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Optimization of Plant Growth Regulators and Bioreactor Systems for Efficient *In Vitro* Shoot Multiplication and Elongation of *Amorphophallus muelleri* Blume

Nur Afni Jayanti, Angelia Krisdanti Nasera Putri, Yosephine Sri Wulan Manuhara* and Anjar Tri Wibowo

Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia

*Corresponding author: yosephine-s-w-m@fst.unair.ac.id

Abstract

Porang (*Amorphophallus muelleri* Blume), an Indonesian endemic species rich in glucomannan, is widely utilized in food industries for its health benefits. However, its traditional vegetative propagation through bulbils produces limited plants, necessitating an efficient *in vitro* multiplication system. This study aimed to optimize plant growth regulator combinations and bioreactor systems for enhanced shoot induction and elongation of *A. muelleri*. During the initiation and induction stages, combinations of thidiazuron (TDZ) with benzylaminopurine (BAP) or kinetin at concentrations ranging from 0 to 1.00 mg l^{-1} were applied to solid MS media and observed for 8 weeks. The combination of TDZ 0.50 mg l^{-1} + BAP 1.00 mg l^{-1} produced the fastest shoot induction (32.34±2.52 days), while TDZ 0.75 mg l^{-1} + BAP 0.75 mg l^{-1} resulted in the highest shoot number (76.33±22.81 shoots explant^{-1}) and 100% shoot formation. TDZ combined with kinetin had no significant effect on induction parameters. For elongation, shoots induced from TDZ 0.75 mg l^{-1} + BAP 0.75 mg l^{-1} medium were cultured in two bioreactor systems: a Temporary Immersion System (TIS) and a Balloon Type Bubble Bioreactor (BTBB). The BTBB significantly outperformed the TIS, yielding 85.2±3.35 shoots explant^{-1} with an average shoot length of 5.67±1.13 mm after 4 weeks. These results indicate that the synergistic use of TDZ and BAP, combined with the BTBB system, substantially improves *in vitro* shoot multiplication and elongation of porang, providing a reliable approach for its large-scale propagation and conservation.

Keywords: bioreactor; conservation; cytokinin; mass propagation; shoot induction

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INTRODUCTION

Porang (*Amorphophallus muelleri* Blume) is an endemic Indonesian plant with significant potential in the health and food industries (Wahidah et al., 2021). Porang tubers are rich in dietary fiber, especially glucomannan, a hydrophilic polysaccharide known to reduce fat storage through intra- and extracellular mechanisms, making them effective in preventing obesity and widely used as traditional food and medicine across Asia (Shi et al., 2019). Furthermore, recent studies have reported that glucomannan from porang tuber flour can be used as a gelatin substitute, fat replacer in cookies, and biodegradable food packaging film (Azhar et al., 2023; Anggraeni et al., 2024; Novianto et al., 2025).

The high glucomannan content in porang (14 to 35%) has increased demand, especially from the food and health sectors both domestically and internationally. Despite the global demand for

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porang, estimated at approximately 9,000 tons of fresh tubers and 1,000 tons of dried tubers annually from countries such as Japan, China, Taiwan, Korea, Europe, and Canada, Indonesia's porang production currently meets only around 10% of this requirement (Nugrahaeni et al., 2021; Winarno et al., 2023). This shortfall is largely due to conventional cultivation methods, which often result in inconsistent tuber sizes, with many weighing under 1 kg, and low yields, with one-hectare fields producing only about 4 tons of wet tubers (Nugrahaeni et al., 2021).

Conventional cultivation of porang is typically carried out through vegetative propagation using stem tubers and bulbils (Wahidah et al., 2021). Seedlings from stem tubers are preferred because they produce larger and heavier tubers than those from bulbils (Wahidah et al., 2021). However, stem tubers are scarce because each porang plant produces only one tuber, and their higher cost compared to bulbils further limits their availability (Harijati and Ying, 2021). Therefore, bulbils are considered the best option for porang propagation due to their lower cost and greater availability (Harijati and Ying, 2021). Conventional propagation using bulbils, however, only yields one plant per bulbil (Hardjo et al., 2023). On the other hand, the three-year waiting period for porang harvest is considered too long by farmers compared to crops such as corn, cassava, and rice. This delay occurs because porang plants enter dormancy during the six-month dry season, especially in their bulbils and tubers, as an adaptive response to environmental conditions with limited water availability (Winarno et al., 2023).

To meet the growing demand for porang seedlings in local and international markets, *in vitro* culture techniques offer a promising alternative by enhancing shoot multiplication through shoot induction (Desai et al., 2022). *In vitro* plant propagation method involves tissue explants from the parent plant, which regenerate into whole plants in a controlled, aseptic environment (Haque et al., 2022). The advantages of *in vitro* culture include accelerated seedling production, resilience to environmental fluctuations, and the capacity to produce large numbers of plants in limited space (Ferziana et al., 2021).

Shoot induction in porang involves several stages: explant selection, initiation and shoot multiplication, shoot elongation, rooting, and acclimatization (Murthy et al., 2023). During the initiation and induction stages, shoot propagation is achieved by culturing explants in a medium containing cytokinin. Cytokinin plays a crucial role in shoot apical meristem (SAM) development and can induce shoot growth from callus (Ohbayashi et al., 2022). Ferziana et al. (2021) reported that MS medium supplemented with 2 mg l^{-1} benzylaminopurine (BAP) produced optimal shoot multiplication in *A. muelleri* bulbil explants (4.8 shoots $explant^{-1}$; mean height 0.73 cm). On the other hand, Normasari (2025) reported that MS medium supplemented with 4 mg l^{-1} kinetin produced optimal shoot multiplication in bulbil *A. muelleri* (22.75 shoots $explant^{-1}$). Furthermore, Imelda et al. (2008) found that a combination of thidiazuron (TDZ) (0.2 mg l^{-1}) and BAP (0.5 mg l^{-1}) yielded substantially higher multiplication (37 shoots $explant^{-1}$). These findings suggest that TDZ, when combined with kinetin and BAP, can markedly enhance shoot multiplication in *A. muelleri* bulbil explants.

