Journal of Tropical Plant Physiology 12(2) (2020):23-32
https://doi.org/10.56999/jtpp.2020.12.2.8

In Vitro Shoot and Root Induction of *Kaempferia parviflora* (Zingiberaceae) Rhizome Using 6-Benzylaminopurine

Nur Adilla Khairudin[1], Zainol Haida[1] and Mansor Hakiman[1,2*]
[1]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[2]Laboratory of Sustainable Resources Management, Institute of Tropical Forestry and Forest Products, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
**Email: mhakiman@upm.edu.my*

Received: 4 September 2020; Revised: 4 December 2020; Accepted: 13 December 2020; Published: 15 December 2020

ABSTRACT

Kaempferia parviflora Wall. ex Baker is a medicinal plant from the Zingiberaceae family. This plant is native to Thailand and currently gaining high popularity in Malaysia. Its popularity is mainly due to the discovery of medicinal properties such as anti-inflammatory, anti-allergies, anti-cancer, anti-plasmodial and anti-fungal. This plant is conventionally propagated via rhizome. However, conventional propagation of *K. parviflora* is time consuming due to the long dormancy period thus difficult to fulfill the market demand for raw materials. To overcome this problem, the tissue culture technique was introduced in this study as a propagation tool to mass-produce and break its dormancy. This study aimed to establish an aseptic culture of *K. parviflora* using different concentrations of Clorox® and to induce the shoot and root of *K. parviflora* buds using different concentrations of 6-benzylaminopurine (BAP). The results showed that 50% Clorox® was the best concentration to reduce the contamination by microorganisms and produce a high survival rate. In the subsequent experiment, the Murashige and Skoog medium supplemented with 1.5 mg/L BAP was found to exhibit the best results on the numbers of shoot, length of shoot and number of roots. In conclusion, this study proved that the rhizome explant was successfully sterilised using Clorox®, and root and shoot formation was induced in the presence of cytokinin, particularly 6-benzylaminopurine.

Keywords: 6-benzylaminopurine; *Kaempferia parviflora*; shoot induction; surface sterilisation.

INTRODUCTION

Kaempferia parviflora Wall. ex Baker*,* also known as black galingale or Cekur Hitam in Malay and Krachaidam in Thailand, is an important medicinal plant from the Zingiberaceae family. *K. parviflora* is a perennial and herbaceous plant with dark purple rhizomes (Figure 1). The leaves of *K. parviflora* is approximately 6 to 8 cm long, oblong to lanceolate in shape with red margins, and produces purple and white flowers (Labrooy et al., 2013; Labrooy et al., 2020). In the Zingiberaceae family, *Zingiber officinale* and *Curcuma longa* are commonly used in culinary to enhance the taste and aroma of the dishes. However, *K. parviflora* is widely used as an alternative medicine in treating various types of diseases including fungal infections, gastrointestinal disorders, and decreased vitality and allergies (Tewtrakul et al., 2008; Trisomboon, 2009). In several studies conducted on *K. parviflora,* the extract of this plant has potential as an anti-inflammatory, anti-allergies, anti-cancer, anti-plasmodial, anti-fungal, anti-HIV-1 protease activity and antispasmodic effects (Sookkongwaree et al., 2006; Wattanapitayakul et al., 2008; Sae-wong et al., 2009; Saokaew et al., 2016).

ISSN No 1985-0484

Figure 1. *K. parviflora* plant (left) and rhizome (right). The scale bar represents 2 cm of the actual size.

Nowadays, natural product derived medicine is preferable to modern medicine because of its low side effects. This phenomenon has led to the demand for medicinal plants including *K. parviflora,* to increase drastically. However, the demand for *K. parviflora* rhizomes in Malaysia can hardly be fulfilled due to the scarcity of planting materials (Labrooy et al., 2013). Propagation of *K. parviflora* via conventional propagation is time consuming due to the long dormancy period after senescence, which is approximately 5 to 7 months during November to May according to the Malaysian climate (Techaprasan et al., 2010). This plant undergoes a vegetative stage for three months and a reproductive stage for two months. This plant produces flowers, but the flowers are inconspicuous and do not produce seeds (Labrooy et al., 2020). The long dormancy period and the inability to set seed affect raw material production for *K. parviflora*. To overcome the limited supply of *K. parviflora* raw materials, plant tissue culture technique was performed. The objectives of this study were to determine the Clorox® concentration for aseptic culture establishment and the 6-benzylaminopurine (BAP) concentrations for *K. parviflora* shoot and root induction.

