Journal of Tropical Biology and Conservation 20: 193–202, 2023 ISSN 1823 3902
E-ISSN 2550-1909

Research Article

Establishment of An in Vitro Mass Propagation System for *Dendrocalamus asper.*

Anis Adilah Mustafa[1], Wilson Thau Lym Yong[1], Julius Kulip[2], Kenneth Francis Rodrigues[1*]

[1]Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.
[2]Botanicals Sabah Sdn. Bhd., 4th Floor, Lot 12, Warisan Square, P. O. Box 10018, 88800 Kota Kinabalu, Sabah, Malaysia.

***Corresponding author:** kennethr@ums.edu.my
Received 19 July 2023|Reviewed 05 September 2023|Accepted 19 September 2023|Published 15 October 2023
Doi: https://10.51200/jtbc.v20i.4644

ABSTRACT

Dendrocalamus asper is a species of bamboo that has high commercial value and is the bamboo of choice for large scale agro-forestry plantations in the tropical regions of the world. Micropropagation using tissue culture is essential to generate uniform clones that are amenable to establishment in industrial agro-forestry projects for bamboo biomass, habitat restoration or in carbon sequestration. This paper reports on the establishment of *D. asper* invitro using commercially available seeds. The seeds were surface sterilized using three different chemical agents which were Sodium Hypochlorite (20%), Mercuric Chloride (0.1%) and Ethanol (70%) followed by shoot initiation on Murashige and Skoog (MS) medium supplemented with 6-Benzylaminopurine (BAP) with a concentration ranging from 1.0 – 5.0 mg/L. Propagules were multiplied on MS media supplemented with different concentration of IBA Indole-3-Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA), and finally rooted and hardened in peat moss. The findings of our study indicate that the sterilization protocol eliminated all the plant pathogens, resulting in an axenic culture. Full strength MS medium supplemented with 5 mg/L BAP yielded the highest number of shoots (11.46 per explant) after four weeks of inoculation. The highest multiplication rate (3.95 shoots per explant) was obtained on MS medium supplemented with 3 mg/L BAP. The time required from initiation to hardening was 70 to 90 days, following which the plantlets were ready for field trials. The findings of this study will facilitate the establishment of plant tissue culture programmes dedicated to the production of *D. asper* locally, thus eliminating the need for imports and the possible entry of plant pathogens that can be detrimental to the local agro-forestry industry.

Keywords: *Dendrocalamus asper;* bamboo; micropropagation; 6 Benzyl aminopurine; Indole-3-Butyric Acid; Naphthalene Acetic Acid; Murashige and Skoog medium

Introduction

Micropropagation of bamboos is accepted as one of the standard methods for the large-scale multiplication of bamboo clones with desirable characteristics. This is achieved by germination of seeds in vitro (Lv et al., 2021) to overcome the low germination rate of bamboo seeds using conventional approaches, by the in vitro establishment of axillary shoots (Sandhu et al., 2018), the induction of callus tissue (Obsuwan et al., 2019; Zang et al., 2016) and organogenesis (Dey et al., 2020). Methods developed for the micropropagation of bamboo must be optimized based on the variety and the type of the tissue (Ara et al., 2020) and this is achieved by the fine tuning of the components of the culture medium as well as the selection of the appropriate concentrations of the carbon sources (Yasodha et al., 2008), the concentration and the ratio of the phytohormones (Venkatachalam et al., 2015), and the mitigation of the activity of polyphenol oxidases in vitro that can contribute to abscission of growing tissue (Huang et al., 2002). Micro propagated bamboo is a downstream product of the biotechnology industry that can be genetically modified for further improvement to improve its growth characteristics (Ranjan, 2021) and contribute to food security (Bhardwaj et al., 2023) in alignment with the United Nations Sustainable Development Goals. One of the major challenges associated with the propagation of bamboo is the lack of seeds. Bamboo stands undergo flowering followed by mass mortality and most of the seeds are not viable. This has led to micropropagation of viable seeds being the method of choice for mass propagation of bamboo. This study set forth to establish a protocol for the large-scale multiplication of a commercial variety of *Dendrocalamus asper* within the context of a limited time frame to ensure that it can be upscaled for industrial production of bamboo clones locally to support the rural economy and contribute to food security. *Dendrocalamus asper* is a fast-growing species of bamboo that is cultivated both for industrial application and consumption. Bamboos are monocarpic and flower only once in 30 to 120 years, thus making it extremely difficult to source seeds for establishment of commercial plantations, per hectare is 9000 kgs per year and the most widely adopted method for propagation is via cuttings or to a lesser extent micropropagation. Previous attempts to establish *Dendrocalamus asper* in vitro relied on the use of a single concentration of MS medium with variable concentrations of 6 BAP (Arya et al., 2008) and this protocol was tested in order to establish the commercial variety of *Dendrocalamus asper* in vitro prior to optimizing the culture conditions.