To obtain a large number of plants, shoot cultures are then transferred to fresh media, ensuring better growth and nutrient supply (Murthy et al., 2023). This process, known as subculturing, involves separating shoots and planting them in new media to meet their nutritional needs and allow for the formation of new organs. Liquid media is particularly effective for subculturing, as it enhances nutrient absorption and promotes better growth compared to solid media, where nutrient uptake is slower (Valdiani et al., 2019; Murthy et al., 2023). In this study, subculturing *A. muelleri* bulbil explants on liquid media using bioreactors without additional cytokinin growth regulators is expected to enhance shoot elongation.

A bioreactor is a controlled environment designed to support the growth, cultivation, and manipulation of living organisms, such as cells or microorganisms, under specific and optimal conditions (Verdú-Navarro et al., 2023). In this study, the bioreactors used in liquid media culture are the Temporary Immersion System (TIS) and Balloon Type Bubble Bioreactors (BTBB). The TIS bioreactor or periodic immersion system has the main advantage that the propagule immersion phase in liquid media (within minutes) and the propagule contact phase with air are longer (within hours). So that cell or tissue damage caused by asphyxia (condition of deficient oxygen supply) and hyperhidricity (physiological malformation resulting excessive hydration, low lignification, impaired stomatal function, and reduced mechanical strength of tissue culture-

generated plants) can be minimized (Lim et al., 2024). BTBB is a bioreactor where explants are submerged in liquid media, providing good aeration and agitation, and is designed to prevent foam formation on the bioreactor walls (Murthy et al., 2023).

Although previous studies have explored cytokinin concentrations (TDZ, BAP, and kinetin) for shoot induction in *A. muelleri* (Imelda et al., 2008; Ferziana et al., 2021; Normasari, 2025), no research has been conducted on optimizing shoot multiplication and elongation using MS liquid media and bioreactor systems. This study was conducted to determine the optimal combination of TDZ, BAP, and kinetin for *in vitro* shoot induction of *A. muelleri* on solid MS medium, and to evaluate the effectiveness of two bioreactor types, the TIS and BTBB for shoot elongation and proliferation. It was hypothesized that specific cytokinin combinations could significantly enhance shoot induction, and that liquid bioreactor systems would promote more efficient shoot growth compared to conventional solid media due to improved nutrient and aeration exchange.

MATERIALS AND METHOD

Experimental setup

This study employed a laboratory-based completely randomized design (CRD) to evaluate the effects of TDZ:BAP and TDZ:kinetin in MS medium, with solid medium used for initiation–induction and liquid medium for elongation. For the initiation–induction stage, 2 independent sets of treatments were tested: (1) TDZ combined with BAP at 5 concentrations (0, 0.25, 0.50, 0.75, and 1.00 mg l^{-1}), and (2) TDZ combined with kinetin at the same 5 concentrations. A total of 25 experimental groups were evaluated, consisting of 24 treatment combinations and 1 control (MS medium without growth regulators). Each treatment was replicated 3 times, with 3 explants per culture bottle. To minimize variability, only bulbils with a diameter of 2 to 3 cm were selected, and explants were randomly assigned to treatments using a CRD to reduce the effect of residual variation.

For the elongation stage, all shoot explants were derived from the most optimal initiation–induction treatment (0.75 mg l^{-1} TDZ + 0.75 mg l^{-1} BAP) to ensure homogeneity. Two types of liquid bioreactors were compared using a simple CRD: a TIS with 5 explant replications per 250 ml of half-strength MS medium, and a BTBB with 10 explant replications per 500 ml of half-strength MS liquid medium per bioreactor.

Explants preparations

Bulbils of porang, measuring approximately 2 to 3 cm in diameter, were collected from Purbalingga, Central Java, Indonesia. Bulbils are obtained from the harvesting of porang plants that are 6 to 8 months old. First, bulbils were peeled and washed thoroughly under running tap water. They were then soaked in a detergent solution for 1 hour, followed by three rinses with running water. Subsequently, the bulbils were immersed in a fungicide solution containing 70% Propineb for 1 hour and rinsed again three times with running water. For surface sterilization, the bulbils were soaked in 70% ethanol for 10 minutes, followed by sequential immersion in 8% sodium hypochlorite (NaOCl) for 10 minutes and 4% NaOCl for another 10 minutes. Finally, the bulbils were rinsed three times with sterile distilled water. This technique was adapted and optimized from Ferziana et al. (2021) to suit the experimental conditions.

Explants planting on solid media

Prepared bulbils were cut into pieces, each measuring approximately 1 cm^3. The explants were then cultured on solid MS media supplemented with various concentrations (0, 0.25, 0.50, 0.75, and 1.00 mg l^{-1}) of the growth regulators TDZ, BAP, and kinetin in different combinations. Each 330 ml culture bottle was filled with two pieces of explants. The cultures were maintained in a growth room at 25±2 °C under continuous LED light for eight weeks.

Explants planting on liquid media in BTBB

The components of the BTBB with a 1 l capacity were assembled, and approximately 500 ml of half-strength MS liquid medium (equivalent to ~50 ml $explant^{-1}$) was dispensed into the culture vessel. Shoots obtained from 8-week-old solid media cultures were aseptically divided into two segments, and 10 explants were transferred into each culture vessel. The BTBB was then placed in a culture chamber and the end of the silicone tubing connected to the air inlet port was attached to an air pump to enable bubbling and continuous aeration. The BTBB containing the shoot explants was maintained in a growth room at 23 to 24 °C under continuous illumination using 20-watt fluorescent lights for 4 weeks. An illustration of the BTBB setup is presented in Figure 1.

