Asian Journal of Pharmacognosy

Research paper

Enchancing tissue culture efficiency and growth of *Bryophyllum pinnatum* (Lam.) Oken with hydrogen peroxide treatment

Vansak He[1], Lina Ly[1], Sophearom Chhea[3], Sokunvary Oeung[1], Huysean Huot[3], Sin Chea[2],Visessakseth So[2*]

[1]*Division of Pharmacognosy, Faculty of Pharmacy, University of Puthisastra, 12000, Cambodia*
[2] *Academic Affairs,University of Puthisastra, Phnom Penh 12000, Cambodia*
[3] *Faculty of Pharmacy, University of Puthisastra, Phnom Penh 12000, Cambodia*

*Corresponding author: svisessakseth@puthisastra.edu.kh
(accepted November 8, 2025)

ABSTRACT

Context: Bryophyllum pinnatum (Lam.) Oken is a well-known medicinal plant in Cambodia, traditionally used for treating various health conditions. *In vitro* tissue culture of this species faces significant challenges from microbial contamination, especially during surface sterilization. *Objective:* to compare the effectiveness of hydrogen peroxide (H_2O_2) and sodium hypochlorite (NaOCl) in reducing contamination and promoting explant growth on Murashige and Skoog (MS) medium. *Methods:* fresh *B. pinnatum* leaves were sterilized by using either 5.5% H_2O_2 for 20 mins or 3% NaOCl for 30 mins. Sterile explants were cultured on Murasige and Skoog medium under aseptic conditions. Contamination and survival rated were record and growth parameters were measure four weeks. *Results:* 5.5% H_2O_2 (20 min) reduced contamination to 58% and supported 14% survival of explants, which developed plantlets reaching 3.4 cm by week 4. In contrast, NaOCl resulted in 100% contamination and no viable explants. *Conclusion:* H_2O_2 treatment is a practical and effective method for improving the micropropagation of *B. pinnatum.*

Keywords: Murashige medium, medicinal plant, Skoog medium, surface sterilization

INTRODUCTİON

Plants are the source of food and medicine for humans and animals, contributing to biodiversity, supporting the preservation, and survival of living things, particularly in developing countries (Carla Guimarães Sobrinho et al., 2022). In recent years, scientists have discovered plant components offering health benefits, including phytochemicals for pharmaceuticals, cosmetics, and nutraceuticals, which are popular due to their accessibility, affordability, and efficacy (Chandran et al., 2020). Thus, scientists have developed natural products from plants, while

eISSN-0128-1119

DOI: http://dx.doi.org/10.71221/ajp.09412

people have shifted their perception towards using healthy products. According to Hasnain et al., 2022, the study reported that the concept of tissue culture techniques was proposed by scientists to publish widely and used annually in bredding genetic method. The tissue culture is a method that refers to the reconstruction of any parts and uses multiplication plants to extract tissues from the mother plant to produce large numbers of plantlets (Chun et al., 2020). This method has received wide recognition for its role in producing secondary metabolites through approaches such as metabolic engineering, organ culture of differentiated cells, and metabolite production (Carla Guimarães Sobrinho et al., 2022). However, several types of culture media influence plant differentiation because various species might also need diverse culture media to induce higher plant regeneration (Long et al., 2022).

Plant medium is also the most important factor involved in growing plant tissue; the frequently used basic media include Murashige and Skoog (MS) medium (Phillips & Garda 2019; Xu et al., 2022). MS medium is a wildly recognized and standard culture medium for tissue culture and various plant biotechnological applications (García-Pérez et al., 2020a). Due to its fundamental nutritional content and balanced mineral content, it makes it frequently utilized in *in vitro* tissue culture research (Bettoni et al., 2021; Chornobrov & Bilous, 2021). This medium was reported as a medium protocol with nutrient concentration for culturing tobacco pith cells (Tabei & Muranaka, 2020).