MATERIALS AND METHODS

Planting materials

K. parviflora plants were obtained from Field 15, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor. The plants were maintained in the glasshouse and watered every morning and evening. Before experimentation, the plants were harvested, the leaves were removed and the rhizomes were cleaned under running tap water to remove the soil. The cleaned rhizomes were brought to the Tissue Culture Laboratory, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor. The buds (approximately 1.5 to 2 cm) from the rhizomes were cut and used for the experiments.

Preparation of basal medium

The Murashige and Skoog (1962) (MS) basal medium was used in this study. The MS medium was supplemented with 30 g/L sucrose, 3 g/L gelrite (Duchefa Biochemie) as a gelling agent and pH was adjusted to 5.75. All MS medium prepared was autoclaved at 121ºC for 20 minutes at a pressure of 1.05 kg/cm^2.

Surface sterilisation of *K. parviflora* using different concentrations of commercial bleach

Surface sterilisation experiment was conducted using commercial bleach (Clorox®) containing 5.25% of sodium hypochlorite. The buds of *K. parviflora* rhizomes were placed in bottle jars containing the Clorox® solution at different concentrations (40, 50, 60, 70 and 80%) with an addition of a few drops of Tween 20. The samples were agitated for 20 min and rinsed with autoclaved distilled water for 3 to 5 times to remove all the disinfectants. The rhizome explants were excised and trimmed to the size of approximately 1 cm x 1 cm before the aseptic rhizomes were inoculated onto the MS medium without plant growth regulators. Data were collected after 2 weeks of culture with five replications for each treatment.

Effect of BAP on shoot and root induction of *K. parviflora*

The four weeks old sterilised explants from the previous experiment were used and the explant was excised to the size of approximately 1 cm x 1 cm. In this experiment, the MS medium was supplemented with various concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L). Data for this experiment were collected after 8 weeks of inoculation with nine replications for each treatment.

Culture maintenance and statistical analysis

All the cultures were incubated in a culture room at 25ºC under 16 h light and 8 h dark using white fluorescence light irradiation of 45 µmol/m^2/s (Philips). The data were analysed by the one-way Analysis of Variance (ANOVA) using statistical software SAS version 9.4 and means were separated using Least Significant Difference (LSD) at $p \leq 0.05$.

RESULTS AND DISCUSSION

Surface sterilisation of *K. parviflora* using different concentrations of commercial bleach

When conducting the propagation of plant via tissue culture technique, sterilisation is the most crucial step that will determine the success in establishing the aseptic culture. In this experiment, the explants were sterilised using different concentrations of Clorox®. As shown in Figure 2, the percentage of contamination was significantly reduced as the concentrations of Clorox® increased. The results showed no significant difference between the treatments of 40, 50 and 60% Clorox® concentrations on the percentage of contamination recorded. The treatment of 40% Clorox® recorded the highest percentage of contamination where 80% of the explants became contaminated.

In the sterilisation step, obtaining an aseptic culture is the main objective. Besides that, the survival rate of the explants is also an important parameter that needs to be considered. A higher concentration of Clorox® might have successfully reduced the contamination. However, the survival rate of the explants could also be affected. In Figure 3, the concentrations of Clorox® were shown to significantly affect the percentage of explants that survived. Application of 50% Clorox® produced the highest percentage of explants. Meanwhile, the treatments of 40 and 80% Clorox® produced the lowest percentage of explants. Based on the results obtained, the application of Clorox® concentration of more than 50% resulted in the decrease of the percentage of explants that survived.