Explants planting on liquid media in TIS

Shoots obtained from 8-week-old solid media cultures were aseptically divided into two segments and subcultured into liquid media using a TIS bioreactor with a 500 ml capacity. Five explants were placed on a support mesh within the bioreactor vessel. Subsequently, 250 ml of half-strength MS liquid medium (approximately 50 ml explant^{-1}) was added to the medium reservoir. The port connecting the air pump to the power source was equipped with a timer to regulate the electrical flow, enabling intermittent immersion cycles. In this study, the immersion was set for 2 minutes every 3 hours, as described by Andriani et al. (2021). The TIS bioreactor containing the shoot explants was maintained in a culture room at a temperature of 23 to 24 °C under continuous illumination using a 20-watt fluorescent lamp for 4 weeks. An illustration of the TIS bioreactor setup is provided in Figure 2.

Data collection

The data obtained from this study consisted of qualitative and quantitative data. For the solid medium treatment, qualitative data were obtained from morphological characteristics (color and texture) and the anatomy of callus and shoots. Quantitative data for the solid medium were obtained from the duration of shoot induction (days) and the number of shoots per explant. Shoot induction time was observed and calculated by measuring the time duration (days) it took for shoots to form on the explants. Calculations began one day after the explants were planted in solid medium. The shoot was considered formed or emerged when a white tip appeared on the explant surface and the shoot height reached at least 2 mm. The number of shoots per explant was obtained by counting the number of shoots produced by each explant in the eighth week after planting in solid medium.

Furthermore, in the liquid medium treatment, qualitative data were obtained from shoot anatomy data. Histological observation of callus and SAM was performed by observing wet preparations of longitudinal sections of callus, nodular callus, and shoots under an Olympus

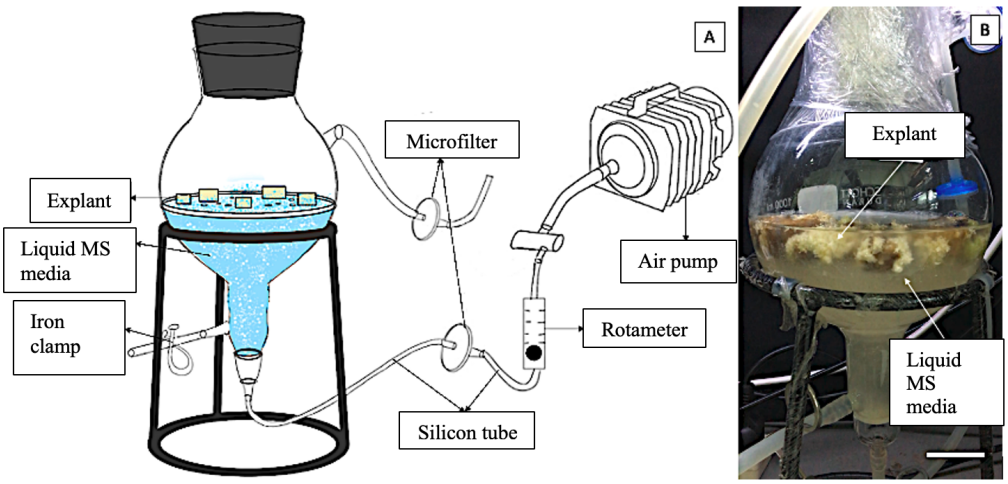

Figure 1. Schematic illustration of the BTBB (A) and fully assembled bioreactor (B)

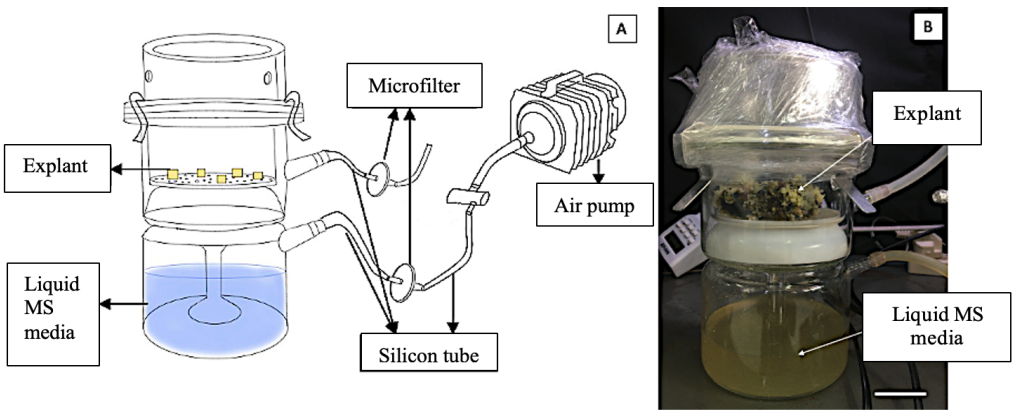

Figure 2. Schematic illustration of TIS (A) and fully assembled bioreactor (B)

U-TV0.5XC-3 inverted microscope with DP20 optics (Olympus, Japan). Quantitative data on liquid medium were obtained from the number of shoots per explant and shoot height (mm). The number of shoots was recorded by counting all shoots produced per explant at 4 weeks after culture initiation. Shoot height was measured from the base to the apex of each shoot using digital calipers, also at 4 weeks post-culture.

Statistical analysis

Data analysis was performed using SPSS version 27. Prior to statistical testing, normality was assessed with the Shapiro-Wilk test. As the data were normally distributed ($p = 0.569 > 0.05$) and homogeneity was confirmed using Levene's test ($p = 0.059 > 0.05$), the Two-Way ANOVA was performed. When significant differences were detected, post-hoc comparisons were conducted using Tukey's Honest Significant Difference (HSD) test at a 5% significance level ($p < 0.05$). For the liquid medium treatment, data normality was verified using the Kolmogorov-Smirnov test ($p = 0.200 > 0.05$), and homogeneity was confirmed using Levene's test ($p = 0.620 > 0.05$ for shoot number; $p = 0.394 > 0.05$ for shoot height). As both assumptions were met, an independent samples t-test was performed. The results showed significant differences between treatments for shoot height ($p = 0.01 < 0.05$) and shoot number ($p = 0.00 < 0.05$).