Material and methods

The micropropagation experiments for the *Dendrocalamus asper* bamboo species were conducted at the plant invitro laboratory located at the Biotechnology Research Institute, Universiti Malaysia Sabah from March to November 2022. The methods of growing bamboos through tissue culture were conducted following a protocol using seeds as a source of explants. The seeds were dehusked and washed under running tap water for 15 min. Surface disinfection was done with 0.1% Mercuric Chloride ($HgCl_2$) for 3 min, 70% Ethanol for 15 min, and followed by 20 % Sodium Hypochlorite (NaOCl) for 10 min with the addition of a 0.1% V/v of the surfactant Tween 20. The seeds were rinsed four times with sterile distilled water and placed on an oscillating shaker to ensure an even complete disinfection of seeds. Sterile filter paper was used to remove the extra moisture on the seed surface. The disinfected seeds were germinated in 100 mL tissue culture flasks containing 35 mL of germination medium [MS medium supplemented with 100 mg/l myo-inositol, 30 g/l sucrose, 5.6 g/L Gelrite (Duchefa Biochemie), and 1.0–5.0 mg/L 6-BAP for a duration of 28 days. Propagules were separated carefully and were transferred on to two types of multiplication media (MS and half-strength MS) on day 22, and sub cultured on fresh media on days 14,21, 35, 46, and 60. Optimization of media for rooting in vitro was done by transferring single shoots to semi solid half-strength MS medium supplemented with different concentration of IBA Indole-3-Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA) for 28 days. After rooting in vitro, the shoots with roots were taken out of the culture bottle carefully without damaging the roots. The roots were washed gently under running tap water to eliminate the culture medium. The plantlets with roots were acclimatized in peat moss (Kekkila, Finland) for hardening and maintained at 25°C ± 2°C under continuous light with an intensity of 30 µmol/ µmol/m2/s provided by cool-white 40 W fluorescent tubes (Philips) for 28 days. The pH of all culture media was adjusted to 5.8 ± 0.1 with 0.1 N NaOH or 0.1 N HCl prior to the addition of Gelrite, followed by autoclaving at 121°C for 20 min at 15 PSI. The experimental design for plant tissue culture was completely randomized and composed of 30 replicates per treatment for shoot initiation and proliferation, while 20 replicates were for rooting. The shoot formation, leaf formation, root formation, and the number of shoots, leaves, or roots were recorded visually and measured using a vernier calliper during each subculture. All the data were subjected to analysis of variance (ANOVA) performed in IBM SPSS statistical software Version 28.0. Significance differences between the means were assessed by Tukey's post hoc test at $p < 0.05$.

Results and Discussion

The process of disinfection of seeds using the three chemical agents in combination with continuous washing in sterile distilled water and de-husking of the seeds to remove the fibrous seed coat resulted in the elimination of microbial contaminant in the culture media. The establishment of an axenic culture of bamboo is critical to the reproducibility of the protocol as the microbes (Banik et al., 2018; Collinge et al., 2021; Djami-Tchatchou et al., 2022) that are cocultured with the target plant can produce a wide range of bioactive compounds that can serve as growth promoters or inhibitors and confound the interpretation of the results. Mercuric chloride at a concentration of 0.1% is ideal for the elimination of microbes and has been reported in other studies (Hypochlorite et al., 2021; Josephine U Agogbua & Bosa E Okoli, 2022), however caution should be exercised when working with Mercuric Chloride as it is a harmful chemical agent that must be eliminated completely from the culture medium by repeated washing followed by proper disposal. The seed husk serves as a matrix for fungal spores that may resist disinfection by chemical agents, and the removal of this outer layer of husk also contributed to the successful establishment of seeds during the first stage.

