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Establishment of Explant Sterilization Protocol and the Effects of BAP and $AgNO_3$ on *In Vitro* Multiplication of *Kaempferia parviflora* Wall. ex Baker

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Continuous supply of *Kaempferia parviflora* plantlets often disrupted by long rhizome bud dormancy period. Rhizome imports from Thailand are required which inflates the cultivation cost. Hence, this study was conducted to establish an alternative cultivation method through *in vitro* propagation. Rhizome buds were surface sterilized with 100% Clorox® for 3 min, 95% ethyl alcohol for 30 s, 5% Clorox® for 15 min and 0.10% mercuric chloride ($HgCl_2$) for 10 min giving the highest disinfection percentage (40%). Then, *K. parviflora in vitro* multiplication were investigated using combinations of benzylaminopurine (BAP) and silver nitrate ($AgNO_3$). Optimum responses were observed in Murashige and Skoog (MS) media with 8 mg/L BAP and 1 mg/L $AgNO_3$ after 30 days, producing shoot number per explant (4.90±0.57), shoot length per explant (5.07±0.33 cm) and root number per explant (9.70±0.58). The results illustrated explants good responses towards BAP and $AgNO_3$ treatments, highlighting *in vitro* propagation as an alternative for *K. parviflora* cultivation.

Keywords: Benzylaminopurine; *Kaempferia parviflora*; micropropagation; silver nitrate surface sterilization

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Kaempferia parviflora is a perennial rhizomatous geophyte belongs to Zingiberaceae family [1]. *Kaempferia parviflora* has long folk history as medicinal and ornamental plants exceeding borders of countries and cultures [2,3]. *Kaempferia parviflora* has been harvested and used medicinally for a long time, but steadily growing demands and depleting supply in the wilds have prompt a treasure hunt for more [4]. The rhizomes, the pharmaceutic plant parts, cost approximately RM 500–1,000 per kilogram [5]. Vegetative propagation of *K. parviflora* is time-consuming and heavily dependent on the rhizomes splitting too. Besides, bud dormancy as a natural survival strategy for *K. parviflora* has meddling with continuous supply of the plant parts for commercial uses [6].

Micropropagation act as a promising mechanism for production of ample high-quality *K. parviflora* plantlets, reducing reliability on wild resources [7]. Efficient micropropagation can be achieved through elimination of microbial contamination through surface sterilization and optimization of culture condition especially plant growth regulators and other additive are crucial. Surface sterilization of *K. parviflora* explant often used ethyl alcohol and Clorox® resulted in unsatisfactory disinfection rate [8]. Therefore, application of another disinfectant such as mercuric chloride ($HgCl_2$) which is highly toxic towards many microorganisms should be explored.

Previous literatures had revealed benzylamino-purine (BAP) as the plant growth regulator of choice for *K. parviflora in vitro* multiplication with satisfactory results [6, 9,10]. Interestingly, another culture additive with boosting effect should be considered to improve multiplication rate. Silver nitrate ($AgNO_3$) is one of the chemicals that its application in micropropagation is widely used mainly for its potency in ethylene inhibition [11]. Ethylene releases from the *in vitro* plants due to stress wounding, eventually accumulates inside the airtight culture vessels [12]. Thus, triggering adverse responses such as leaves etiolation, epinasty

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and hyperhydricity among species and growth environments [13,14]. So, application of $AgNO_3$ should be further explored.

Just as the BAP and $AgNO_3$ facilitate positive development on plant growth, so as inhibition. The counterproductive effects happen because of cellular concentration of such plant growth regulators in the plant parts go beyond limit of its optimal range by way of impeding protoplasmic streaming [15]. Hence, it is crucial to determine the ideal concentration of BAP and $AgNO_3$ to achieve favourable *in vitro* responses. The effect of BAP and $AgNO_3$ in *K. parviflora* micro-propagation have not been reported up to the present day. Hence, this study intends to identify the best surface sterilization protocol and determine the effect of BAP and $AgNO_3$ in Murashige and Skoog (MS) culture medium for *K. parviflora* micropropagation.