RESULTS AND DISCUSSION

Initiation-induction stage

Shoot formation in bulbil explants of *A. muelleri* occurs through the process of organogenesis, which can be classified as either direct or indirect (Desai et al., 2022). In direct organogenesis, explants respond to inducing hormones and undergo organogenesis without requiring a dedifferentiation phase. In contrast, indirect organogenesis involves an initial callus formation stage prior to the development of roots or shoots (Desai et al., 2022). Observations during the shoot development phase (Figure 3) revealed that shoot formation in *A. muelleri* bulbil explants was preceded by the emergence of yellowish-white, compact callus with nodular structures, characteristics of indirect organogenesis. The substituted phenylurea nature of TDZ enhances cell regeneration by stimulating the accumulation of endogenous cytokinins and facilitating auxin translocation, thereby regulating the auxin-to-cytokinin ratio to support callus development (Ashokhan et al., 2020). The yellow hue suggests active cell division and indicates metabolically active callus at a mature stage. Furthermore, the compact texture with nodules suggests a high potential for the callus to regenerate into shoots (Du et al., 2025).

The mechanism of the role of cytokinin in organogenesis of *A. muelleri* bulbil explants is related to the study of cytokinin signaling pathways. Histidine-phosphotransfer (HPt) protein has been reported to play a pivotal role in regulating CKs expression through the transfer of the phosphate signal, thereby enhancing cell differentiation, proliferation, multiplication, and regeneration (Bidabadi and Jain, 2020). The initiation of cytokinin signaling is facilitated by membrane-associated histidine kinase receptors, which subsequently transduce the signal through a phosphorelay system (Yang et al., 2021). TDZ frequently exhibits superior activity in promoting shoot multiplication when compared to other adenine-type cytokines. This superiority is attributed to its ability to induce a high level of expression in the *BrCKX* and *BrIP* genes (van Voorthuizen et al., 2021). TDZ has been shown to promote axillary bud proliferation in strawberry by enhancing auxin signaling transduction, a critical process for cell division and shoot initiation. Furthermore, the expression levels of *CRE1*, *AHP*, and *type-A ARR* genes exhibited a significant increase following TDZ application (Wang et al., 2025).

Shoot induction progresses through three phases: (1) the dedifferentiation of cells, establishing morphogenic competence; (2) the cellular response to exogenous growth regulators; and (3) organ morphogenesis that occurs independently of additional phytohormones (Kruglova et al., 2023). The regulation of shoot regeneration depends on a finely tuned balance between auxin and cytokinin activity. Auxin initiates the acquisition of pluripotency, while cytokinins drive the organogenic program toward shoot formation (Šmeringai et al., 2023). Elevated cytokinin levels rapidly suppress auxin signaling in a concentration-dependent manner, resulting in the loss of root identity (Pernisova et al., 2018). Disruption of auxin signaling has been shown to be essential for subsequent shoot induction on shoot-inducing medium (Ohbayashi et al., 2022). Such perturbation may occur through reduced auxin biosynthesis or impaired transport, as cytokinin signaling downregulates auxin biosynthetic genes in the central meristem, thereby lowering auxin levels (Šmeringai et al., 2023).

In this study, dedifferentiation in *A. muelleri* explants was evident from callus formation observed during the third week after inoculation. Differentiation was also observed in the longitudinal sections of the callus, which revealed clusters of meristematic cells in the central zone (CZ), as shown in Figures 4D and 4E. The next phase involves the response to exogenous growth regulators, which induce the formation of shoots. This is evident in the subsequent development of the SAM from the callus (Figure 4), occurring in response to combinations of TDZ + kinetin as well as TDZ + BAP, confirming successful shoot organogenesis.

The interaction between TDZ and BAP showed a strong synergistic influence on both shoot induction time and number of shoots, as confirmed by Two-Way ANOVA and Tukey's HSD post-hoc test (Table 1). The fastest shoot induction was observed in the treatment with TDZ 0.50 mg l^{-1} + BAP 1.00 mg l^{-1}, with an average induction time of 32.34±2.52 days, while the highest average number of shoots (76.33±22.81 shoots explant^{-1}) was obtained from the TDZ 0.75

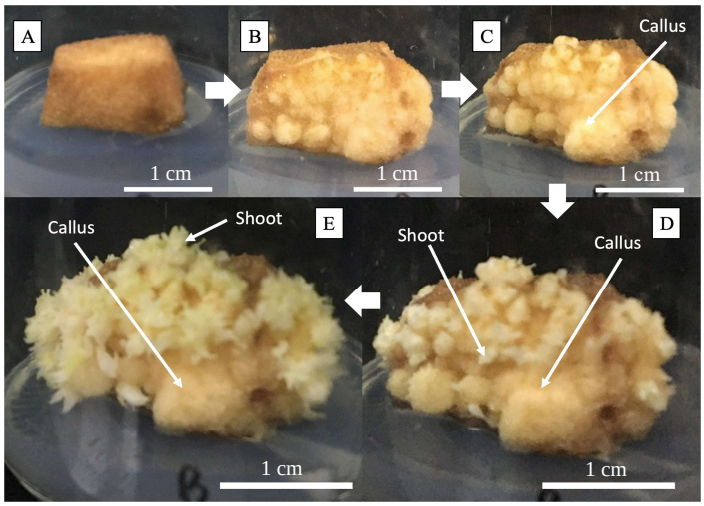

Figure 3. Stages of shoot development from *A. muelleri* bulbil explants cultured on MS medium supplemented with 0.75 mg l^{-1} TDZ and 0.75 mg l^{-1} BAP for 8 weeks. (A) Initial bulbil explants; (B) Explant swelling observed during the second to third week; (C) Formation of yellowish-white callus from the third to fourth week; (D) Emergence of initial buds between the sixth and eighth week; (E) Adventitious bud growth at the eighth week