MS medium was used at two different concentrations in accordance with the experimental design. The full-strength MS medium (1X) was found to be ideal for initiation of shoots during germination, however, continued use of this concentration led to browning of tissue (**Figure 1**) that can be attributed to abscission as has been reported in other studies (My Ngan et al., 2020). This study did not revert to the usage of additional antioxidants such as Salicylic acid (Ali, 2021), because these would add to the complexity of the culture medium.

Figure 1. Evidence of abscission in *Dendrocalamus asper* plantlets cultivated on full-strength MS medium 10 days after subculture.

The problem of tissue necrosis in long-term culture was reduced significantly by using half-strength MS medium. The reason for the reduction in necrosis and abscission in lower strength of MS media is the presence of high concentration of micronutrients. One of the possible alternatives will be to optimize a novel medium for bamboo using response surface methodology.

The results of the germination of seeds followed by the development of seedlings using the cytokinin BAP after four weeks of culture are documented in **Table 1.** The number of shoots were determined to be independent of the concentration of 6-BAP. A similar observation was made with reference to the number of leaves at the tested concentration of 6 BAP.

Table 1. Effect of BAP concentrations in MS medium on development of shoot and leaves from single seed. Data recorded after 4 weeks of culture.

6-BAP concentration (mg/l)	Shoot length (cm)	Number of shoots	Number of leaves
0.0	2.93±1.11a	1.0±0.0d	5.37±1.94a
1.0	2.47±0.85a	2.06±0.77d	4.65±1.51a
2.0	1.52±0.72b	4.89±0.57c	2.62±0.91b
3.0	1.48±0.41b	7.2±1.44b	2.29±0.82b
4.0	1.08±0.20bc	10.86±0.85a	1.70±0.55bc
5.0	0.96±0.23c	11.46±0.47a	1.49±0.50c

Values are means ± SE (Standard Error) of 30 replicates. Data scored after 4 weeks in culture.
Means followed by the same alphabet (a, b, c) do not differ significantly by Tukey's test ($p < 0.05$)

The objective of the first stage of tissue culture was to increase the number of shoots, without compromising the number of leaves, and it was concluded that the optimum concentration of BAP was 4 mg/l that yielded a lower number of shoots (10.86) with an increased number of leaves. Studies in other species of bamboo have revealed that 6 BAP is the ideal for mass propagation of bamboo and can be used in combination with the auxin Kinetin (Khare et al., 2021), however for the purpose of the development of this protocol, Kinetin was eliminated due to the high cost, making it not suitable for commercial projects. The previous study (Arya et al., 2008) reported that full strength MS medium could be utilized throughout the process of micropropagation, but based on the observations made during this study, the use of half strength MS medium resulted in a decrease in the formation of necrotic tissue. One of the possible explanations for this is that seeds derived from different locations are likely to have genetic and epigenetic variations that can influence the process of invitro establishment and propagation. Thus, future research work must consider the geographic origin of the germplasm prior to establishment in vitro.

The new shoots were separated into individual propagules comprising one shoot and its associated root from the in vitro-developed shoots for subculture into different concentrations of BAP for 14 days. The highest multiplication rate of 3.95 shoots per propagule was recorded in MS medium with 3 mg/L (**Table 2**).

Table 2. Effect of BAP concentration in MS medium on shoot multiplication. Propagules consisting of one shoot and its associated root were cultured and data recorded after 4 weeks of inoculation (± SE). Three propagules were inoculated in a single tissue culture jar.

