Micropropagation of *Arisaema* spp. (*filiforme* and *brinchangense*): explant selection and surface sterilization insights

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Abstract. *Arisaema filiforme* and *A. brichangense* are a perennial herbaceous plant (family Araceae) found distributed in mossy forest, Cameron Highlands, with the elevation of 1,900 meters above sea level (a.s.l). The unique inflorescence formation resembling cobra has given this plant the name Cobra lilies, and suitable to be planted as ornamental plant. In addition, it has been used traditionally as a herb. However, the population of these two species are very limited, only thrive in higher elevation and also considered as an endangered. Therefore, realizing its potential in the future as one of the new ornamental plant and materials for the herb bioindustry, a micropropagation approach was employed to produce these species in mass production. Seeds, rhizomes, and petioles were used as the explant materials, cultured onto Murashige and Skoog (MS) media supplemented with different concentrations (0, 0.5, 1, 2 mg L^{-1}) of 6-Benzylaminopurine (BAP). The findings revealed rhizomes and seeds to be significant explants for micropropagation, where the survival rate for these two are more than 80%. Petioles had 0% of survivability after week eight of culture due to the fungi infection and tissue necrosis. This study provides an insight into explant selection, where different plant organs have different survival rate due to the tissue mechanical strength. Also, optimum surface sterilization process is very critical in micropropagation to avoid the contamination of the culture and also necrotizing.

1. Introduction

Arisaema is the fourth largest genus out of 150 accepted genera of the Araceae family [1]. The genus *Arisaema* is a perennial monocotyledon herbaceous plant, also known as Cobra-lilies and Jack-in-the-Pulpit. The genus consists of approximately 207 species, with a range that stretches from Central and East Africa to Southeast Asia, via Yemen, Oman, Pakistan,

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Afghanistan, the entire Himalayan range, India, China, Korea, Japan, and Siberia, and finally to North America [1]; [2]; [3]. Peninsular Malaysia is a home to eight species of *Arisaema*: *A. anomalum*, *A. filiforme*, *A. fimbriatum* subs. *fimbriatum*, *A. laminatum*, *A. roxburghii*, *A. scortechninii*, *A. wrayi* and *A. brinchangense* [3]; [4].

Borneo was the initial location where *A. filiforme* was discovered, followed by Peninsular Malaysia. In Sabah, the species' spathe is green, whereas in Peninsular, the spathe is red and occasionally stained with green [3]. This morphological variation resembles the specimen discovered in Brinchang. In addition, *A. brichangense* is a newly described species originating in Peninsular Malaysia. The species is comparable to *A. anomalum*, but its spathe has a distinct morphology. *A. brinchangense* is endemic to the Cameron Highlands, Pahang, where the majority of recent occurrences occurred near the summit of Mt. Brinchang, which is protected by the Batu Gangan Permanent Forest Reserve [4].

Similar to other genera in the Araceae family, *Arisaema* species exhibit a diverse range of applications and properties. For instance; (i) Use as a food source. According to [5], boiled juvenile leaves of *A. peninsulae* in Korea and dried leaves of *A. jacquemontii* in Nepal are consumed as vegetables, whereas in Southern Ethiopia, the species serves as an essential famine-food resource. (ii) Use as a traditional medicinal herb. Since primordial times, *Arisaema* has been utilised in both traditional Chinese medicine and Ayurvedic medicine. [6] state that *A. tortuosum* (tuber) was utilised to treat liver infection, microbial contamination, and tumor growth. According to a report by [7], the tuber of *Arisaema* sp. has been used to treat constipation, abdominal pain, and dysentery. More recently, chemical constituents from various *Arisaema* parts, including alkaloids, phenols, terpenes, flavonoids, lectins, saponins, glycosides, triterpenoids, campesterols, oxalates, etc., were discovered. Antioxidant, antifungal, antibacterial, insecticidal, antimicrobial, anticancer, and antitumor properties have been discovered in the plant [8];[9]. (iii) Use as an ornamental plant. Araceae are best known as ornamental plants and among the most significant foliage ornamentals cultivated and sold for display on a commercial scale. The pseudostem in *Arisaema* is frequently beautifully variegated or marked with various colours, which can make it quite ornamental.