EXPERIMENTAL

Explant Preparation

Kaempferia parviflora rhizomes were purchased from local farmer in Jengka, Pahang. Buds sprouted from rhizomes were used as explants. Bud sprouting method was adopted from literature with some modifications [6]. The rhizomes were gently cleaned from any visible dirt using soft brush and running tap water until visible soil and dirt were removed. The rhizomes were soaked in 10% (v/v) fungicide solution (Benomyl 50%) for 1 h with occasional agitation. After that, the rhizomes were rinsed with tap water to remove fungicide residue. The rhizomes were airdried before incubated on moist perlite to produce buds at room temperature in the dark. After 4 weeks, buds around 2–3 cm were collected and used as explants (Figure 1).

Explant Surface Sterilization

Surface sterilization technique was adopted from literature with some modifications [16]. Rhizome buds (2–3 cm) were collected and washed gently under running tap water for 1 h to remove any visible dirt and then soaked in a clean container filled with distilled water and 3 drops of Decon90 for 15 min. After rinsing with distilled water, the buds were treated with 3% (v/v) fungicide solution (Benomyl 50%) for 1 h with constant agitation. Later, the buds were rinsed with distilled water to remove fungicide residue before being transferred into laminar air flow.

Under aseptic condition, the buds were treated in 100% (v/v) Clorox® (5.25% sodium hypochlorite) solution for 3 min followed by 95% (v/v) ethyl alcohol for 30 s. Then, the buds were soaked in 5% Clorox® for 15 min. After that, the buds were treated with 0.1% (v/v) mercuric chloride ($HgCl_2$) with 5 immersion times (0, 3, 7, 10 and 12 min). Bud treatment without $HgCl_2$ were used as control. Two drops of Tween® 20 were applied with each disinfectant solution, except for ethyl alcohol to increase surface contact between disinfectants and buds [17]. The buds were also thoroughly rinsed 5 times with sterilized distilled water after each sterilizing steps. Finally, the buds were trimmed to 0.8–1.0 cm sizes by removing the outer leaves sheath and base of the buds.

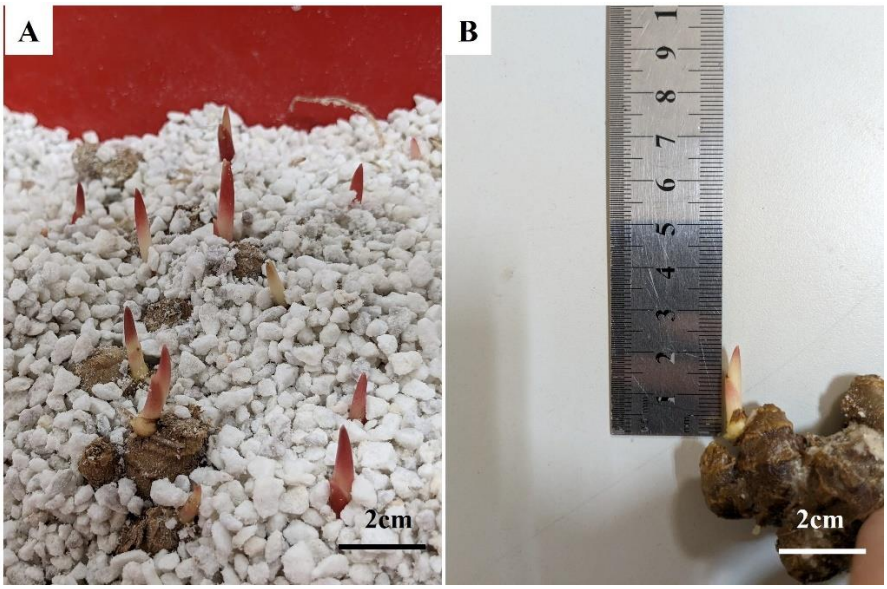

Figure 1. Buds sprouting of *Kaempferia parviflora* (A) buds sprouted after 4 weeks of incubation in moist perlite (B) size of rhizome buds selected as explants.