6-BAP concentration (mg/l)	Shoot length (cm)	Number of shoots
0.0	3.37±0.86^{a}	1.0±0.0^{e}
1.0	2.86±0.51^{b}	6.68±0.89^{d}
2.0	1.75±0.61^{c}	18.37±1.012^{d}
3.0	1.55±0.35^{cd}	28.46±0.45^{a}
4.0	1.23±0.24^{de}	25.9±0.97^{b}
5.0	1.11±0.22^{e}	24.23±1.12^{b}

Green multiple shoots with 3 propagules were further sub-cultured into different concentrations of 6 -BAP for 14 days. Values are means ± SE of 30 replicates. Data scored after 4 weeks in culture.
Means followed by the same alphabet (a, b, c) do not differ significantly by Tukey's test ($p < 0.05$)

Therefore, 3 mg/L BAP was selected for further proliferation. Subculturing was initiated every 2 weeks. The increase in shoot number was observed until the fourth subsequent subcultures, after which it declined in the fifth subculture (as shown in **Table 3**). In the present study, BAP was found to be effectual for shoot multiplication. These findings are consistent with those reported by (Suwal et al., 2020) and (Hiswan et al., 2020). To conclude, the use of a single auxin at lower concentration was determined to be ideal for the *D. asper* variety selected for this study.

Table 3. Effect of subsequent subculture on multiple shoot proliferation in MS basal medium supplemented with 3.0 mg/L BAP.

Subcultures	Number of shoots	Length of shoots (cm)	Multiplication fold
1st subculture (14d)	9.98±0.28e	1.55±0.82e	3
2nd subculture (21d)	20.91±0.098d	2.12±0.43d	2.09
3rd subculture (35d)	26.58±0.19c	3.81±0.51c	1.27
4th subculture (46d)	35.33±0.13a	6.61±0.29a	1.16
5th subculture (60d)	30.3±0.11b	5.01±0.74b	1.13

Means followed by the same alphabet (a, b, c, d, e) do not differ significantly by Tukey's test ($p < 0.05$)

The rooting stage is crucial for the establishment of plants in the field and the induction of a large network of roots can be promoted by the usage of auxins. Two auxins, 1-Naphthaleneacetic acid (NAA) and Indole-3-butyric acid (IBA) were used in various combinations (**Table 4**) (**Figure 2**) and it was determined that the optimum concentration was 0.5 mg/l of IBA based on the rooting

percentage (90%), the average number of roots in a clump (16.65) and the length of the roots (4.15 cm).

Table 4. Effect of different concentration of IBA and NAA on rooting of in vitro raised shoots *D. asper*. Data recorded after 4 weeks of inoculation (± SE).

Plant growth regulator (mg/ l)		Rooting (%)	No. roots in clump (Mean ± SE)	Root length (cm)
IBA	NAA			
0	0	0	0.00^{h}	0.00^{i}
0.5	-	96	16.65±0.52^{a}	4.15 ± 0.75^{a}
1.0	-	81	9.9±0.30^{bc}	3.35 ± 0.27^{b}
3.0	-	82	7.7±0.45^{e}	2.45 ± 0.51^{c}
5.0	-	75	3.83±0.24^{f}	2.0 ± 0.49^{cd}
7.0	-	63	2.82±0.36^{fg}	1.25 ± 0.31^{de}
10.0	-	54	1.75±0.61^{g}	0.62 ± 0.37^{gh}
-	0.5	60	3.75±0.53^{f}	0.45 ± 0.30^{hi}
-	1.0	68	8.05±0.44^{de}	1.0 ± 0.29^{fg}
-	3.0	80	10.85±0.58^{b}	2.37 ± 0.49^{c}
-	5.0	76	9.3±0.69^{cd}	1.37 ± 0.50^{ef}

Values are means ± SE of 20 replicates. Data scored after 4 weeks in culture.
Means followed by the same alphabet (a, b, c, d, e, f, g, h, i) do not differ significantly by Tukey's test ($p < 0.05$).