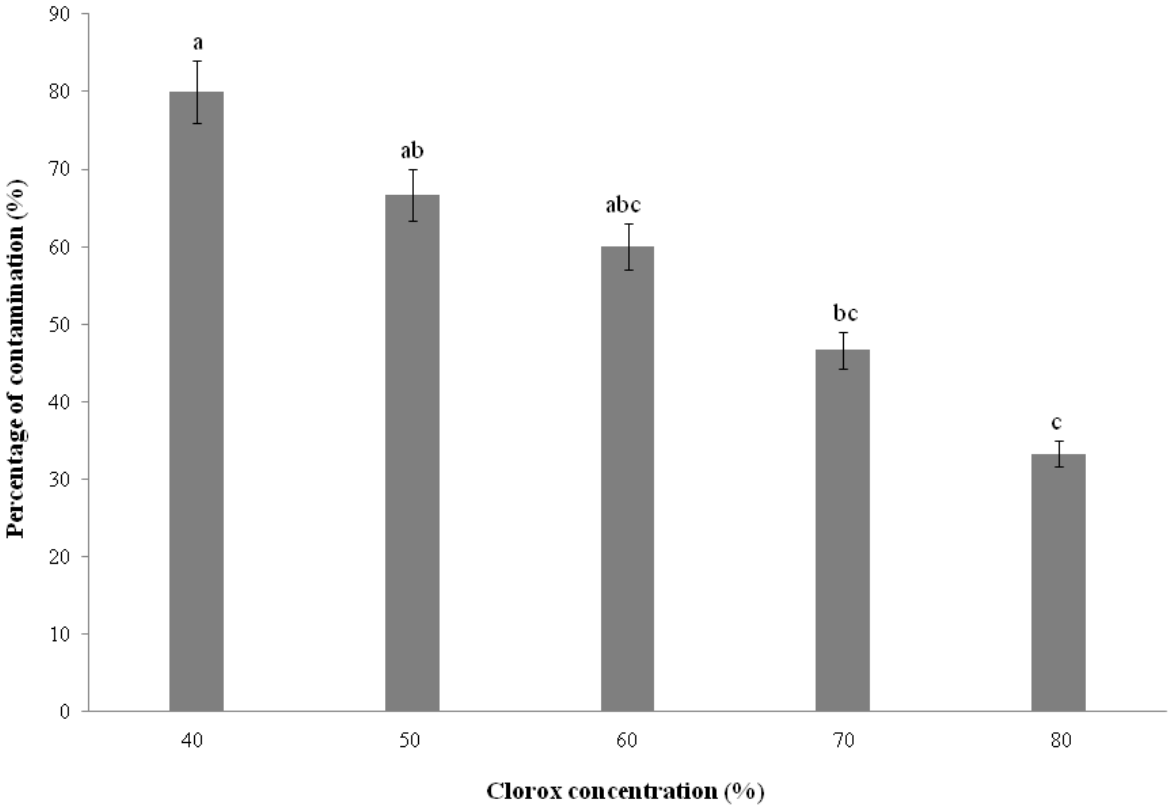

Figure 2. The effect of different concentrations of Clorox® on the percentage of contamination