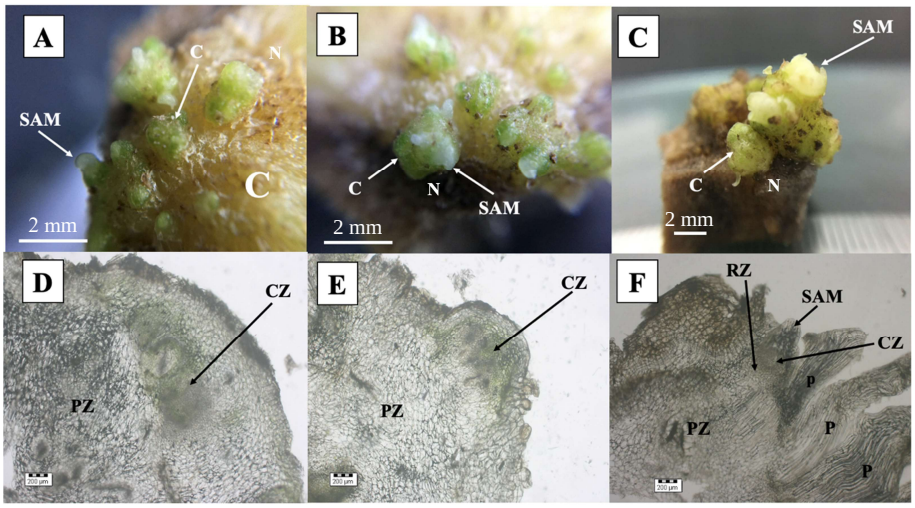

Figure 4. Morphological and histological features of callus and shoot development in *A. muelleri* bulbil explants. (A–C) Morphology of callus, nodules on callus, and shoots; (D) Longitudinal section of callus; (E) Nodules on callus; (F) SAM

Note: C = Callus; CN = Nodule in callus; SAM = Shoot apical meristem; CZ = Central zone; PZ = Peripheral zone; RZ = Rib zone; P = Primordial leaf

mg l^{-1} + BAP 0.75 mg l^{-1} treatment. As shown in Figure 5, the combined use of TDZ and BAP resulted in better shoot induction time and number compared to their individual applications, indicating a possible synergistic effect.

According to Velda et al. (2023), TDZ promotes cytokinin activity by increasing biologically active free bases through ribonucleotide dephosphorylation and by inducing de novo synthesis of purine-based cytokinins such as BAP. No shoot formation was observed in the control and BAP 0.25 mg l^{-1} treatments. This suggests that *A. muelleri* explants are slow to respond to inductive signals when growth regulators are absent or present at low concentrations, resulting in browning and no visible response following 8 weeks of culture. These results are consistent with previous studies, which have shown that BAP, a cytokinin of the adenine type, is typically effective only at higher concentrations (Leto et al., 2025). This aligns with the findings of Ferziana et al. (2021), who reported optimal shoot induction in *A. muelleri* at a BAP concentration of 2 mg l^{-1}.

In the TDZ 0.75 mg l^{-1} + BAP 0.50 mg l^{-1}, 66.7% of explants exhibited callus formation by the fourth week after inoculation (Table 1), but no shoot emergence was observed after 8 weeks of culture. Although all plant cells possess totipotency, their ability to express it varies among species, varieties, and even explants from the same individual (Long et al., 2022). A key factor influencing shoot induction and organogenesis is explant age. Bulbil explant selection in this study was based on a diameter of approximately 2 to 3 cm; however, it is possible that some explants were physiologically older despite having similar sizes, which may have contributed to unresponsiveness or delayed responses to inductive signals.

Table 1. Average length of time for shoot induction, number of shoots, and percentage of explants forming shoots on *A. muelleri* bulbil explants in solid MS medium with the addition of a combination of TDZ and BAP concentrations

Concentration of plant growth regulators (mg l^{-1})		Length of shoot induction time	Average number of shoots	Percentage of explants forming shoots
TDZ	BAP	(days)	(shoots explant^{-1})	(%)
0.00	0.00	No shoot^{a}	0.00±0.00^{a}	0.0
	0.25	No shoot^{a}	0.00±0.00^{a}	0.0
	0.50	59.00±0.00^{h}	0.00±0.00^{a}	0.0
	0.75	54.34±1.15^{g}	4.34±4.92^{a}	100.0
	1.00	54.34±1.15^{g}	2.00±1.73^{a}	100.0
0.25	0.00	36.00±0.00^{ab}	38.67±9.50^{c}	100.0
	0.25	43.34±1.73^{e}	21.00±11.53^{b}	100.0
	0.50	51.00±1.73^{f}	9.67±2.52^{a}	100.0
	0.75	47.34±2.31^{ef}	13.34±1.15^{ab}	100.0
	1.00	42.34±2.52^{de}	7.67±1.52^{a}	100.0
0.50	0.00	54.34±1.15^{g}	19.34±12.50^{b}	100.0
	0.25	40.67±1.15^{cd}	23.00±25.71^{b}	100.0
	0.50	52.67±0.58^{fg}	12.67±4.93^{ab}	100.0
	0.75	52.67±2.52^{fg}	9.67±6.81^{a}	100.0
	1.00	32.34±2.52^{ab}	30.00±23.58^{bc}	100.0
0.75	0.00	36.67±1.15^{b}	42.34±28.29^{c}	100.0
	0.25	42.67±1.15^{de}	36.33±10.02^{c}	100.0
	0.50	55.34±2.31^{g}	11.67±14.57^{ab}	66.7
	0.75	38.00±1.73^{bc}	76.33±22.81^{d}	100.0
	1.00	36.00±1.73^{ab}	42.33±15.50^{c}	100.0
1.00	0.00	40.00±1.73^{cd}	20.00±1.73^{b}	100.0
	0.25	36.00±1.73^{ab}	60.33±21.94^{d}	100.0
	0.50	42.34±2.51^{de}	40.00±13.86^{c}	100.0
	0.75	36.00±1.73^{ab}	60.33±21.93^{d}	100.0
	1.00	38.67±2.89^{bc}	63.33±23.86^{d}	100.0