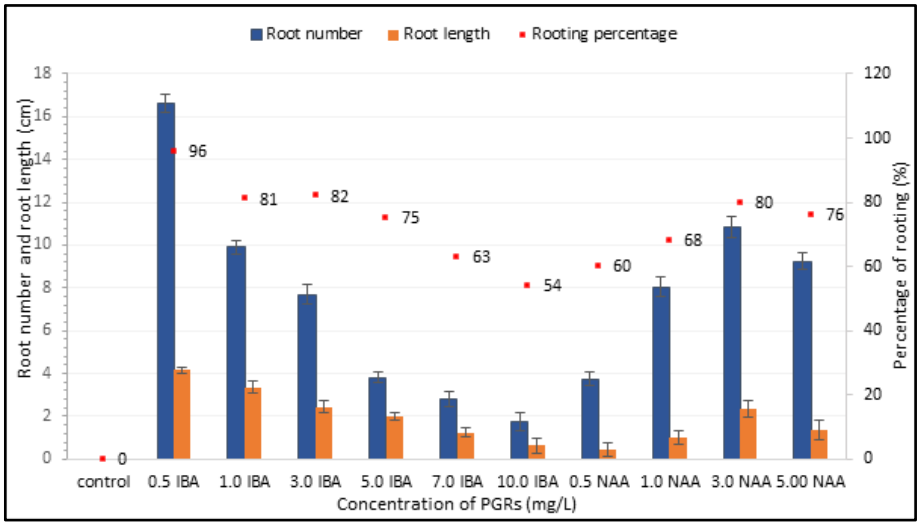

Figure 2. Effect of different concentrations of IBA and NAA on rooting of in vitro raised shoots *D. asper*. for 4 weeks. Values are Mean ±SE of 20 replicates. Bars denote the standard error (SE).

We concluded that IBA is more effective than NAA in establishing a high number of roots per clump. Studies involving other species of bamboo are indicative that the concentration of auxin varies from species to species (Yasodha et al., 2008)

(Sharothi et al., 2022) and this may be dependent on the tissue type as well as the genetic factors that are likely to differ based on the species.

The final stage involved hardening of the tissue culture plantlets in a climate-controlled greenhouse and finally transferring the propagated plantlets for field testing. The complete process from the establishment of seeds to the final stage of hardening is summarised in **Figure 3**. The methods established in this study, can be applied for the mass propagation of *D. asper* in Sabah and can be extended to other species of bamboo.