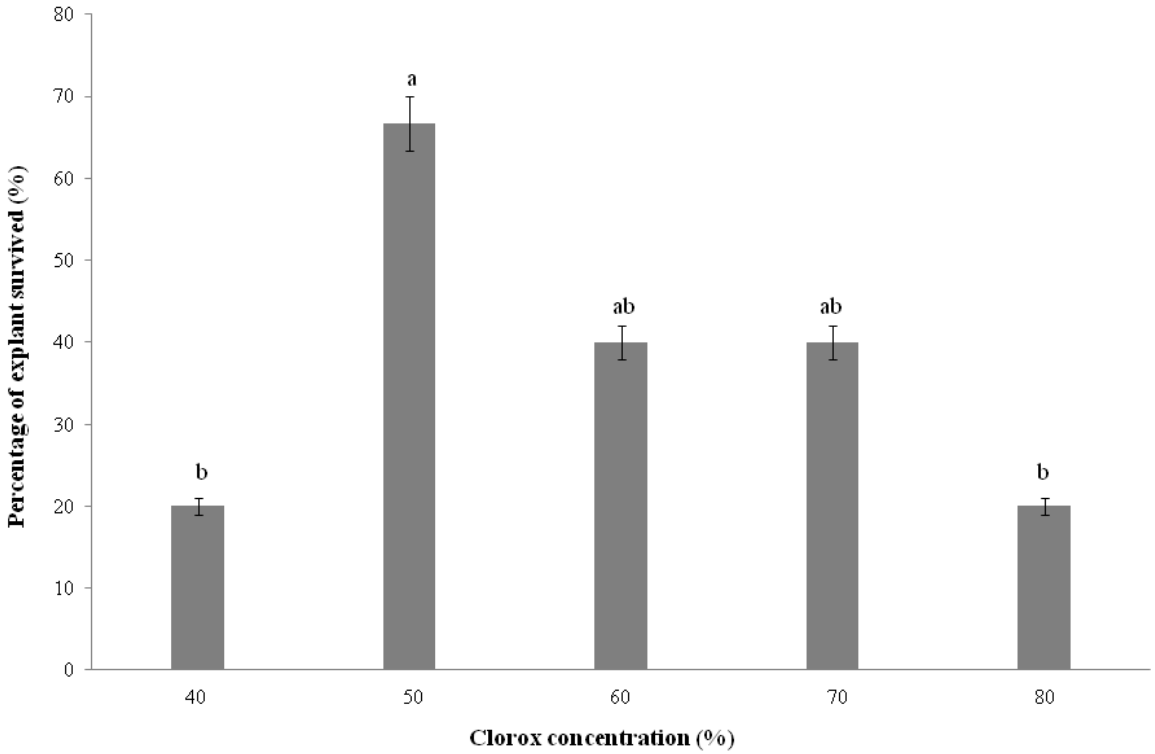

Figure 3. The survival rate of explant at different concentrations of Clorox®

Based on the results obtained, Clorox® at a concentration of 50% was found to exhibit the highest percentage of explants survived despite a recorded high percentage of contamination. In this experiment, fungal contamination was observed after 4 days of inoculation and contamination by bacteria was observed after 7 days of inoculation. For sterilisation of the rhizome part, mercury chloride is preferable to bleach because the active chemical present in mercury chloride is stronger than bleach. However, mercury chloride is very dangerous, hazardous and toxic to humans and plants despite its effectiveness in reducing contamination (Poobathy et al., 2019). Hence, commercial bleach such as Clorox® was used in this study. The underground explant source such as rhizome is the most challenging part to sterilise due to high contamination rates caused by bacteria, fungi and viruses present in the soil (Yildiz et al., 2012). Even though smaller explant size could reduce the contamination rate, a small explant takes a longer time to respond compared to a large explant. This is because larger explants contain an endogenous plant growth regulator and nutrients reserve that the explant can utilise to grow (Sathyagowri and Seran, 2011). Azhar et al. (2018) observed that 80% aseptic explants of *Boesenbergia rotunda* (Zingiberaceae) were obtained when the rhizome was immersed in double sterilisation of 60% commercial bleach (30 min) followed by 20% commercial bleach (15 min). Different findings were observed in a study by Hamirah et al. (2010) who obtained 75% of aseptic cultures when the rhizome explants of *Zingiber montanum* were immersed in 20% of Clorox® for 20 min. In the current study, a low concentration of Clorox® was not sufficient to produce an aseptic culture of *K. parviflora*. This difference could be due to the different locality where the plant was grown which would contain different types of microorganisms.

Effect of BAP on shoot and root induction of *K. parviflora*

Shoot formation is induced directly on cultured explants influenced by factors such as plant growth regulators. Cytokinin is one of the most common plant growth regulators used to induce shoot in plant tissue culture techniques. Cytokinin promotes cell division and facilitates the shoot induction and multiplication process of explants (Hartmann et al., 2002). In this study, different concentrations of BAP were tested on shoot induction of *K. parviflora.* Based on the analysis of variance conducted, there was a significant difference between BAP concentrations and the number of shoots produced. The treatment of 1.5 mg/L BAP was found to produce the highest number of shoots (Figure 4). However, the value was not statistically different from the treatment of 0.5, 1.0, 2.0 and 3.0 mg/L BAP. Based on the observation, the fastest shoot was induced from the treatment of 1.5 mg/L BAP, which took 11 days. Meanwhile, treatment of MS medium without supplementation of BAP took 19 days to induce the shoot.

Based on the shoot length graph trend in Figure 5, MS medium without BAP supplementation produced the shortest shoot length of 2.19 cm. The shoot length was increased as the BAP concentration increased to 1.5 mg/L at 4.3 cm. The addition of higher BAP concentrations of more than 1.5 mg/L resulted in decreasing *K. parviflora* shoot lengths. However, analysis of variance conducted showed no significant difference between the BAP concentrations and the shoot length of *K. parviflora.*