Table 1. List of different combinations of BAP and $AgNO_3$ supplemented into MS media for *in vitro* multiplication of *K. parviflora.*

Treatment	BAP (mg/L)	$AgNO_3$ (mg/L)
T1 (control)	0	0
T2	0	1
T3	4	0
T4	4	1
T5	8	0
T6	8	1
T7	12	0
T8	12	1

The cleaned buds were inoculated inside a sterile glass jar filled with MS media with 30 g/L sucrose as carbon source, pH of 5.8 and solidified with 5 g/L Gelrite®. The medium was supplemented with 8 mg/L BAP, the optimized culture initiation media for *K. parviflora* by Prathanturarug et. al. [10]. All cultures were incubated in a 16/8 h (light/dark) photoperiod at the light intensity of 80 µmol/m²/s¹ provided by white fluorescent tubes with temperature of 25±2 °C. Each treatment was replicated 3 times with 10 explants in each replication cultured individually in culture media. Data were collected after 3 weeks of incubation. After 3 weeks, aseptic cultures were separated and transferred to MS media void of plant growth regulator for 4 weeks before being used for *in vitro* multiplication experiment.

In vitro Multiplication

Aseptic *in vitro* shoots were cultured individually in each glass jar to test *in vitro* multiplication responses towards 8 different combinations of BAP, whether individually or in combination with 1 mg/L $AgNO_3$ in MS media (Table 1). The MS media were supplemented with 30 g/L sucrose and 5 g/L Gelrite®. The pH was set at 5.8. All cultures were incubated in a 16/8 h (light/dark) photoperiod at the light intensity of 80 µmol/m²/s¹ provided by white fluorescent tubes at 25±2 °C. Each treatment was consisted of 10 replications with an explant per glass jar. Three growth parameters (number of shoots, length of shoots and root numbers) were observed after 30 days of incubation.

Statistical Analysis

All experiments were carried out in a Completely Randomized Design (CRD). Comparison between treatments were analysed using Analysis of Variance (ANOVA). The mean values were further separated using the Tukey's b multiple range test at $p \leq 0.05$ using the IBM SPSS (Statistical Package for the Social Sciences) Statistics Software (version 27) for significant difference determination. All data obtained are expressed as mean ± standard error.

RESULTS AND DISCUSSION

Surface Sterilization of Bud Explants

Table 2 shows the observation of aseptic, contaminated and dead cultures percentages for surface sterilization of *K. parviflora* after 3 weeks. Five immersion time of 0.1% $HgCl_2$ (0, 3, 7, 10, 12 min) were tested while variables for other disinfectants remain constant. Treatment without $HgCl_2$ (0 min) was assigned as control treatment. All data were expressed in the form of mean values and standard errors. The highest percentage of aseptic cultures (40%) was achieved when *K. parviflora* bud explants were treated with 0.1% $HgCl_2$ for 10 min. The highest contamination (100%) was observed in control treatment without $HgCl_2$. The highest dead cultures percentage (26.67%) was obtained from the longest $HgCl_2$ immersion time which was 12 min. No dead cultures were observed in 0 min and 3 min of immersion in 0.1% $HgCl_2$. Comparison between immersion times and explants responses were illustrated in Figure 2.

The ANOVA test was carried out for determination of significant differences between all treatments. The test revealed that different immersion times of 0.1% $HgCl_2$ significantly affected the percentage of aseptic, contaminated and dead cultures for surface sterilization of *K. parviflora*. There were significant differences between the optimum 0.1% $HgCl_2$ immersion time (10 min) with control treatment in terms of aseptic, contaminated and dead culture. This indicated the importance of incorporating 0.1% $HgCl_2$ into surface sterilization protocol of *K. parviflora*.

Table 2. The percentages of aseptic, contaminated and dead cultures obtained from different 0.1% $HgCl_2$ immersion time during surface sterilization of *K. parviflora*.