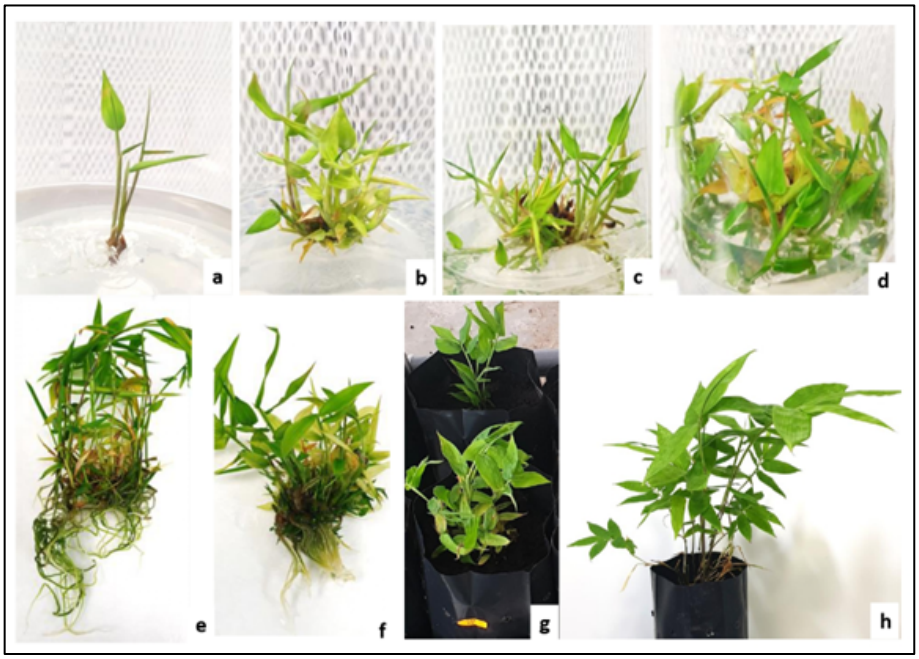

Figure 3. (a) Seedlings growth in MS medium (no Plant Growth Regulators). (b) Initiation of multiple shoots on MS + 3 mg/L BAP. (c) Proliferation of numerous shoots after 2 months on MS + 3.5 mg/l BAP. (d) Clusters of adventitious shoots after 3 months on MS + 3.5 mg/l BAP. (e) Root induction on MS + 0.5 mg/L IBA. (f) Root induction on MS + 3.0 mg/L NAA. (g) 1 month old, hardened plant in greenhouse. (h) 4 months old, hardened plant.

Conclusion

This study successfully developed a protocol for the large-scale production of *D., asper* from commercial seeds that were reproducible and yielded clones within a period of two months. Future research will focus on field trials and the

selection of field grown plants with desirable phenotypes for the establishment of elite lines of plants.

Funding information: This research was funded by Universiti Malaysia Sabah under a niche grant (DN20091) entitled the "Identification and Selection of Endemic Bamboo Varieties of Sabah for Mass Propagation using Tissue Culture".

References

Ali B. 2021. Salicylic acid: An efficient elicitor of secondary metabolite production in plants. In *Biocatalysis and Agricultural Biotechnology*, ***(Vol. 31)***. https://doi.org/10.1016/j.bcab.2020.101884.

Ara MT, Nomura T, Kato Y, Ogita S. 2020. A Versatile Liquid Culture Method to Control the <i>in Vitro</i> Development of Shoot and Root Apical Meristems of Bamboo Plants. *American Journal of Plant Sciences*, ***11*** (02). https://doi.org/10.4236/ajps.2020.112020.

Arya S, Satsangi R, Arya ID. 2008. Direct regeneration of shoots from immature inflorescences in Dendrocalamus asper (edible bamboo) leading to mass propagation. *The Journal of the American Bamboo Society*, ***21*** (1).

Banik A, Pandya P, Patel B, Rathod C, Dangar M. 2018. Characterization of halotolerant, pigmented, plant growth promoting bacteria of groundnut rhizosphere and its in-vitro evaluation of plant-microbe protocooperation to withstand salinity and metal stress. *Science of the Total Environment*, ***630***. https://doi.org/10.1016/j.scitotenv.2018.02.227.

Bhardwaj DR, Rana S, Kumar D, Sharma P. 2023. Nutritive value of tender shoots of different bamboo species in relation to harvesting height in mid-hills of north-western Himalayas. *Applied Food Research*, ***3*** (1). https://doi.org/10.1016/j.afres.2022.100244.

Collinge DB, Gregersen PL, Thordal-Christensen H. 2021. The Induction of Gene Expression in Response to Pathogenic Microbes. In *Mechanisms of Plant Growth and Improved Productivity*. https://doi.org/10.1201/9781003210252-11.

Dey B, Verma A, Tewari S, Dubey A. 2020. Organogenesis optimization in Dendrocalamus asper. *Journal of Non Timber Forest Products*, ***27*** (2). https://doi.org/10.54207/bsmps2000-2020-zd4xvn.

Djami-Tchatchou AT, Li ZA, Stodghill P, Filiatrault MJ, Kunkel BN. 2022. Identification of Indole-3-Acetic Acid-Regulated Genes in Pseudomonas syringae pv. tomato Strain DC3000. *Journal of Bacteriology*, ***204*** (1). https://doi.org/10.1128/JB.00380-21.

Hiswan J, Larekeng SH, Gusmiaty W, Putri SD. 2020. In vitro shoot induction of batik bamboo using BAP and NAA. *Plant Archives*, ***20*** (2): 2343–2349.

Huang LC, Lee YL, Huang BL, Kuo CI, Shaw JF. 2002. High polyphenol oxidase activity and low titratable acidity in browning bamboo tissue culture. *In Vitro Cellular and Developmental Biology - Plant*, ***38*** (4). https://doi.org/10.1079/IVP2002298.