Bryophyllum pinnatum (Lam.) Oken, locally known as *kabellaphoahs*" (កបិលភ័ស) in Cambodia, is a type of herbaceous plant in the *Kalanchoe* genus and Crassulaceae family (Chassagne et al., 2016). It is also well-known as a garden ornamental with several common name including life plant, or miracle leaves with traditional uses to various illness such as body pain, skin issues, fever, headache, and stomachache (Saurabh et al., 2024). This plant has medicinal value such as antibacterial, antioxidant, and immune regulation functions to develop the drug (Liu et al., 2023). This plant is widely used in folkloric medicine and treated skin problems, respiratory system, pain, inflammation, and gastrointestinal (Emeka, 2021, Elufioye et al., 2022). In Cambodia, it's used to treat ankle injuries, while Hmong people use it in soup for tonic and muscular-skeletal issues in Thailand (Fernandes et al., 2019; Nguanchoo et al., 2019). *B. pinnatum* was cultured on MS medium to measure the growth, the result showed that they grew in good condition and produced many plantlets (Saidu & Tahir 2021). However, research on this tissue culture of *B. pinnatum* remains slim, particularly regarding explant treatment and its effects on plant growth. The article of Lozano-Milo et al., 2020, reviewed the use of plant tiisues culture in *Bryophyllum* spp. for secondary metabolite production, but detailed studied on explant handling and growth repsonses are scarce. In tissue culture, contamination is a major concern that can be brought on by both bacteria and fungus. So, an appropriate sterilization agent is key to eradicating contamination from plant tissue culture (Adebomojo & AbdulRahaman, 2020). The selection of surface sterilization agents provides an efficient treatment in plant tissue culture, using hydrogen peroxide (H_2O_2) and sodium hypochlorite (NaClO) (Davoudpour et al., 2020). Accordingly, several substances were used to create an axenic culture with H_2O_2 (5.25-12.25%) and NaClO (0.54-1.26%) (Gammoudi et al., 2022). Although, H_2O_2 and NaClO have been used as surface strilants in other plant species, there are not pervious study has systematically compareed their effects on *B. pinnatum* expants. Our findings demonstrate for the first time that optimized H_2O_2 treatment is superior to NaClO

eISSN-0128-1119

DOI: http://dx.doi.org/10.71221/ajp.09412

for establishing aseptic cultures of this species. The objective of this study is to apply the appropriate surface sterilization procedure method and to investigate the effect of the difference between H_2O_2 and NaClO on the *B. pinnatum*'s leaf explants in tissue culture.

METHODS

Plant materials and surface sterilization

Fresh *B. pinnatum* leaves were collected from the University of Puthisastra botanical garden. Leaves were pre-cleaned with running water and sterilized using 3 percentages (%) NaClO (weight per volume, w/v) for 20 mins (min), followed by triple rinsing with sterile water (Masumoto & Degawa, 2019). They were immersed in 95% ethanol for 1 min and cut into 1-3 cm segments (Boonsnongcheep et al., 2019). For comparison, another set of explants was treated with 5.5% (w/v) of H_2O_2 for 20 min. Explants were rinsed again with sterile water before culturing (Handayani et al., 2022).

Agar medium preparation

MS basal medium was prepared by dissolving 2.2 grams (g) of MS powder and 15 g of sucrose in 400 milliliters (ml) distilled water (DW). The pH was adjusted to 5.5 using 10% of sodium hydroxide (NaOH) by pH meter, and agar (5 g) was added. The final volume was adjusted to 500 ml, autoclaved at 121 degrees celsius (°C) and stoired for one week before use (Amanda et al., 2020,Samantaray & Singh, 2021).

Cultivation technique

Sterile explants were tranferred to the MS medium under laminar airflow using flame-sterilzed tools (AL-Alwani & Mohammed 2023, García-Pérez et al., 2020a). Culture were incubated in a dark room, and growth parameters were observed weekly for 4 weeks.