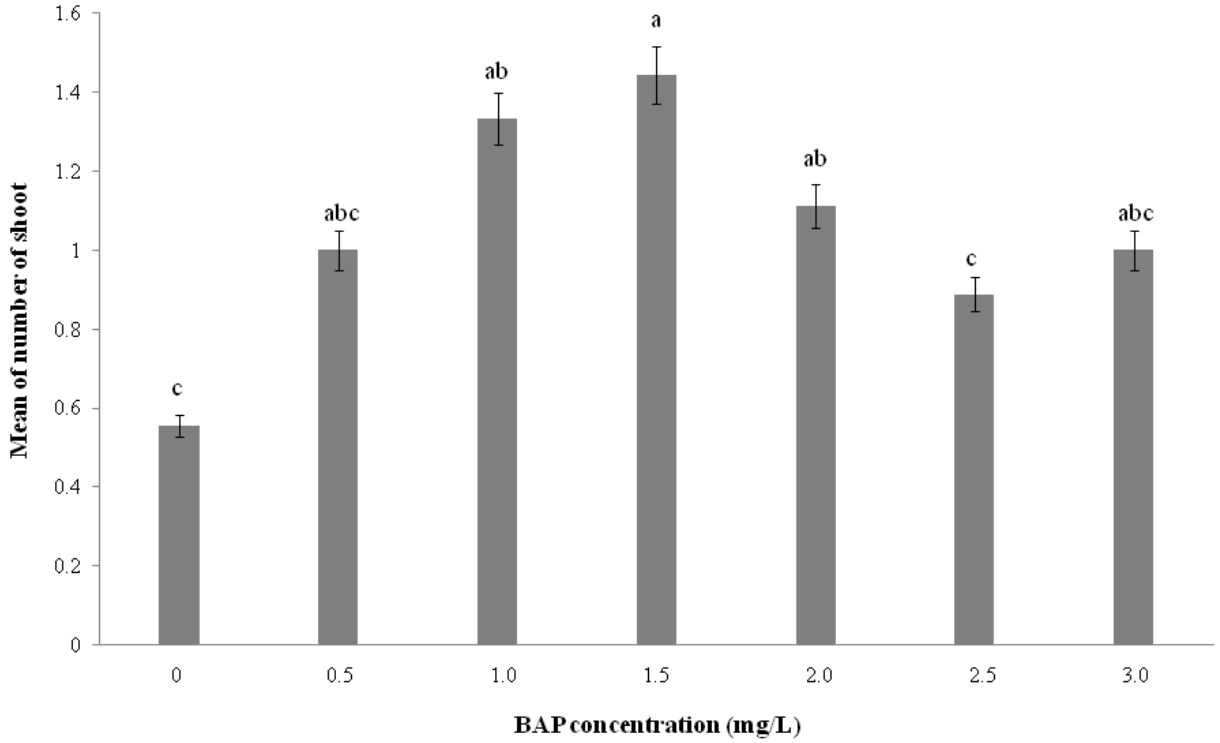

Figure 4. The effect of different concentrations of BAP on shoot induction of *K. parviflora*