Hypochlorite S, Singh BK, Ali MN, Samanta S, Mandal N. 2021. A Comparative Analysis among Different Surface Sterilisation Methods for Rice Invitro

Culture. *International Journal of Plant & Soil Science*. https://doi.org/10.9734/ijpss/2021/v33i1730559.

Josephine U Agogbua, Bosa E Okoli. 2022. Procedure for in vitro seed sterilization, germination and aseptic seedling establishment of Zehneria capillacea (Schumach) C. jeffrey. *GSC Biological and Pharmaceutical Sciences*, ***19*** (1). https://doi.org/10.30574/gscbps.2022.19.1.0135

Khare SR, Kharate PS, Sahu RK, Jha Z. 2021. Rapid in-vitro micropropagation of Bamboo (*Dendrocalamus strictus*) and its genetic fidelity testing using ISSR markers. *Environment Conservation Journal, 22* (3): 69–77. https://doi.org/10.36953/ecj.2021.22308.

Lv Z, Zhu F, Jin D, Wu Y, Wang S. 2021. Seed Germination and Seedling Growth of Dendrocalumus brandisii in vitro, and the Inhibitory Mechanism of Colchicine. *Frontiers in Plant Science*, *12*. https://doi.org/10.3389/fpls.2021.784581

Ngan HTM, Tung HT, Van Le B, Nhut DT. 2020. Abnormal symptoms in micropropagation and strategies to overcome. *Vietnam Journal of Biotechnology*, ***18*** (1): 23–39. https://doi.org/10.15625/1811-4989/18/1/15258.

Obsuwan K, Duangmanee A, Thepsithar C. 2019. In vitro propagation of a useful tropical bamboo, Thyrsostachys siamensis Gamble, through shoot-derived callus. *Horticulture Environment and Biotechnology*, ***60*** (2). https://doi.org/10.1007/s13580-018-00119-z

Ranjan A. 2021. Bamboo Tissue Culture - Commercial Applications and Environment. *Acta Scientific Agriculture*, ***5*** (10). https://doi.org/10.31080/asag.2021.05.1061

Sandhu M, Wani SH, Jiménez VM. 2018. In vitro propagation of bamboo species through axillary shoot proliferation: a review. In *Plant Cell, Tissue and Organ Culture, **(Vol. 132, Issue 1)***. https://doi.org/10.1007/s11240-017-1325-1.

Sharothi P, Raju RI, Hossain MT. 2022. In vitro Propagation of an Ornamental Bamboo (Bambusa tuldoides Munro). *Plant Tissue Culture and Biotechnology*, ***32*** (2). https://doi.org/10.3329/ptcb.v32i2.63550.

Suwal MM, Lamichhane J, Gauchan DP. 2020. Regeneration Technique of Bamboo Species through Nodal Segments: A Review. *Nepal Journal of Biotechnology*, ***8*** (1). https://doi.org/10.3126/njb.v8i1.30209.

Venkatachalam P, Kalaiarasi K, Sreeramanan S. 2015. Influence of plant growth regulators (PGRs) and various additives on in vitro plant propagation of Bambusa arundinacea (Retz.) Wild: A recalcitrant bamboo species. *Journal of Genetic Engineering and Biotechnology*, ***13*** (2). https://doi.org/10.1016/j.jgeb.2015.09.006.

Yasodha R, Kamala S, Kumar SPA, Kumar PD, Kalaiarasi K. 2008. Effect of glucose on in vitro rooting of mature plants of Bambusa nutans. *Scientia Horticulturae*, ***116*** (1). https://doi.org/10.1016/j.scienta.2007.10.025.

Zang Q, Zhou L, Zhuge F, Yang H, Wang X, Lin X. 2016. Callus induction and regeneration via shoot tips of *Dendrocalamus hamiltonii*. *SpringerPlus*, ***5*** (1). https://doi.org/10.1186/s40064-016-3520-7