Data analysis

Contamination and surival rate were calculated as percentages of the toal number of explants inoculated foolowing standard tissue culture practice (Davoudpour et al., 2020, Hashim et al., 2021, Sifa et al., 2020). Data analysis was performed using Microsoft Excel 2019. The contamination rate (%) was determined as:

$$\frac{\text{Number of contaminated explants}}{\text{Toral explants inoculated}} \times 100$$

The survival rate (%) was calculated as:

$$\frac{\text{Number of strile and live explants}}{\text{Total explants inoculated}} \times 100$$

Research approval

The study was approved by University of Puthisastra Committee (Ref: 19IR24).

eISSN-0128-1119

DOI: http://dx.doi.org/10.71221/ajp.09412

RESULTS AND DİSCUSSİON

Surface sterilization outcomes

The study evaluated the effectiveness of two surface sterilization agens, H_2O_2 (5.5% w/v for 20 min) and NaOCl (3% w/v for 30 min) on *B. pinnatum* leaf explants. Observations were made over four weeks post-inoculation to assess contamination rates and explant viability. As presented in Table 1, H_2O_2 treatment yielded significantly better outcomes compared to NaOCl. The contamination rate was reduced to 58% with growth. In contrast, NaOCl treatment resulted in 100% contamination with no viable explants. These findings underscore the superior sterillization efficiency of 5.5% H_2O_2 under tested conditions.

Table 1. Surface sterilization data of *B. pinnatum* explant treated between H_2O_2 and NaOCl

	Duration (min)	Contamination rate (%)	Sterile and dead rate (%)	Sterile and live rate (%)
5.5% H_2O_2	20	58	42	14
3% NaOCl	30	100	-	-

Initial signs of contamination were observed by day 5 to become more prominent after four weeks. Figure 1 showed typical contamination patterns and plantlet health under both treatments. H_2O_2 treated explants (Figure 1A and 1B) showed active root and shoot development with minimal fungal growth while NaOCl treated explants (Figure 1C and 1D) were overwhelmed by fungal contamination. These findings are consistent with Sifa et al., 2020, who reported 5-25% viability in *Staurogyne repens* Kuntze (Nees) was treated with similar concentration of H_2O_2, and Amarasinghe et al., 2018, who achieved high germination and low contamination using H_2O_2 on *Rhododendron wardii* (Huang bei du juan).

Plantlet growth in culture

Plantlet growth from H_2O_2 treated explants developed roots within week 1 and visible shoot elongation from week 2 onwards, and plantlets reached an average height of 2.7 cm. By week 4, planlets reached a maximum length of 3.4 cm, indicating successful establishment in Ms medium. The average weekly growth from week two to four was 0.7 cm. Figure 2 showed progressive development of the plantlets over a four-week period from root emergence (week 1) to shoot elongation and full plantlet formation (week 4). Figure 3 shows the growth trend over time. The plantlets were initially cultured in the test tubes but were transferred to glass bottles in week 3 to accommodate increased growth. These results align with the observations of García-Pérez et al., 2020b, who reported 3.5 to 5.5 cm shoot heights after 12 weeks in MS medium for *B. pinnatum*. Similarly, Sparjan Babu et al., 2019, noted a growth of 2.05 cm in *Elaeis guneensis* Jacq. var. *dura* embryo after 30 days, highilighting the MS medium's versatility across species.