Immersion times (min)	Mean percentage of aseptic cultures (%)	Mean percentage of contaminated cultures (%)	Mean percentage of dead cultures (%)
0 (control)	0.00 ± 0.00^{c}	100.00 ± 0.00^{a}	0.00 ± 0.00^{c}
3	6.67 ± 0.05^{cb}	93.33 ± 0.05^{a}	0.00 ± 0.00^{c}
7	26.67 ± 0.08^{ab}	66.67 ± 0.09^{b}	6.67 ± 0.05^{bc}
10	40.00 ± 0.09^{a}	36.67 ± 0.09^{c}	23.33 ± 0.08^{ab}
12	33.33 ± 0.09^{a}	40.00 ± 0.09^{c}	26.67 ± 0.08^{a}

Note: Each value represents means ± standard error (SE), n = 30 (3 replication with 10 explants per replicate). Significant difference ($p \leq 0.05$) between treatments displayed by different superscript of means inside column according to Tukey's b multiple range test.

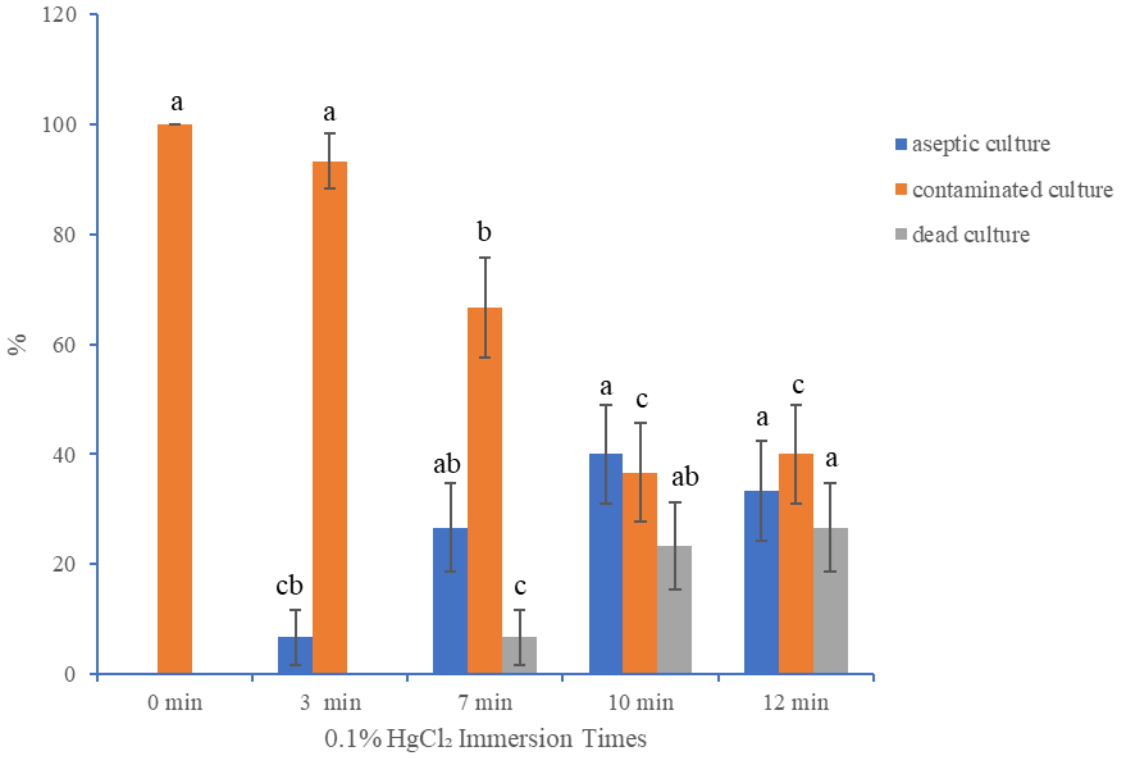

Figure 2. The percentage of aseptic, contaminated and dead cultures using different immersion times of 0.1% $HgCl_2$ after 3 weeks.