Note: Means followed by the same letter are not significantly different at $p < 0.05$ (Tukey's HSD)

The shortest average shoot induction time and highest shoot number were observed in the TDZ-only medium (Table 2). The fastest shoot induction occurred with TDZ 0.25 mg l^{-1}, averaging 36.00±0.00 days, while the highest shoot number was observed with TDZ 0.75 mg l^{-1}, averaging 42.34±28.29 shoots explant^{-1}. Among the TDZ and kinetin combinations, TDZ 1.00 mg l^{-1} + kinetin 0.25 mg l^{-1} yielded the best response, with an average induction time of 36.00±1.73 days and 35.67±10.69 shoots explant^{-1}. The Two-Way ANOVA indicated that both TDZ and kinetin concentrations affected shoot induction time, with TDZ exerting a stronger influence. Treatments without TDZ or containing only kinetin displayed extremely long induction periods (≥ 105 days). These findings confirm that TDZ acts as a potent cytokinin regulator enhancing early morphogenesis. However, statistical analysis showed no significant difference among these treatments, suggesting that, unlike BAP, the addition of kinetin did not significantly enhance shoot multiplication. Interestingly, while kinetin did not significantly improve shoot numbers, it appeared to promote shoot elongation. MS medium supplemented with kinetin produced fewer shoots than with BAP, but the shoots were longer. These results align with findings by Kashyap et al. (2022), who reported that BAP is more effective for shoot induction and multiplication, whereas kinetin is more effective for elongation. Similarly, Imelda et al. (2008) found kinetin to be less effective than BAP and TDZ in shoot multiplication of young *A. muelleri* explants.

TDZ is known to promote shoot regeneration even at low concentrations. In this study, a single treatment of TDZ 0.25 mg l^{-1} produced 36.00±0.00 days induction time and 38.67±9.50

Table 2. Average length of time for shoot induction, number of shoots, and percentage of explants forming shoots on *A. muelleri* bulbil explants in solid MS medium with a combination of TDZ and kinetin concentrations

Concentration of plant growth regulators (mg l^{-1})		Length of shoot induction time	Average number of shoots	Percentage of explants forming shoots
TDZ	Kinetin	(days)	(shoots explant^{-1})	(%)
0.00	0.00	No shoot^{a}	0.00±0.00^{a}	0.0
	0.25	129.00±0.00^{f}	0.00±0.00^{a}	0.0
	0.50	149.00±0.00^{g}	0.00±0.00^{a}	0.0
	0.75	105.00±1.73^{e}	0.00±0.00^{a}	0.0
	1.00	38.67±1.15^{b}	3.00±2.00^{a}	100.0
0.25	0.00	36.00±0.00^{b}	38.67±9.50^{d}	100.0
	0.25	53.34±2.89^{cd}	16.67±11.37^{b}	100.0
	0.50	41.67±2.86^{b}	6.34±4.72^{ab}	100.0
	0.75	38.00±0.00^{b}	22.00±19.28^{c}	100.0
	1.00	55.00±0.00^{cd}	8.67±2.081^{ab}	100.0
0.50	0.00	54.34±1.15^{cd}	19.34±12.50^{c}	100.0
	0.25	54.00±1.73^{cd}	3.00±2.00^{a}	100.0
	0.50	36.34±2.31^{b}	13.67±2.08^{b}	100.0
	0.75	53.67±1.15^{c}	6.00±1.00^{ab}	100.0
	1.00	55.00±0.00^{cd}	26.34±10.69^{cd}	100.0
0.75	0.00	36.67±1.15^{b}	42.34±28.29^{d}	100.0
	0.25	54.34±3.06^{cd}	5.67±4.93^{ab}	66.7
	0.50	44.00±0.00^{bc}	20.34±15.14^{c}	100.0
	0.75	44.00±0.00^{bc}	23.00±19.97^{c}	100.0
	1.00	57.00±2.00^{cd}	2.67±4.62^{a}	33.3
1.00	0.00	40.00±1.73^{b}	20.00±1.73^{c}	100.0
	0.25	36.00±1.73^{b}	35.67±10.69^{d}	100.0
	0.50	37.00±5.29^{b}	5.67±5.51^{ab}	66.7
	0.75	49.34±1.73^{bc}	7.34±6.51^{ab}	100.0
	1.00	43.00±1.73^{bc}	27.34±17.39^{cd}	100.0

Note: Means followed by the same letter are not significantly different at $p < 0.05$ (Tukey's HSD)

shoots explant^{-1}. This result aligns with Velda et al. (2023), who noted that low TDZ levels could enhance regeneration comparably or better than other cytokinins. Media without added cytokinins, and single kinetin treatments–kinetin at 0.25, 0.50, and 0.75 mg l^{-1}–showed no positive response by the eighth week. These explants turned brown, indicating cell death, likely due to insufficient levels of growth regulators. Browning is associated with phenolic compound accumulation, typically caused by wounding during explant preparation. This activates oxidase enzymes and phenylalanine ammonia-lyase, triggering phenylpropanoid production, which leads to browning and cell death (Widhiastuty et al., 2023).

Elongation stage

High concentrations of cytokinins result in the formation of small shoots that often fail to elongate (Velda et al., 2023). Therefore, transferring shoots from the multiplication phase to medium without cytokinins is essential to promote proper elongation (Ali et al., 2022). This observation aligns with the findings of this study, where most shoots produced from TDZ + BAP and TDZ + kinetin treatments remained short following eighth week of culture (Figure 3E). Consequently, cytokinin-free medium was used for the elongation phase to eliminate residual hormonal effects. The use of liquid medium significantly affects nutrient uptake and overall explant growth (Padilha et al., 2021). Since plant species and explant types differ in their physiological requirements, the internal environment and bioreactor design must be optimized accordingly for effective micropropagation (Murthy et al., 2023). Therefore, two bioreactor types, TIS and BTBB, were tested to determine the most suitable system for *in vitro* shoot propagation and elongation of *A. muelleri*.