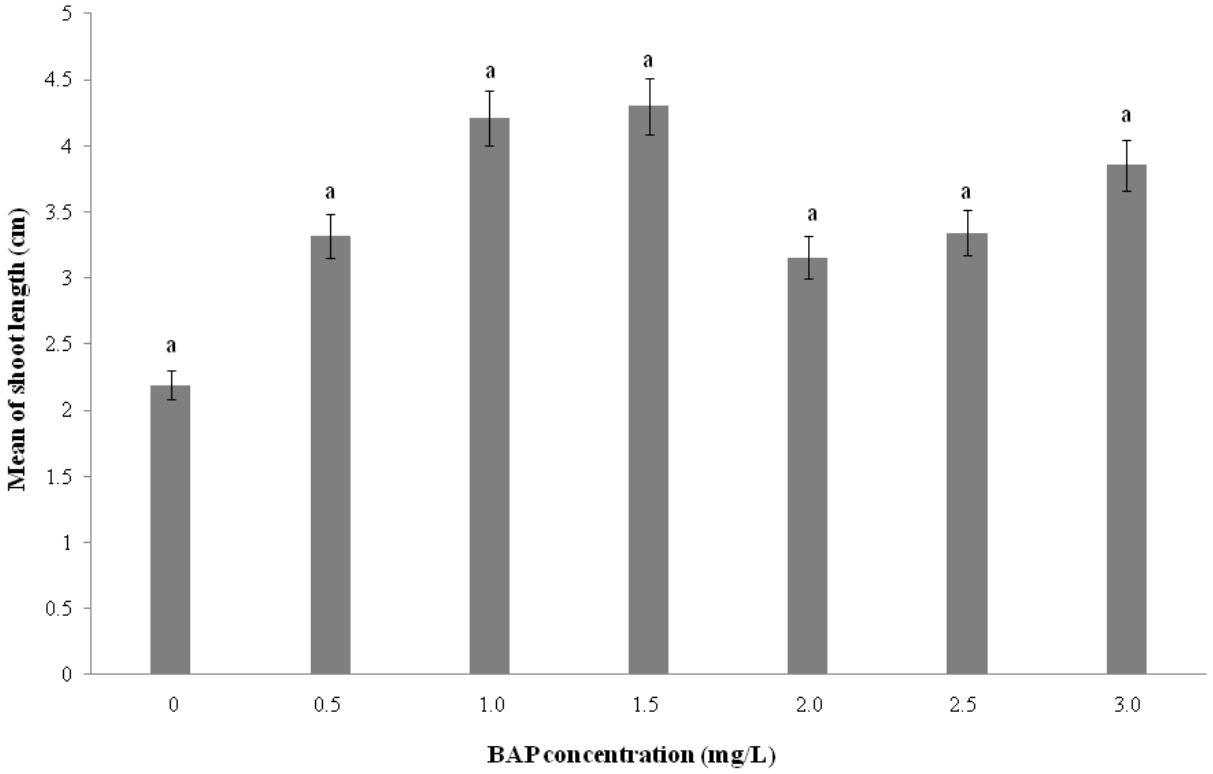

Figure 5. The effect of different concentrations of BAP on shoot length of *K. parviflora*

In this study, root induction spontaneously occurred during the shoot induction process. The earliest root induced was observed from the treatments using 1.5, 2.0 and 3.0 mg/L BAP at 10 days after inoculation. Based on the observation, the root formed faster than the shoot. The treatments using 0.5, 1.0 and 2.0 mg/L BAP produced more than seven roots after 8 weeks of inoculations (Figures 6 and 7). This result was in agreement with previous study where root induction occurred spontaneously with the shoot induction and multiplication of ginger (*Z. officinale* var. *rubrum*) (Zuraida et al., 2016). The simultaneous shoot and root induction phenomenon is commonly observed in Zingiberaceae plant species (Bharale et al., 2005). From the results, roots emerged in MS basal media devoid of auxin. However, the presence of endogenous auxin in the explant could be the reason for root induction (Kochuthressia et al., 2010). An adequate number of roots per plantlet were required to successfully transplant and survive the *in vitro*-raised plantlets of *K. parviflora*.

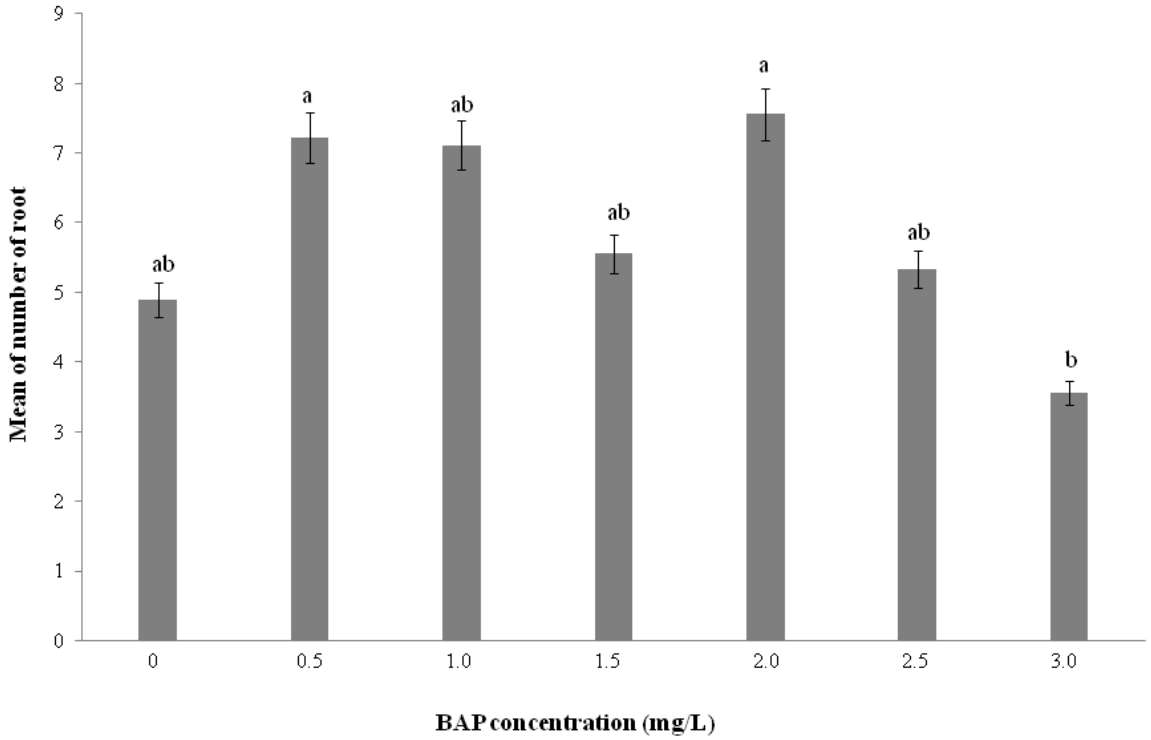

Figure 6. The effect of different concentrations of BAP on the number of roots of *K. parviflora*

