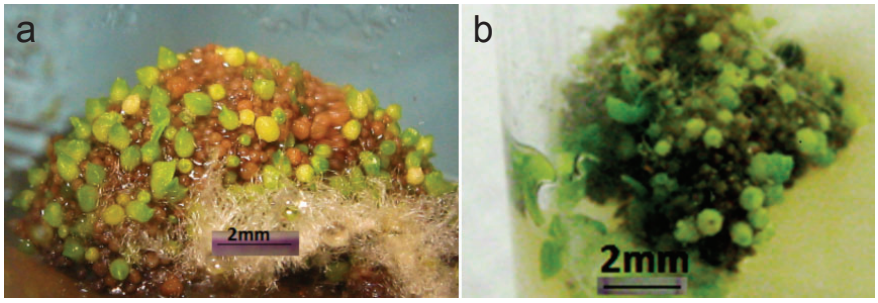

Figure 4. (a) PLB formation in *Beclardia macrostachya* on media with BAP (1.0mg/L) and (b) subsequent plantlet development.

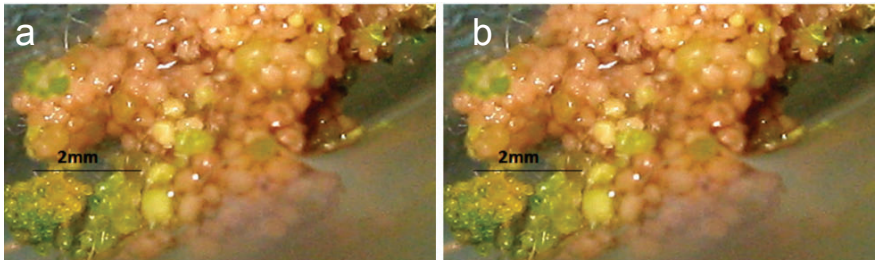

Figure 5. (a) PLB development on media with 0.1 mg/L TDZ and (b) 0.3 mg/L TDZ showing a high level of necrosis.

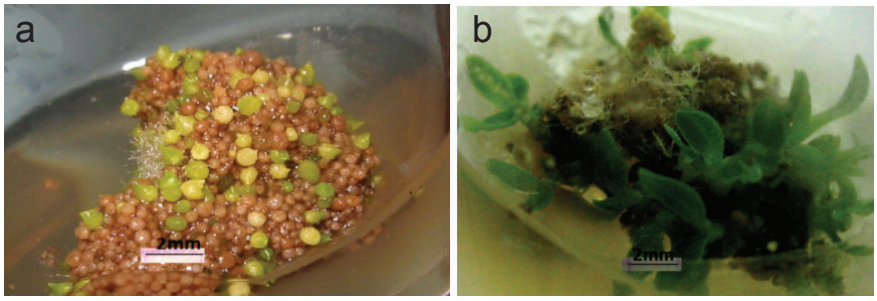

Figure 6. (a) PLB formation on medium without PGR and (b) subsequent plantlet formation.

cytokinins like zeatin and kinetin. Even at a very low dose, TDZ treatment showed high embryogenesis and PLB formation. Nevertheless, keeping the PLBs in the same media for a longer time showed increased vitrification and necrosis. This means that after a certain stage (globular embryo formation), it is advisable to transfer the embryos to basal media. For *Beclardia macrostachya*, TDZ at 0.3 mg/l was lethal with time, even though it stimulated fast embryo development during the first month.[11] It was also reported in Oncidium that, even though TDZ favored PLB formation and proliferation, maximal spontaneous shoot proliferation was obtained on basal media without any growth regulators.[14,15] BAP was also reported to induce PLB formation in Alstromea and Vanilla planiflora.

A higher dose of BAP (1.0 mg/L) initiated faster embryo development, but did not result in higher plantlet formation (Figure 4a,b), whereas BAP at 0.5 mg/L was very effective and yielded faster plantlet formation and development for *Beclardia macrostachya*. Thus, the results suggest that the modified ½ MS medium supplemented only with coconut water sufficed to stimulate germination and plantlet formation, but each ovule would yield a single plantlet and no callus and PLB proliferation would be observed. TDZ did not yield plantlets, but callus and PLB proliferation was observed (Figure 5).

Conclusions

In vitro techniques for conservation purposes of endangered plant species, require tedious literature search to understand optimal growth conditions due to limited explant quantities and availability (yearly for seeds). For *Beclardia macrostachya*, hand-pollinated plants have yielded capsules that were mass propagated. Results suggest that digital photography was a safe and reliable way to assess *in vitro* growth. BAP at a low dose was very effective for embryo development and subsequent plantlet formation. The modified ½ MS medium supplemented only with coconut water also generated plantlets (Figure 6).

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