The bioreactor type significantly affected both the number and length of porang shoots (Table 3). The BTBB yielded the highest shoot number and length, averaging 85.2±3.35 shoots explant^{-1} and 5.67±1.13 mm, respectively. These findings are consistent with a previous study by Esyanti et al.

Table 3. Average number and height of porang shoots on ½ MS liquid medium in the TIS and BTBB

Treatments	Average number of shoots (shoots explant^{-1})	Average height of shoots (mm)
TIS	62.6±4.98^{a}	3.63±0.86^{a}
BTBB	85.2±3.35^{b}	5.67±1.13^{b}

Note: Means followed by notations indicate significant differences at a significance level of 0.05 according to the independent sample t-test

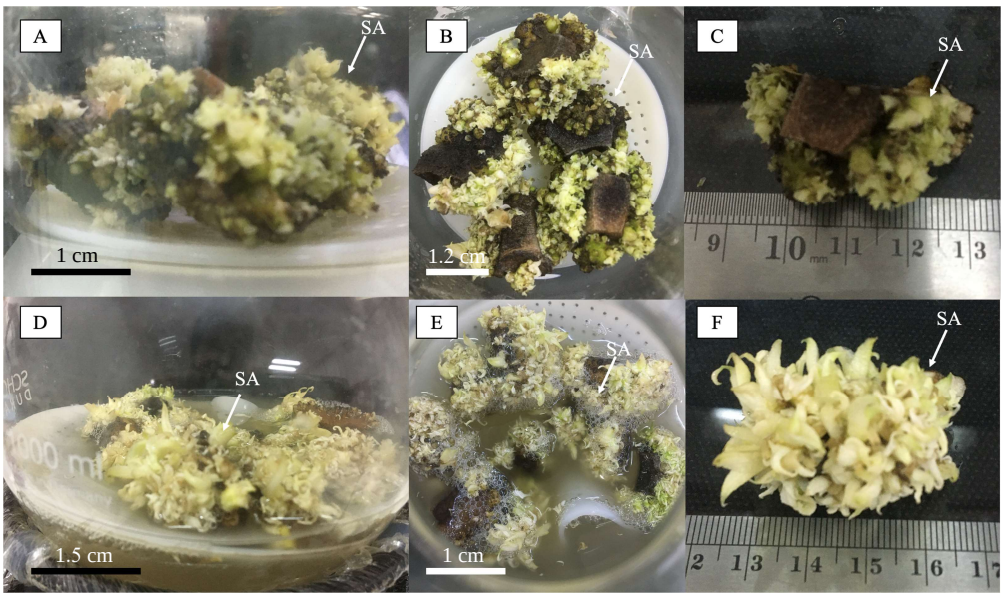

Figure 5. Shoot morphology of *A. muelleri* bulbil explants cultured in the TIS and BTBB during the fourth week is shown. (A) Side view of the shoot explants in the TIS; (B) Top view of the shoot explants in the TIS; (C) Shoot morphology in the TIS; (D) and (E) Side and top views, respectively, of the shoot explants in the BTBB; (F) Shoot morphology in the BTBB

Note: SA = The shoots

(2019), which showed that the BTBB bioreactor applied to *Aquilaria malaccensis* explants produced optimal regeneration with 5.12 shoots explant^{-1}. These differences reflect the species-specific responses to bioreactor conditions. The results suggest that *A. muelleri* metabolism is not negatively affected by continuous contact with liquid medium. The BTBB design ensures constant medium contact, thereby enhancing nutrient, sugar, and water uptake, which promotes optimal elongation (Wongsa et al., 2023).

Visual comparison in the fourth week (Figure 5) revealed that shoots in the TIS bioreactor grew in clusters with residual callus tissue visible (Figure 5C), while those in the BTBB developed without visible callus formation (Figure 5F). This suggests that the BTBB offers better nutrient distribution, allowing callus to differentiate into shoots fully. Additionally, shoots from the BTBB appeared larger and taller than those from the TIS system.

Further comparison was made by observing shoots left on solid medium until week 12 and those subcultured into liquid media bioreactors for 4 weeks (Figure 6). Shoots maintained on solid medium (Figure 6A) remained in clusters with minimal change in size, only transitioning in color from yellowish to green due to chlorophyll development under fluorescent light. In contrast, shoots subcultured in medium without cytokinins exhibited significant elongation and increased size (Figures 6C and 6D). Shoots in the BTBB (Figure 6D) showed greater elongation than those in solid medium (Figure 6B), supporting findings by Seliem et al. (2025) that cytokinin-free media promote shoot elongation.

One concern for liquid culture systems is the potential for hyperhydricity or asphyxia, which may cause abnormal growth (Polivanova and Bedarev, 2022). Morphological observations (Figure 7) revealed no signs of hyperhydricity in either bioreactor system. Shoots in both TIS and BTBB showed firm, non-brittle leaves. Leaves in solid medium and the TIS bioreactor were whitish (Figures 7A and 7C), while those in the BTBB appeared yellowish-white (Figure 7B). Morphological indicators of hyperhydricity, such as thickened stems, short internodes, and brittle or translucent leaves, were also absent (Polivanova and Bedarev, 2022; Ersali, 2024). Histological analysis further confirmed the absence of hyperhydricity. Compared to healthy controls, hyperhydric plants usually exhibit disrupted cell membranes, thinner cell walls, higher vacuolation, and altered vascular structure (Polivanova and Bedarev, 2022). However, cell structure and wall thickness in all BTBB and TIS treatments were consistent and normal.