According to a study by Alveno et al. (2012), young shoots of *K. parviflora* cultured on MS medium supplemented with 3.75 mg/L BAP produced the highest number of shoots with 1.77 shoots after 4 weeks of inoculation. In a recent study by Labrooy et al. (2020), MS medium supplemented with a high concentration of BAP (8 mg/L) produced the highest number of roots (17.8 roots), shoots (22.4 shoots) and leaves (29.27 leaves) after 9 weeks of inoculation. These findings were in agreement with the current study where BAP was suitable for shoot induction of *K. parviflora.* However, the different results obtained from the studies might be due to the different maturity of the mother plant rhizome used and other factors such as plant maintenance and climate, which directly affect the dormancy of the mother plant. In this current study, all of the BAP treatments, including the control treatment (BAP-free MS medium), induced shoots of *K. parviflora*. The results were contrary to the findings by Rao et al. (2011), who found that zeatin treatment produced a significantly higher number of shoots of *Alpinia galanga* (Zingiberaceae) compared to BAP, thidiazuron and kinetin treatments. The effectiveness of zeatin in some Zingiberaceae species could be due to its higher biological activity than the other cytokinin types, which are synthetic. However, in most shoot induction and multiplication studies, BAP was preferred compared to zeatin. This is because zeatin

as natural cytokinin is more expensive than BAP, kinetin and thidiazuron and not stable for a long time in the basal medium. The effectiveness of BAP for shoot induction in the present study could be due to its resistance to cytokinin oxidase cleavage, which ensured its stability in the culture medium (Kieber and Schaller, 2018).

Figure 7. Root induction of *K. parviflora* in MS medium supplemented with 2.0 mg/L of BAP after 8 weeks of inoculation. The scale bar represents 1 cm of the actual size.

CONCLUSIONS

This study showed that the rhizomes of *K. parviflora* were successfully established in the *in vitro* culture by surface sterilisation in 50% of Clorox®. Shoot induction of *K. parviflora* was induced in different concentrations of BAP. By investigating different concentrations of BAP, 1.5 mg/L positively influenced the shoot induction rate. It indicated that cytokinin, mainly BAP can be successfully adopted for micropropagation of *K. parviflora*. However, studies on the effect of other cytokinin types should also be conducted to examine their effectiveness in *K. parviflora* tissue culture.

AUTHORS CONTRIBUTION

MH conceived and designed the work. NAK and ZH performed the analysis. ZH wrote the paper, and MH checked and approved the submission.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

FUNDING

The authors wish to thank the Ministry of Education Malaysia and Universiti Putra Malaysia for financing the present study through Translational Research Program, 5526700 and Putra Grant – Putra Young Initiative, 9629000.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to the Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia for their support to this research.

REFERENCES

Alveno, V. Khumaida, N. and Ardie, S. W. (2012). *In vitro shoot induction of Kaempferia parviflora Wall. Ex. Baker*. [Undergraduate thesis, Bogor Agricultural University]. Institutional Repository at Bogor Agricultural University. Available at: http://repository.ipb.ac.id/handle/123456789/60147.

Azhar, S. Z. A., Ghani, K. A. and Yusuf, N. A. (2018). *In vitro* induction of adventitious root from shoot bud of *Boesenbergia rotunda* (Zingiberaceae): effect of plant growth regulators. *Science International*, 30, 147-151.

Bharalee, R., Das, A. and Kalita, M. C. (2005). *In vitro* clonal propagation of *Curcuma caesia* Roxb. and *Curcuma zedoaria* Rosc from rhizome bud explants. *Journal of Plant Biochemistry and Biotechnology,* 14(1), 61-63.

Hamirah, M. N., Sani, H. B., Boyce, P. C. and Sim, S. L. (2010). Micropropagation of red ginger (*Zingiber montanum* Koenig), a medicinal plant. *Asia Pacific Journal of Molecular Biology and Biotechnology*, 18, 125-128.

Hartmann, H. T., Kester, D. E., Davies, Jr., F. T. and Geneve, R. L. (2002). *Hartmann and Kester's plant propagation: principles and practices.* 7th ed. New Jersey: Pearson Education.

Kieber, J. J. and Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development*, 145, 1-7.