As is the case with the majority of tuberous plants, *Arisaema* can be propagated in two main ways: (i) By vegetative proliferation and (ii) From seeds [3]. However, the slow rate of vegetative multiplication complicates the conventional propagation of these two species. In addition, the growth and propagation conditions for Araceae are extremely critical, necessitating the control of moisture and temperature. According to [10], micropropagation through tissue culture is a method for overcoming these limitations. Studies on micropropagation of Araceae was conducted by several researchers such as, [11] has reported on *in vitro* propagation of five *Alocasia* species, whereas, [12] has reported on the novel technique for *in vitro* propagation of Araceae. The modern technique of tissue culture represents a promising method for mass-producing the plant. Tissue culture has advantages for the propagation of uncommon and endangered species with extremely low parent stock. In addition, tissue culture can solve the problem of infertility in plant [3]. The use of tissue culture in germplasm conservation is essential for the conservation of endangered plant species and is also significant to this study.

Explant selection is crucial in plant tissue culture for the following reasons: (i) Genetic integrity; (ii) Regeneration potential; (iii) Contamination control; (iv) Explant size and vigor; and (v) Multiplication potential. Micropropagation in plant tissue culture is also affected by the surface sterilization technique [13]. Tissue culture methods in *Arisaema* are less developed, this project, therefore, describes study on the micropropagation of *A. filiforme* and *A. brichangense*, as a beginning to more detailed studies on the propagation of *Arisaema* in general.

2. Methodology

2.1 Plant material collection

The Mossy Forest Scientific Expedition was held in Cameron Highlands, Pahang, from 10 to 14 March 2023. During the survey, two species of *Arisaema* were found; *A. filiforme* and *A. brinchangense*, thriving in the montane forest at the altitude of 1800 to 2000 meter above sea level (a.s.l). The identification of these species was done through references to pertinent articles and books, including works by [3]; [4]; [14], as well as through discussions with experts in Araceae, notably Peter C. Boyce. It flourishes on the moist forest floor covered with thick organic litter under deep to semi-shaded conditions. The collected live specimen of *A. filiforme* and *A. brinchangense* were carefully placed in sampling bags to preserve their freshness, moisture, and prevent damage. In-situ location pictures were taken for documentation purposes, as shown in Figure 1.

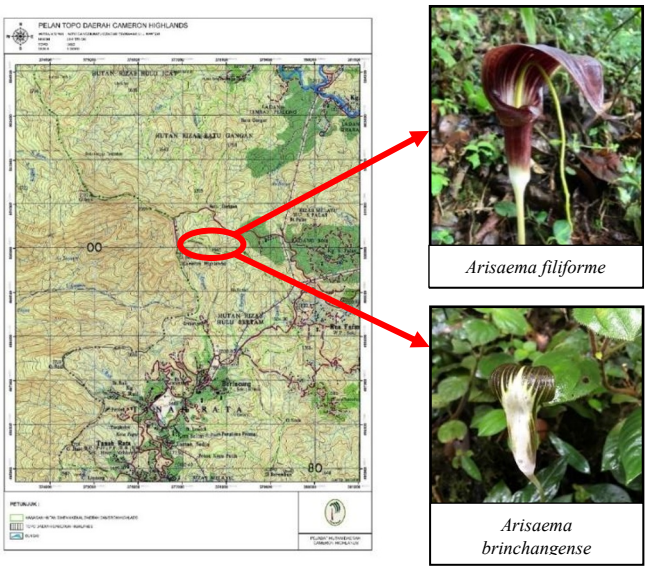

Fig. 1. (Left) Map of the sampling area, whereas, (Right, above) Image of *A. filiforme* inflorescence and (Right, bottom) Image of *A. brinchangense* inflorescence

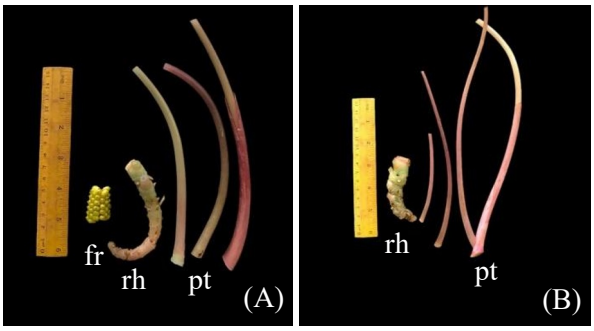

Fig. 2. The explants selected for micropropagation, (A) *A. filiforme* and (B) *A. brinchangense*, where: fr = infructescence; rh = rhizome; pt = petioles

2.2 Experimental design

The design of tissue culture experiments included a sufficient number of replicates for statistical analysis. Each treatment had 30 replicates. The experiments utilised a completely randomised design (CRD). The survival rate (%), browning (%), and the condition (callus/ germination/ response/ contamination) was reported as the outcome.

