Research article

In Vitro Propagation and Genetic Fidelity Assessment of *Hedychium longicornutum* Griff. ex Baker, a Vulnerable Zingiberaceous Plant of Thailand

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Abstract

Hedychium longicornutum Griff. ex Baker is a Zingiberaceous plant that has been used for traditional medicines and ornamental purposes. Though this plant is a vulnerable plant of Thailand, it has a high potential to be promoted as a new economic ornamental plant due to its exotic inflorescences. However, the usage of this valuable plant on a large scale is restricted since the population of *H. longicornutum* in natural habitat is limited. This current study established a micropropagation protocol to ensure sustainable use of this plant for commercial and conservation purposes. *In vitro* axenic plants of *H. longicornutum* were initiated from seeds. Optimum shoot multiplication medium was evaluated by culturing leafy-shoot bases on Murashige and Skoog's (MS) medium supplemented with 0-35 µM of N^6-benzyladenine (BA) or thidiazuron (TDZ). BA supplemented media were better than those TDZ media since no abnormalities of shoot morphology, shoot elongation, and root regeneration were found. MS supplemented with 15 µM BA was revealed to be the most effective medium for shoot multiplication (9.75 leafy-shoots/explant with 9.54 cm height). Regenerated shoots spontaneously rooted on MS medium. The *in vitro* raised plants were successfully grown *ex vitro* and showed the highest survival rate (100%) on planting substrate consisting of 2 garden soil mixture: 1 smashed charcoal. The genetic fidelity of the micropropagated plants compared with mother plants grown in the field was assessed by random amplified polymorphic DNA (RAPD) technique. The results consistently demonstrated the genetic uniformity of the regenerants. This is the first report of an established *in vitro* true-to-true type propagation in *H. longicornutum*.

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1. Introduction

Hedychium J.Koenig (Zingiberaceae) is a perennial terrestrial or epiphytic medium-sized plant which its rhizome can grow to about 1-2 m. *Hedychium* composes of 70-80 accepted species worldwide, and the distribution of its members ranges from Madagascar, tropical and temperate regions of Asia, Australia, Fiji, New Caledonia, New Guinea, New Hebrides, and Samoa to the Solomon Islands [1]. Southern China to the Himalayan regions shows the high diversity of this genus [2]. In Thailand, 21 species (2 endemic, 1-2 introduced) and 3 natural hybrids were recorded [Suksathan, manuscript in preparation for Flora of Thailand], and the highest *Hedychium* diversity was found on the mountain areas in the northern parts [3]. *Hedychium* presents various advantages and is considered as one of the economically important plants. Rhizome and inflorescence of this genus usually produce fragrance, representing as the good raw material sources of essential/volatile oils for perfume making [4]. *Hedychium* aerial stem, such as *H. coronarium*, contains about 42-48% cellulose that can be used in paper-making industries [5]. Various *Hedychium* species also show significant biological activities including anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antimicrobial, antioxidant, cytotoxic/antitumor, hepatoprotective, insecticide [5], and antiglycation [6]. With these presented properties, they have been used in traditional medicine systems of several countries for treating illnesses and also used for producing cosmetic products. The biological activities of *Hedychium* are probably due to the presence of different types of diterpenes [5]. Rhizomes, flowers, and young shoots of some species in this genus are consumed as vegetables in different parts of the globe [7, 8]. Some *Hedychium* species are also known as an important horticultural crop due to their beautiful inflorescences with fragrant scent and attractive foliage. Consequently, they are used for ornamental purposes in gardens in several countries and *H. longicornutum* Griff. ex Baker is one of the ornamental gingers from this genus [2].

Hedychium longicornutum (Hornbill's ginger) is distributed in tropical rain forests at 1,000 m above mean sea level in Sumatra, Malaysia, and only in Yala and Narathiwat (Hala-Bala) Provinces of Thailand [9]. Its habitat can be terrestrial or epiphytic [3]. *Hedychium longicornutum* produces fiery-multicolored inflorescences that resemble a hornbill. Its inflorescence form is exotic and looks like a pointy tiara or Medusa's head (Figure 1A). With the inflorescence appearances, it is used as an ornamental ginger [2] and is sold in various e-commerce ventures. Therefore, it has high potential to be used as an ornamental plant for large-scale commercial. *Hedychium longicornutum* is also used in the traditional medicine system in Thailand. Roots of this plant are used to treat earache; while pounded roots or whole plants are a remedy for intestinal worms [10]. Moreover, this plant plays an important role in the ecological system. As an epiphytic plant, the root masses of *H. longicornutum* are excavated and used as a roost site for spotted-winged fruit bat (*Balionycteris maculata*) [11]. Though *H. longicornutum* provides many benefits, there is no conservation strategy and no efficient large-scale propagation method for using this valuable species without disruption of its natural habitat. Human have interfered with this plant in many ways such as smuggling plants out of the forest, over-harvesting, and trying to develop it into an agricultural product, which lead to the destruction of its habitat. Global warming is another cause of the decline in the numbers of this plant in nature. As a result, the International Union for Conservation of Nature (IUCN) assigned the status of 'near threatened species' to *H. longicornutum* [12] whereas its status in Thailand was classified as a vulnerable plant [9]. Conservation of *H. longicornutum* in order to prevent the species from becoming extinction and to promote its sustainable use are imperative. Conventional propagation methods cannot produce the large numbers of plants needed for germplasm conservation or supporting large-scale usage. Since *Hedychium* generally produced few seeds per inflorescence while conservation of the genus using rhizomes may carry diseases and pests [13]. Currently, biotechnological approaches are to overcome these limitations, and *in vitro*

Figure 1. *H. longicornutum*: an inflorescence (A) and seeds (B)

propagation is one of the most useful techniques for multiplication, conservation, and sustainable use of plants.