eISSN-0128-1119

DOI: http://dx.doi.org/10.71221/ajp.09412

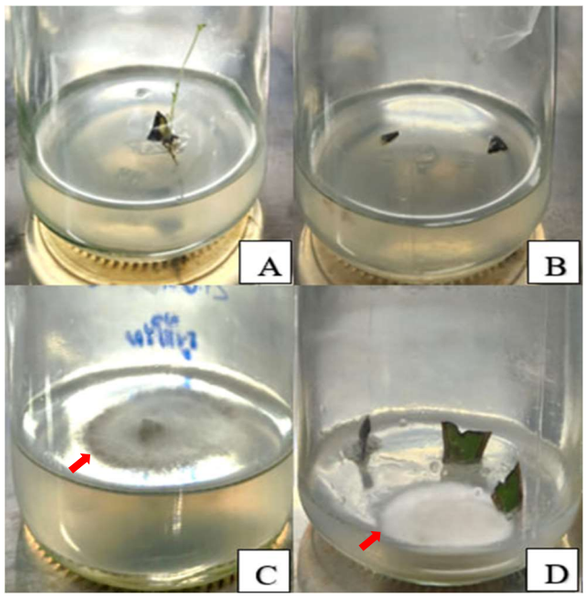

Figure 1. Surface sterilization outcomes
The growth of *B.pinnatum* explant (A and B) was treated with H_2O_2 (5.5%, w/v) for 20 min, and others (C and D) explant were treated with NaOCl (3%, w/v) for 30 min. Red arrow indicated the fungal contamination on the agar medium.

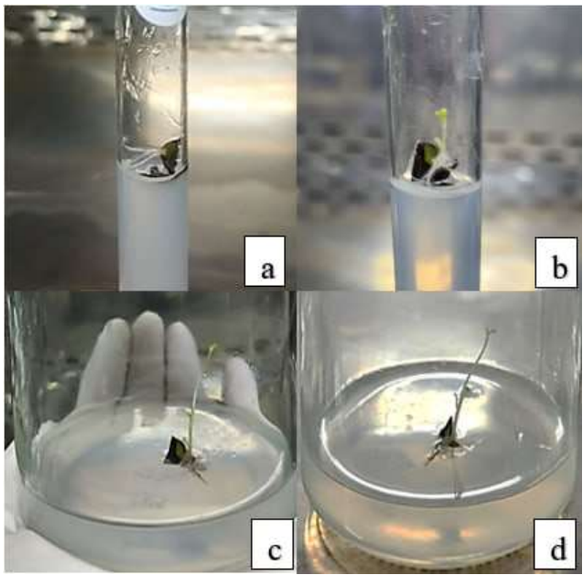

Figure 2. Plantlet growth in culture
Plantlets of *B.pinnatum* were cultivated from 1st (a), 2nd week (b), 3rd week (c), and 4th week (d)

eISSN-0128-1119

DOI: http://dx.doi.org/10.71221/ajp.09412

Comparative literature analysis

The variable efficacy of surface sterilization agents across plant species is well documented. While NaOCl is commonly used, its effectiveness can vary significantly. For example, Hashim et al., 2021, found that increasing NaOCl concentations up to 40% showed no significant difference in baterial contamination control in *Clinacanthus nutans* (Sabah snake grass). In contrast, Gammoudi et al., 2022, optimized H_2O_2 use for *Pistacia vera* L. (Pisachio) explants and demonstrated superior disinfection results. In our study, H_2O_2 proved effetive at a moderated concentration (5.5% for 20 min), offering a balance between microbial eimination and explant viability. In contrast, NaOCl at 3% was too harsh or ineffective for *B.pinnatum*, resulting in complete tissues loss. However, a limitation of this study is that only two sterilization agents were tested at fixed concentration and durations; testing a braoder range of agents concentrations, or exposure time could provide more comprehensive insights.

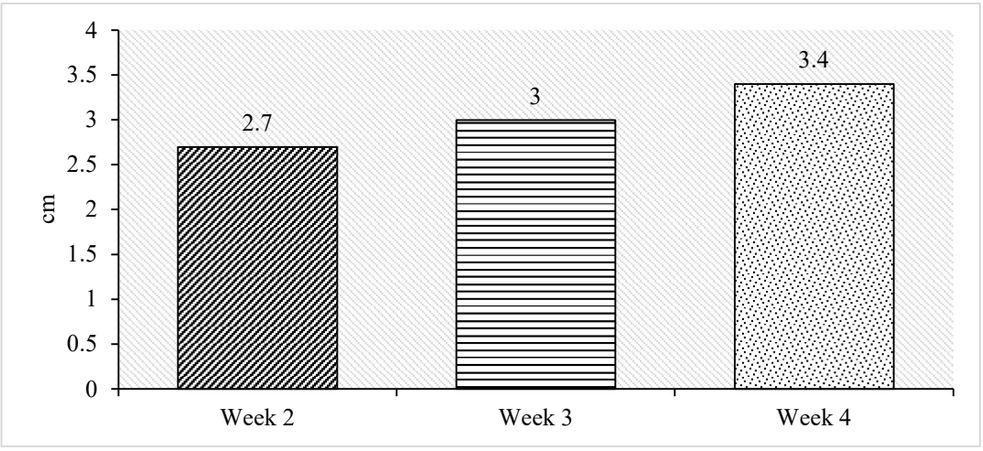

Figure 3. *B. pinnatum* growth in MS medium from week 2 to week 4.