Table 1. Murashige & Skoog (MS) media supplemented with different concentrations of 6-Benzylaminopurine (BAP) for micropropagation of *Arisaema* spp. (*filiforme & brichangense*)

Code	Treatments
TZ	Control (without BAP)
TM	MS + 0.5 mg L^{-1}BAP
TN	MS + 1 mg L^{-1}BAP
TO	MS + 2 mg L^{-1}BAP

2.3 Surface sterilization

Prior to surface sterilization, the samples (Figure 2) were washed with tap water to remove the debris. The samples were then placed in a beaker covered with aluminium foil for laminar flow transfer. For the surface sterilization technique, samples were initially immersed for 5 minutes in 70% ethanol (EtOH). The samples were then disinfected in 30% Chlorox ® (containing 5.3% sodium hypochlorite (NaOCl) with one drop of Tween-80 solution for 3 minutes, followed by three rinsings in sterile distilled water. Again, samples were immersed for 1 minute in 70% EtOH and then rinsed with sterile distilled water.

2.4 Culture incubation & growth room conditions

All instruments' beakers, and media were autoclaved at 104 kPa and 121°C (Atherton Equipment, maximum specifications: 500 kPa, 134°C) for 15 minutes. The laminar flow was routinely sterilised with 70 % EtOH. The plant material for the cultivation of *A. filiforme* and *A. brinchangese* were handled and prepared using long forceps and scalpel blades. As explants, the rhizomes and petioles were cut approximately 0.5 cm × 0.5 cm horizontally and vertically, whereas the seeds were cultivated as a single organ. In the laminar flow cabinet, instruments were re-sterilised between procedures using a heated glass bead steriliser (Sanap). The explants were grown on Murashige & Skoog (MS) media supplemented with different concentrations of BAP, as shown in Table 1 section 2.2. The cultures were stored in the culture room with a temperature of 18 to 28 °C, photoperiod of 12 to 16 hours, and a light intensity of 30 – 50 $\mu mol.m^{-2}. s^{-1}$ (PAR) provided by cold white fluorescent tubes.

3. Result and discussion

The results of this study on the *in vitro* micropropagation of *Arisaema* spp. (*filiforme* and *brinchangense*) revealed important insights into the explant selection and surface sterilization technique on the success of tissue culture. As shown in Table 2, the current study indicates that the rhizome and fruit have a higher survival rate than the petiole. This suggested that both explants are substantially suitable for selection as the micropropagation explant. Based on the study, the survivability of the explants was influenced by two factors, these are (i) the condition of the donor plant; and (ii) the sterilization technique – causing tissue necrosis/ contamination. As mentioned in section 2.1, the plant materials were collected from different sites, where *in situ* habitat has mild and cold temperatures around 18-22 °C, and grow at high altitudes. It took several days before the plants were brought to the laboratory. Therefore, it was suggested the plant's conditions should be maintained and acclimatized before being

subjected to TC. The status of the donor plant is crucial in ensuring the success of TC. This is complying with [13] who mentioned the condition and health of the donor plants are extremely important for the success of TC studies. Besides, it was determined that the protocol for surface sterilization of the petiole must be differed from that of the rhizomes and seeds due to the varied tissue hardiness. Upon exposure to the disinfectant, the petiole, which is predominantly composed of ground tissue, namely parenchyma cells, as well as sclerenchyma, xylem, and phloem components [15], exhibited susceptibility to chemical damage due to the soft character of its tissue, affecting the survivability. Throughout the process, no browning effect was detected. The media and the explants were cleaned from the phenolic contamination suggesting no treatment required such as the use of activated charcoal in the media.