Most of contaminations were caused by fungi and emerged as early on the fifth day of culture. Creamy white growth came from explant tissues indicating bacterial contamination were reported on the eighth day of culture. Dead cultures due to over sterilization showed slight discoloration and no growth after 3 weeks (Figure 3). Microbial elimination plays a huge role for successful aseptic culture establishment yet remains a big problem for *in vitro* propagation. Rhizome bud is commonly used as explant in the initiation of aseptic cultures of *K. parviflora* though it demonstrates higher contamination risk [6,8,9]. Buds taken from rhizomes carried heavy microbial loads which made surface sterilization very challenging [18,19]. Similar choice of explant was observed in other Zingiberaceae plants such as *Zingiber officinale* [20,21] and *Curcuma zedoaria* [22].

Smaller explant size usually is harder to induce under *in vitro* conditions, however in this study, small bud explants of *K. parviflora* could give good responses towards *in vitro* stimulation. Bud sprouting was done to obtain new young plant tissue as explant. A few prophylactic measures prior to explant

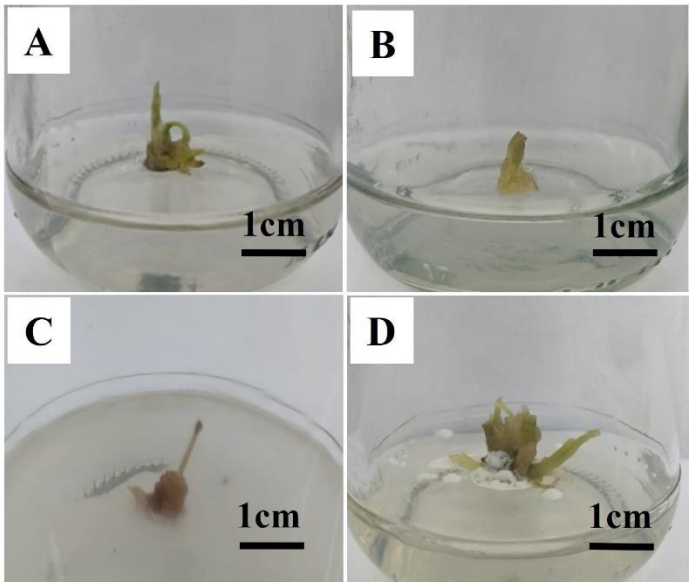

Figure 3. The responses observed on *K. parviflora* explants after surface sterilization with 10 min immersion time of 0.1% $HgCl_2$ showed (A) aseptic culture (B) dead culture (C) contaminated culture with bacteria (D) contaminated culture with fungi after 3 weeks of incubation.

surface sterilization were implemented to improve decontamination. Fungicide treatment (10% Benomyl) on intact rhizomes and bud sprouting on soilless medium (perlite) were done to minimize the presence of soil borne microorganisms on new axillary buds that later being used as explants. Similar soilless approach for bud sprouting was also reported by Labrooy et. al. [6] and Bhattacharya and Sen [23].

Surface sterilization of *K. parviflora* rhizome bud explants with only ethyl alcohol and sodium hypochlorite was not sufficient for microbial decontamination as observed in the control as there was no explant survived during culture with 100% contamination. The use of highly deleterious chemical, especially $HgCl_2$ was adopted for rhizome bud explants with extra caution due to its wide spectrum of toxicity against microorganisms. Extensive use of $HgCl_2$ had been reported in various Zingiberaceae plants such as *Kaempferia angustifolia* [24], *Kaempferia galanga* [25], *Curcuma longa* [26], *Zingiber officinale* [27] and *Zingiber zerumbet* [28]. Mercuric chloride usually paired with other disinfectants such as ethyl alcohol and sodium hypochlorite, providing multiple steps sterilization procedure to improve decontamination of *K. parviflora* and other Zingiberaceae plants [6, 29].