CONCLUSİON

Surface sterilization is an essential method of plant tissue culture. In this study, successful surface sterilization of *B. pinnatum* was achieved using H_2O_2. Bacterial contamination is generally a major challenge in the process of plant tissue culture. However, it should be noted that using a high concentration of H_2O_2 can be toxic to plants and may cause the explants to die. In this study, direct comparison demonstrates the practical superiority of 5.5% H_2O_2 (20 min) over NaClO (30 min), the full factorial optimization was not conducted. Future work will therefore evaluate a broader matrix (H_2O_2 3-7.5% $\times$10-30 min; NaClO 0.5-1.5% $\times$ 10-20 min) to refine the optimum for *B.pinnatum*. These findings are significant as they provide valuable insights into improving surface sterilization techniques and enhancing plant tissue culture for *B. pinnatum*. By fine-tuning the concentration and duration of H_2O_2 treatment, researchers can achieve more effective surface sterilising methods, thereby increasing success rates in plant tissue culture experiments. Overall, this study contributes to our understanding of surface sterilizing methods and provides useful recommendations for future research on

eISSN-0128-1119

DOI: http://dx.doi.org/10.71221/ajp.09412

plant tissue culture techniques specifically related to *B.pinnatum*. In Cambodia, improving tissue culture method can support both conservation and sustainable use, as the techniques contribute to preserving plants, including *B. pinnatum* to facilitate plants integration into traditional health systems. Also, this study advances plant tissue culture methodology and underscores the broader health and economic significance of *B. pinnatum* in Cambodia.

ACKNOWLEDGEMENTS
The authors would like to thank Division of Pharmacognosy, Faculty of Pharmacy, University of Puthisastra for support and encourage for the research.

CONFLİCT OF İNTEREST DİSCLOSURE
The authors of this study declare that they have no conflic of interest.

DECLARATİON OF HONOR
We declare on our honor that our research are not fake and make up.

AI ASSISTANCE DISCLOSURE
The authors used [ChatGPT/GPT-5] to improve the clarity and readability of the manuscript. The authors carefully reviewed and edited the content to ensure accuracy and take full responsibility for the final text.

REFERENCES
Al-Alwani, A. A. M., Mohammed, M. A. (2023) Propagation of Chia Plants Using Plant Tissue Culture Technique. Journal of Biotechnology Research Center, 17(1): 66–80.

Amanda, P., Putri, A., Masruchin, N., Banar Kusumaningrum, W., Surya Ningrum, R. (2020) Autoclave-assisted deacetylation: a rapid method to recycling cigarette buttsinto celluliose. Jurnal Sains Materi Indonesia, 22(1): 32-39.

Amarasinghe, R. M. N. T., Wang, J.-H., Xie, W.-X., Peng, L.-C., Li, S.-F., Li, H. (2018) Seed-sterilization of *Rhododendron wardii* for micropropagation. Sri Lanka Journal of Food and Agriculture, 4(1), 9–14.

Adebomojo, A., AbdulRahaman, A. (2020) Surface sterilization of Ocimum seeds and tissues with biosynthesized nanosilver and its effects on callus induction. IOP Conference Series: Materials Science and Engineering, 805(1), 012024.

Boonsnongcheep, P., Sae-foo, W., Banpakoat, K., Channarong, S., Chitsaithan, S., Uafua, P., Putha, W., Kerdsiri, K., Putalun, W. (2019) Artificial color light sources and precursor feeding enhance plumbagin production of the carnivorous plants *Drosera burmannii* and *Drosera indica*. Journal of Photochemistry and Photobiology B: Biology, 199, 111628.