Kochuthressia, K. P., Britto, S. J., Raj, L. J. M., Jaseentha, M. O. and Senthilkumar, S. R. (2010). Efficient regeneration of *Alpinia purpurata* (Vieill.) K. Schum. plantlets from rhizome bud explants. *International Research Journal of Plant Science*, 1(2), 43-47.

Labrooy, C., Thohirah, L. A., Johnson, S., Nur Ashikin, P. A. and Maheran, A. A. (2013). Morphological description for kunyit hitam *(Kaempferia parviflora*) and breaking bud dormancy with BAP and Ethephon treatment. *Transactions of the Malaysian Society of Plant Physiology*, 22, 139-141.

Labrooy, C., Abdullah, T. L. and Stanslas, J. (2020). Influence of N6-Benzyladenine and sucrose on *in vitro* direct regeneration and microrhizome induction of *Kaempferia parviflora* Wall. Ex Baker, an important ethnomedicinal herb of Asia. *Tropical Life Sciences Research*, 31(1), 123-139.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco cultures. *Physiologia Plantarum,* 15, 473-497.

Poobathy, R., Zakaria, R., Murugaiyah, V. and Subramaniam, S. (2019). Surface sterilization and micropropagation of *Ludisia discolor*. *Biocatalysis and Agricultural Biotechnology*, 22, 101380.

Rao, K., Chodisetti, B., Gandi, S., Mangamoori, L. N. and Giri, A. (2011). Direct and indirect organogenesis of *Alpinia galanga* and the phytochemical analysis. *Applied Biochemistry and Biotechnology*, 165, 1366-1378.

Sae-wong, C., Tansakul, P. and Tewtrakul, S. (2009). Anti-inflammatory mechanism of *Kaempferia parviflora* in murine macrophage cells (RAW 264.7) and in experimental animals. *Journal of Ethnopharmacology*, 124(3), 576-580.

Saokaew, S., Wilairat, P., Raktanyakan, P., Dilokthornsakul, P., Dhippayom, T., Kongkaew, C. and Chaiyakunapruk, N. (2016). Clinical effects of krachaidum (*Kaempferia parviflora*): A systematic review. *Journal of Evidence-Based Complementary and Alternative Medicine,* 22(3), 413-428.

Sathyagowri, S. and Seran, T. H. (2011). *In vitro* plant regeneration of ginger (*Zingiber officinale* Rosc.) with emphasis on initial culture establishment. *International Journal of Medicinal and Aromatic Plants*, 1(3), 195-202.

Sookkongwaree, K., Geitmann, M., Roengsumran, S., Petsom, A. and Danielson, U. H. (2006). Inhibition of viral proteases by Zingiberaceae extracts and flavones isolated from *Kaempferia parviflora*. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, 61(8), 717-721.

Techaprasan, J., Klinbunga, S., Ngamriabsakul, C. and Jenjittikul, T. (2010). Genetic variation of *Kaempferia* (Zingiberaceae) in Thailand based on chloroplast DNA (psbA-trnH and petA-psbJ) sequences. *Genetics and Molecular Research*, 9(4), 1957-1973.

Tewtrakul, S., Subhadhirasakul, S. and Kummee, S. (2008). Anti-allergic activity of compounds from *Kaempferia parviflora. Journal of Ethnopharmacology*, 116(1), 191-193.

Trisomboon, H. (2009). *Kaempferia parviflora*, a Thai herbal plant, neither promote reproductive function nor increase libido via male hormone. *Thai Journal of Physiological Sciences*, 21, 83-86.

Wattanapitayakul, S. K., Chularojmontri, L., Herunsalee, A., Charuchongkolwongse, S. and Chansuvanich, N. (2008). Vasorelaxation and antispasmodic effects of *Kaempferia parviflora* ethanolic extract in isolated rat organ studies. *Fitoterapia*, 79(3), 214-216.

Yildiz, M., Fatih, Ö. S., Kahramanogullari, C. and Tuna, E. (2012). The effect of sodium hypochlorite solutions on the viability and *in vitro* regeneration capacity of the tissue. *The Natural Products Journal,* 2, 328-331.

Zuraida, A. R., Mohd Shukri, M. A., Erny Sabrina, M. N., Ayu Nazreena, O., Che Radziah, C. Z., Pavallekoodi, G. and Sreeramanan, S. (2016). Micropropagation of ginger (*Zingiber officinale* var. *rubrum*) using buds from microshoots. *Pakistan Journal of Botany*, 48, 1153-1158.