In this study, incorporation of $HgCl_2$ into the surface sterilization protocol decreased the contamination percentage in the immersion time-dependent manner. The optimum $HgCl_2$ immersion time for *K. parviflora* surface sterilization was 10 min and slight increase in contamination percentage and decrease in aseptic culture percentage were observed beyond that time limit. The optimum 10 min immersion time of $HgCl_2$ was in accordance with Devi et. al. [29] on *Curcuma angustifolia* and Miri [30] on *Zingiber officinale* with 100% and 65% of aseptic cultures, respectively. The present study found that shorter immersion times of $HgCl_2$ (3 min and 7 min) did not gave satisfactory decontamination for *K. parviflora*, with only 6.67% and 26.67% aseptic cultures, respectively.

Higher immersion times were needed for aseptic culture establishment despite over sterilization on some cultures. Immersion times on 10 min and 12 min showed better decontamination rate with 40.00% and 33.33% of aseptic culture, respectively. However, the prolonged immersion time might cause *K. parviflora* cell death indicated by the increase in dead culture percentage in 10 min and 12 min immersion times. Many cultures were successfully decontaminated in those treatments but showed slight discolouration and died after 3 weeks with no visible growth.

In contrast, there were reports on surface sterilization done in shorter $HgCl_2$ immersion time in a few studies. Labrooy et. al. [6] treated *K. parviflora* explants with 0.1% $HgCl_2$ for 3 min resulted in 88% decontamination while Keng and Hing [31] gained 62–81% decontamination on various Zingiberaceae plants treated with 0.1% $HgCl_2$ for 5 min. This difference in optimum $HgCl_2$ immersion times might be contributed by the different locality and cultivation practices of *K. parviflora* stock plants where diversified micro-organism populations were possible. Nevertheless, endophytes presented in the explant vascular system could further limit the successful rate of aseptic cultures establishment as disinfectants mostly targeted at the explant surfaces [32].

Table 3. The effects of BAP (0, 4, 8 and 12 mg/L) and $AgNO_3$ (0 and 1 mg/L) on *in vitro* multiplication of *K. parviflora* after 30 days of culture.

BAP	$AgNO_3$	Treatment	Mean number of shoots per explant	Mean shoot length per explant (cm)	Mean root number per explant
0	0	T1	0.80 ± 0.13^{e}	1.84 ± 0.31^{c}	0.90 ± 0.31^{d}
	1	T2	1.80 ± 0.25^{de}	3.71 ± 0.09^{b}	5.20 ± 0.44^{c}
4	0	T3	3.80 ± 0.25^{abc}	3.85 ± 0.26^{ab}	6.40 ± 0.45^{bc}
	1	T4	4.00 ± 0.33^{abc}	4.16 ± 0.23^{ab}	8.80 ± 0.70^{a}
8	0	T5	4.60 ± 0.48^{ab}	4.38 ± 0.17^{ab}	10.30 ± 0.42^{a}
	1	T6	4.90 ± 0.57^{a}	5.07 ± 0.33^{a}	9.70 ± 0.58^{a}
12	0	T7	2.90 ± 0.53^{cd}	3.55 ± 0.62^{b}	8.70 ± 0.94^{a}
	1	T8	3.20 ± 0.33^{bcd}	4.44 ± 0.09^{ab}	8.20 ± 0.59^{ab}

Note: Each value represents means ± standard error (SE), n = 10 (each treatment consisted of 10 replicates). Means within columns followed by the different superscript are significantly different at $p \leq 0.05$ according to Tukey's b multiple range test.