Bettoni, J. C., Bonnart, R., Volk, G. M. (2021) Challenges in implementing plant shoot tip cryopreservation technologies. Plant Cell, Tissue and Organ Culture (PCTOC), 144(1), 21–34.

Carla Guimarães Sobrinho, A., Silva Corpes, R., Isabel Portilho dos Santos, K., Maria Menezes Barra, I., Kiyoshi Miyagawa, H., Silva Santos, A. (2022) Untargeted GC-MS metabolomics applied to wild leaves and callus produced by plant tissue culture of *Hibiscus sabdariffa* L. Arabian Journal of Chemistry, 15(9), 1–8.

Chandran, H., Meena, M., Barupal, T., Sharma, K. (2020) Plant tissue culture as a perpetual source for production of industrially important bioactive compounds. Biotechnology Reports, 26(3), 1–10.

eISSN-0128-1119

DOI: http://dx.doi.org/10.71221/ajp.09412

Chassagne, F., Hul, S., Deharo, E., Bourdy, G. (2016). Natural remedies used by Bunong people in Mondulkiri province (Northeast Cambodia) with special reference to the treatment of 11 most common ailments. Journal of Ethnopharmacology, *191*, 41–70.

Chun, S. C., Gopal, J., Iyyakannu, S., Muthu, M. (2020) Current endeavours and future perspectives. TrAC Trends in Analytical Chemistry, 126(2), 115843.

Chornobrov, O., Bilous, S. 2021. *In vitro* plant regeneration of Christmas cactus (*Schlumbergera truncata* (Haw.) Moran) by indirect morphogenesis. Folia Forestalia Polonica, 63(1), 68–73.

Davoudpour, Y., Schmidt, M., Calabrese, F., Richnow, H. H., Musat, N. (2020) High resolution microscopy to evaluate the efficiency of surface sterilization of Zea Mays seeds. PLOS ONE, 15(11), e0242247.

Elufioye, T. O., Oyedeji, A. O., Habtemariam, S. (2022) A review of the traditional uses, phytochemistry and pharmacology of *Bryophyllum pinnatum* (Lam.) (Crassulaceae). Journal of Biologically Active Products from Nature, 12(3), 190–222.

Emeka, P. M. (2021) In-vitro studies of *Bryophyllum pinnatum* crude extract on blood coagulation indices: an investigation on its traditional medicine use. Pharmacognosy Research, 13(4), 268–273.

Fernandes, J. M., Cunha, L. M., Azevedo, E. P., Lourenço, E. M. G., Fernandes-Pedrosa, M. F., Zucolotto, S. M. (2019) *Kalanchoe laciniata* and *Bryophyllum pinnatum*: An updated review about ethnopharmacology, phytochemistry, pharmacology and toxicology. Revista Brasileira de Farmacognosia, 29(4), 529–558.

Gammoudi, N., Nagaz, K., Ferchichi, A. (2022) Establishment of optimized in vitro disinfection protocol of *Pistacia vera* L. explants mediated a computational approach: multilayer perceptron–multi−objective genetic algorithm. BMC Plant Biology, 22(1), 324.

García-Pérez, P., Lozano-Milo, E., Landin, M., Gallego, P. P. (2020a) From ethnomedicine to plant biotechnology and machine learning: the valorization of the medicinal plant Bryophyllum sp. Pharmaceuticals, 13(12), 444.

García-Pérez, P., Lozano-Milo, E., Landin, M., Gallego, P. P. (2020b) Machine learning unmasked nutritional imbalances on the medicinal plant Bryophyllum sp. cultured *in vitro*. Frontiers in Plant Science, 11, 576177.

Hashim, S. N., Ghazali, S. Z., Sidik, N. J., Chia-Chay, T., Saleh, A. (2021) Surface sterilization method for reducing contamination of *Clinacanthus nutans* nodal explants intended for *in-vitro* culture. E3S Web of Conferences, 306, 1-8.