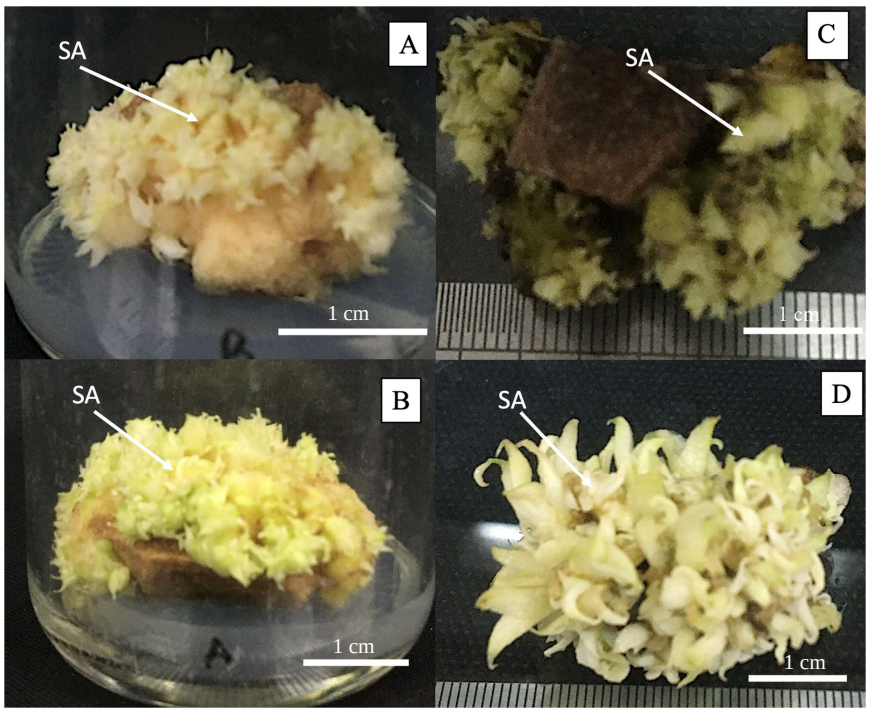

Figure 6. Comparison of shoot morphology on solid and liquid medium. (A) Shoots on solid medium at 8 weeks old; (B) Shoots on solid medium at 12 weeks old; (C) Shoots subcultured on liquid medium in the TIS bioreactor; (D) Shoots subcultured on liquid medium in the BTBB
Note: SA = Shoot

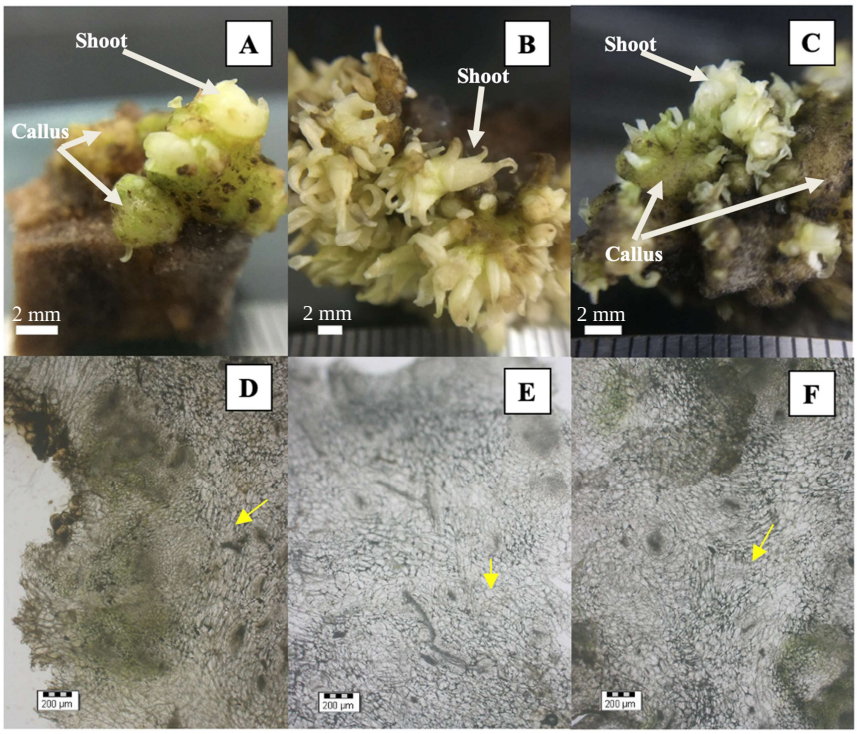

Figure 7. Comparison of morphology and histology of *A. muelleri* shoots on solid and liquid medium. (A) Shoot morphology on solid medium; (B) Shoot morphology on BTBB liquid medium; (C) Shoot morphology on TIS liquid medium. Histological observations: (D) Solid medium; (E) BTBB liquid medium; (F) TIS liquid medium

Note: Observations were made under an Olympus U-TV0.5XC-3 inverted microscope with DP20 optics (Olympus, Japan) at 40× magnification. Yellow arrows indicate the cell walls in the shoots

No increased intercellular spaces or vacuolation were observed, indicating that neither bioreactor induced hyperhydricity in the cultured shoots. In this study, the use of bioreactors was able to enhance the growth of *A. muelleri* shoots because the nutrients in the liquid medium could be easily accessed by the explants. Additionally, the oxygen requirements of the explants can be met by air bubbles blown through a pump in BTBB. Meanwhile, in TIS, the periodic nutrient transport allows the explant an opportunity to obtain oxygen when it is not submerged in liquid medium.

CONCLUSION

The growth regulators TDZ and BAP significantly influenced the shoot induction time, number of shoots, and percentage of explants forming shoots in A. muelleri bulbil explants. In contrast, the combination of TDZ and kinetin did not significantly affect these parameters. The fastest average shoot induction time was obtained from combination concentration of TDZ 0.50 mg l^{-1} and BAP 1.00 mg l^{-1} with an average shoot induction time of 32.34 days. The highest number of shoots was obtained from the addition of concentration of TDZ 0.75 mg l^{-1} and BAP 0.75 mg l^{-1} with an average number of shoots of 76.33 shoots $explant^{-1}$ with a percentage of explants forming shoots of 100%. Among the bioreactor systems tested, the BTBB produced the highest average number and length of shoots, so in future research the scale-up of the micropropagation of *A. muelleri* can use the BTBB.

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