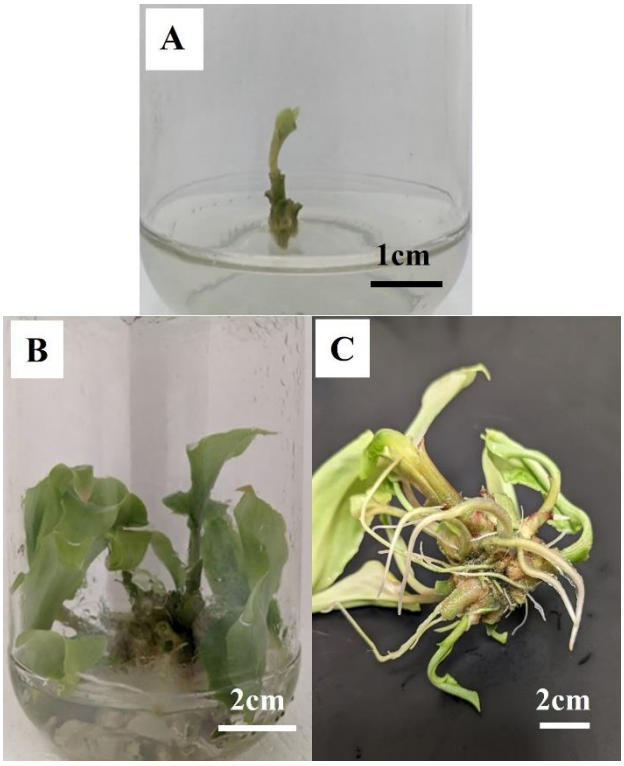

Figure 4. *In vitro* shoot development of *Kaempferia parviflora* (A) after 10 days (B) after 30 days of culture in T6 (8 mg/L BAP + 1 mg/L $AgNO_3$). (C) *in vitro* rooting in T5 (8 mg/L BAP) after 30 days of culture.

In vitro Multiplication

Table 3 shows the observation of *K. parviflora in vitro* multiplication based on 3 growth parameters (shoot number per explant, shoot length per explant and root numbers per explant) after being cultured in MS media with addition of different concentration of BAP, with or without 1 mg/L $AgNO_3$ for 30 days. Supplementation of MS media with 8 mg/L BAP + 1 mg/L $AgNO_3$ (T6) resulted in the highest number of shoots (4.90±0.57) and shoot length (5.07±0.33 cm) (Figure 4A,B).

Murashige and Skoog (MS) media fortified with BAP alone showed gradual increase in shoot multiplication and elongation as its concentration increased until reaching the optimum level which was 8 mg/L. Decline in growth rate was observed when explants treated in 12 mg/L BAP. Number of shoots decreased from 4.60±0.48 to 2.90±0.53, while shoot length decreased from 4.38±0.17 cm to 3.55±0.62 cm when cultured in MS media with 12 mg/L BAP. Shoot elongation was observed as early on day-4 of culture while rooting on day-12 of culture. The application of

BAP, whether singly or in combination with $AgNO_3$ displayed a positive effect on *in vitro* root development of *K. parviflora*. The highest root numbers per explant was observed in T5 (8 mg/L BAP) with 10.3±0.42 root numbers per explant. Observation on the adventitious roots after 30 days in T5 looked healthy and thick (Figure 4C).

After a week of culture period, the explants swelled very slightly and whitish bud grew from the basal part of explants and later developed into dark red colour buds and eventually grew into shoots. Statistical analysis found that BAP has significant effect on the number of shoots, shoot length and root numbers of *K. parviflora*. Meanwhile, $AgNO_3$ only significantly affected the shoot length and root numbers. Combination of BAP and $AgNO_3$ did not gave significant effect on all growth parameters except for root numbers.

All explants showed responses in all treatment, however explants failed to multiply in control treatment devoid of BAP and $AgNO_3$ and displayed very minimal growth rate in all parameters. Shoot elongation started early on fourth day of culture period, and this may be due to the use of aseptic shoots as explants for *in vitro* multiplication experiment instead of sterilized buds, as the *in vitro* plants already recovered from the harsh chemical treatment during surface sterilization.

Augmentation of MS media with BAP showed gradual increase in shoot multiplication and elongation as its concentration increased until reaching the optimum level which was 8 mg/L. The growth rate for both parameters started to decline when BAP was supplemented beyond that concentration. Benzylaminopurine has been widely used for shoot multiplication of *K. parviflora* at various concentration 1.5 mg/L–8.0 mg/L [6,10,33,34]. Present finding on the optimum concentration of BAP (8 mg/L) for shoot multiplication was also reported by Labrooy et. al. [6] and Prathanturarug et. al. [10], producing 22.40±1.84 and 47.30±3.40 shoots per initial explant, respectively. However, lower number of shoots per explant (4.90± 0.57) was obtained in the present study because of shorter culture period and lack of repeated subculture. Indeed, BAP is preferable for *in vitro* propagation of *K. parviflora* and other Zingiberaceae plants such as *Curcuma aeruginosa* [35], *Zingiber officinale* [36,37] and *Alpinia zerumbet* [38]. Benzylaminopurine, as a synthetic cytokinin stimulates shoot induction by overcoming of shoot apical dominance [39].