Handayani, E., Irsyadi, M. B., Alawiyah, R. L. M. N., Aris, I. (2022) Effect of explants sterilization and plant growth regulators on embryo culture of Kepel (*Stelechocarpus burahol*). IOP Conference Series: Earth and Environmental Science, 985(1), 012016.

Hasnain, A., Naqvi, S. A. H., Ayesha, S. I., Khalid, F., Ellahi, M., Iqbal, S., Hassan, M. Z., Abbas, A., Adamski, R., Markowska, D., Baazeem, A., Mustafa, G., Moustafa, M., Hasan, M. E., Abdelhamid, M. M. A. (2022) Plants in vitro propagation with its applications in food, pharmaceuticals and cosmetic industries; current scenario and future approaches. Frontiers in Plant Science, 13, 1009395.

Liu, Y., Li, Y., Yuan, Y. Y., Geng, Z. P., Li, J. L., Wang, M. J., Wang, J. L. (2023) Elucidation of the potential molecular mechanism of the active compounds of *Bryophyllum pinnatum* (L. f.) Oken against gastritis based on network pharmacology. Chinese Journal of Analytical Chemistry, 51(1), 1–11.

Long, Y., Yang, Y., Pan, G., Shen, Y. (2022) New insights into tissue culture plant-regeneration mechanisms. Frontiers in Plant Science, 13, 926752.

eISSN-0128-1119

DOI: http://dx.doi.org/10.71221/ajp.09412

Lozano-Milo, E., García-Pérez, P., Gallego, P. P. (2020) Narrative review of production of antioxidants and anticancer compounds from Bryophyllum spp. (Kalanchoe) using plant cell tissue culture. Longhua Chinese Medicine, 3, 18–18.

Nguanchoo, V., Wangpakapattanawong, P., Balslev, H., Inta, A. (2019) Exotic plants used by the Hmong in Thailand. Plants, 8(11), 500.

Phillips, G. C., Garda, M. (2019) Plant tissue culture media and practices: an overview. In Vitro Cellular and Developmental Biology - Plant, 55(3), 242–257.

Saidu, A., Tahir, S. M. (2021) *In vitro* propagation of *Bryophyllum pinnatum* using leaf explant. KASU Journal of Biochemistry and Biotechnology, 2(2), 14–19.

Saurabh D, Tejas N, Mahesh S, Kiran D, Krushna D. (2024). View of Exploring the Therapeutic Potential: Phytochemistry and Pharmacology of *Bryophyllum pinnatum*. Journal of Drug Delivery and Therapeutics, 14(2), 171–177.

Sifa, M., Kose, H., Dogan, M., Sadi, G. (2020) Surface sterilization of *Staurogyne repens* (Nees) Kuntze with hydrogen peroxide. Bull Biotechnol, 1(2), 39-42.

Samantaray, U. S., Singh, T. (2021) Optimization of modified Murashige and Skoog (MS) medium promoting efficient callus induction in *Zinnia elegans*. International Journal for Research in Applied Sciences and Biotechnology, 8(3).

Sparjan Babu, D, S., Naveen Kumar, P., Krishna, M, S, R., Ramajayam, D., Susanthi, B. (2019) Effect of activated charcoal, culture media and plant growth regulators on *in vitro* germination and development of Dura oil palm (*Elaeis guneensis* Jacq.) zygotic embryos. Plant Cell Biotechnology and Molecular Biology 20(7&8):314-323. ISSN: 0972-2025.

Tabei, Y., Muranaka, T. (2020) Preface to the special issue "technology in tissue culture toward horizon of plant biotechnology." Plant Biotechnology, 37(2), 117–120.

Xu, K., Lin, C., Lee, S. Y., Mao, L., Meng, K. (2022) Comparative analysis of complete Ilex (Aquifoliaceae) chloroplast genomes: insights into evolutionary dynamics and phylogenetic relationships. BMC Genomics, 23(1). 21-14.

eISSN-0128-1119

DOI: http://dx.doi.org/10.71221/ajp.09412