However, in the fourth week after the culture process, it was observed that the plates were contaminated with fungi. As reported by [16], contamination is the most frequently observed factor affecting the effectiveness of plant tissue culture (PTC). The contamination is likely due to a technical issue – a growth room condition. During the experiment, a roof leak caused water to cascade onto the plates. [17] also mentioned in the PTC laboratory, proper controlled environmental conditions with the basic facilities are required in maintaining the PTC's success. Besides, the internal contamination is believed due to the presence of the endophytic microorganism in the tissue [18]. The endophytic microorganism cannot be removed through surface sterilization. Special treatment such as the application of an antibiotic (e.g., ampicillin, penicillin, tricarcillin), copper sulfate, or fungicide [19]; [20]; [21] is capable to resolve the issue. A sub-culture process was implemented to address contamination issues, where the explants were re-sterilized. However, this resulted in increased tissue necrosis, particularly in the petiole. It was observed that the smaller cutting size (0.5 cm × 0.5 cm) used as explants led to higher rates of tissue necrosis. To overcome this, the size of the explants should be increased to approximately 2 to 3 cm, as conducted by [12].

Contamination control was an important aspect of the study, where proper surface sterilization is a critically important procedure in PTC. Test with surfactant; sodium hypochlorite (NaOCl) and Tween-80 should be carried out to establish which treatment time should be chosen for surface sterilization of explants. Increasing exposure times are believed to elevate necrosis, consistent with the findings by [22]. Their study indicated that shorter exposure durations resulted in death due to microbial contamination, while prolonged durations led to tissue death due to chemical-induced damage. Therefore, sterilizing solutions should effectively eliminate fungal or bacterial contaminants while preserving the functional integrity of plant tissues [23]. Other factors such as careful handling and precautionary steps were necessary during subculture and environmental conditions need to be considered too in order to maintain sterile conditions.

Table 2. Percentage of survival rate, browning, and condition of the *Arisaema filiforme* and *A. brinchangense* explants after eight weeks in culture

Treatments	Explants		Survival Rate (%)	Browning (%)	Conditions
MS + 0 mg/L BAP	*A. filiforme*	rh	100	0	NC; NR
		fr	100	0	NG; NR
		pt	0	0	NC; NR; N (100%); CF (15%)
MS + 0.5 mg/L BAP		rh	100	0	NC; NR
		fr	100	0	NG; NR
		pt	0	0	NC; NR; N (100%); CF (10%)
		rh	96.67	0	NC; NR; CF (4%)

MS + 1 mg/L BAP	*A. brinchangense*	fr	100	0	NG; NR
		pt	0	0	NC; NR; N (100%); CF (15%)
MS + 2 mg/L BAP		rh	93.33	0	NC; NR; CF (7%)
		fr	100	0	NG; NR
		pt	0	0	NC; NR; N (100%); CF (10%)
MS + 0 mg/L BAP		rh	93.33	0	NC; NR; CF (7%)
		pt	0	0	NC; NR; N (100%); CF (8%)
MS + 0.5 mg/L BAP		rh	100.00	0	NC; NR
		pt	0	0	NC; NR; N (100%); CF (12%)
MS + 1 mg/L BAP		rh	96.67	0	NC; NR; CF (4%)
		pt	0	0	NC; NR; N (100%); CF (9%)
MS + 2 mg/L BAP		rh	83.33	0	NC; NR; CF (16.67%)
		pt	0	0	NC; NR; N (100%); CF (10%)

* fr = infructescence; rh = rhizome; pt = petioles; NC = no callus; NG = no germination; NR = no response; N = Necrosis; CF = contaminated by fungi

The findings show, even after the eight weeks of the culture, no callus formation was detected, the seeds were not germinated and no other response of the explants was observed. It is important to acknowledge the limitations and challenges encountered during the study. Future research should address these limitations and explore modifications to enhance the efficiency of the micropropagation protocol. The potential applications of the developed protocol for the commercial production of *Arisaema* species should be further investigated.

4. Conclusion

As conclusion, the selection of rhizomes and seeds as the explants for TC is significant as both have a high survivability rate. The type of explants use, condition, and health status of donor plants combined with proper surface sterilization technique is critically important in ensuring the success in plant tissue culture. The establishment of these surface sterilization and plant cultivation techniques could lead to more in-depth research and a greater understanding of *Arisaema* in general. The findings will have implications for the conservation and cultivation of these plant species and contribute to the field of plant tissue culture.

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