Interestingly, further boost in shoot multiplication and development were observed when $AgNO_3$ at 1 mg/L were added with BAP at all concentration level tested. The number of shoots increased from 4.60±0.48 to 4.90±0.57 and the shoot length increased from 4.38±0.17 cm to 5.07±0.33 cm with incorporation of 1 mg/L $AgNO_3$ into culture media with 8 mg/L BAP. Similar effect of BAP and $AgNO_3$ combinations were reported in *Hypericum perforatum* [40] and *Prosopis cineraria* [41]. Once $AgNO_3$ combines with BAP, better *in vitro* multiplications were achieved due to cell growth-promoting mechanisms provided by both factors. Ethylene, when accumulates in culture vessels, induces many physiological disorders towards *in vitro* plants such as leaf senescence, shoot tip necrosis and stunted shoot growth [42]. Plant *in vitro* studies adopted $AgNO_3$ into their protocols because it acts as a potent ethylene inhibitor [43]. Silver nitrate suppress ethylene accumulation in culture vessels and promotes better *in vitro* responses [44]. Easier translocation and assimilation of nutrients in culture media by the explant happens when ethylene is inhibited by $AgNO_3$, thus facilitating cell proliferation and growth [45]. Silver ions in $AgNO_3$ also efficient in decreasing ethylene sensitivity in plants [46].

As mentioned before, during culture, explants displayed slight swelling and later bud growth can be seen from the basal part which turned into shoots. It is presumes that BAP stimulates plant growth through cell wall extensibility by creating osmotic potential in plant cells thus promotes water uptake and reducing turgor for cell growth and expansion [47]. Rooting was simultaneously happened with shoot development irrespective of treatments, except in control treatment. Spontaneous rooting is a common phenomenon documented during *in vitro* propagation of *K. parviflora* [6,10] and other plants such as *Bambusa vulgaris* [48] and *Alpinia calcarata* [49].

After shoot development, endogenous auxin produced in shoot meristem stimulates spontaneous rooting in *in vitro* plants [50]. A separate auxin rooting treatment is not necessary, hence elucidating the added value provided by application of *in vitro* propagation in *K. parviflora* in term of cost, time and labour saving. Nevertheless, *K. parviflora in vitro* plants were treated with 2 µM Indole-3-butyric acid (IBA) and obtained better quality of adventitious and lateral roots [9].

CONCLUSION

In vitro multiplication of *K. parviflora* from rhizome bud explants was successfully achieved in this study. Contamination was the major challenge faced during aseptic culture establishment of *K. parviflora*. Nevertheless, multi-step surface sterilization using 100% Clorox® for 3 min, 95% ethyl alcohol for 30 s, 5% Clorox® for 15 min and 0.1% $HgCl_2$ for 10 min managed to control contamination and produced acceptable percentage of aseptic cultures. Better explants such as meristem tissue with lower microbial load should be used to minimize loss of culture materials due to contamination, for future improvement of surface sterilization. Rapid cell proliferation of meristems inhibits contaminants travel throughout plant vascular system. Besides that, identification of contaminants will be very useful for selection of better disinfectant agent.

Regarding shoot multiplication of *K. parviflora*, supplementation of culture media with 8 mg/L BAP + 1 mg/L $AgNO_3$ resulted in the most optimum shoot multiplication and elongation from aseptic shoot explants. This finding can provide a basic understanding on relationship between plant growth regulator and other culture media additives which are beneficial for *in vitro* propagation of *K. parviflora*. Spontaneous rooting was also reported in the regenerant plants which can be exploits for cost-cutting purposes. Further research on the effect of $AgNO_3$ at other concentrations is recommended so that a more optimum *in vitro* responses can be achieved.

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