In vitro propagation is a technique based on totipotency theory [14]. Various plant parts/organs can be used in proliferation process, resulting in a large number of plant production independently of the season [15]. Moreover, this technique can rapidly generate morphologically, genetically, and biochemically axenic plants by using a minimum of plant material with little impact on the wild population [7, 15, 16]. This cost-efficient technique can produce vigorous plants that can be reintroduced to the wild or grown *ex vitro*. Moreover, micropropagation has also been used to aid the reproduction of some self-incompatible plant species [14]. With these benefits of plant tissue culture, this technique has been applied in the propagation of various *Hedychium* species including *H. bousigonianum* [17], *H. coccinieum* [18], *H. coronarium* [7-9, 15, 16, 19-29], *H. ellipticum* [18]*, H. flavescens* [30], *H. forrestii* [31], *H. gardnerianum* [32], *H. muluense* [33], *H. neocarneum* [30], *H. roxburghii* [34], *H. spicatum* [35-37], and *H. stenopetalum* [6, 30]. These *Hedychium* micropropagation was reported to be used in tissue culture technique for multiple purposes including plant conservation, microrhizome induction, secondary metabolite production, plant breeding, synthetic seed production, and rapidly plant production for the use of regenerated plants in the cosmetic, medicinal, and ornamental industries. Therefore, sustainable use and conservation of *H. longicornutum* should take place if an *in vitro* propagation protocol for this plant is established.

Micropropagation efficiency is affected by various factors, and the type and concentration of plant growth regulators (PGRs) using during *in vitro* plant propagation are major factors affecting success rate. Cytokinin is a PGR that is widely used in *Hedychium* micropropagation. It positively regulates the expression of the gene encoding the master regulator of shoot meristem stem cell identity, *WUSCHEL* (*WUS*) [38]. Therefore, it can stimulate cell division and differentiation, resulting in shoot regeneration. Meanwhile, cytokinin is also involved in break bud dormancy, withdrawal apical dominance, development of adventitious shoot buds, and the control of stem elongation [14]. N^6-benzyladenine (BA), an adenine cytokinin, was used in various *Hedychium* micropropagations reported by various investigators [6, 8, 18, 20, 24-27, 31, 32, 37]. BA is involved in shoot regeneration by increasing the accumulation of N^6(2-isopentenyl)adenine (iP) and N^6(2-isopentenyl)adenosine (iPR) [39], the substrates for cytokinin biosynthesis. BA also increases and lengthens photosynthesis rate [40], which leads to the increases of energy sources needed for shoot development. Gavinlertvatana and Prutpongse [41] suggested that herbaceous plants are highly responsive to BA by producing robust and well-formed shoots. For these reasons, BA is commonly used during *in vitro* propagation of *Hedychium*, which is also a genus of herbaceous plant. Besides BA, thidiazuron (TDZ), a phenylurea cytokinin, is a synthetic cytokinin that has been widely used

in *Hedychium* micropropagation [7, 23, 28, 30, 35]. TDZ promotes strong cytokinin activity similar to that of N6 substituted adenine derivatives [42]. The efficient shoot morphogenesis from TDZ may be attributed to its ability to induce endogenous cytokinin accumulation [43]. TDZ is stable and biologically active even at low concentration [44] and its growth-promoting activity is accompanied by high acid-phosphate level [45]. With the ability of BA and TDZ to regenerate the shoots of *Hedychium* as described in previous reports, the possibility of using these PGRs to establish a cost-efficient micropropagation method of *H. longicornutum* was investigated.

In vitro propagation generates new plants through an asexual process (mitotic cell division), so variations should not occur [46]. However, some factors during tissue culture including prolonging culture, even under optimal culture conditions [47], or the presence of PGRs, may induce stress leading to genetic variation among the *in vitro* regenerated plants [46, 48]. Somaclonal variation may be considered as an obstructive and worthless phenomenon because it leads to loss of genetic fidelity [48]. Achievement of true-to-type and genetic stability propagules are major concerns of micropropagation [7, 15, 16] and are critical for commercial micropropagation [49] to maintain the essential characteristics of the mother plant. Therefore, assessment of the genetic fidelity of the regenerated plants after a large-scale *in vitro* propagation is essential for establishing a reliable micropropagation protocol. Clonal fidelity assessment of *in vitro* regenerated plants using morphological characters has limitations since these parameters are often influenced by environmental factors and do not always represent a change in the plant genetic [7]. On the contrary, molecular markers are not affected by environmental factor interference. In this study, random amplified polymorphic DNA (RAPD) was used as a molecular marker for evaluating genetic stability. Since RAPD is simple, fast, cost-effective, reliable, and does not require DNA sequence information [50], it has been used to assess genetic fidelity in various *in vitro* culture reports of *Hedychium* [15, 19, 22].

Plant tissue culture helps in producing large number of clonal plants, which is clearly evident in the micropropagation reports of various *Hedychium* species. However, there is no report on true-to-true type micropropagation for *H. longicornutum*. Therefore, this present study was carried out to establish an efficient *in vitro* propagation protocol for this rare ginger plant by comparing the effects of BA and TDZ on plant regeneration. Most importantly, the genetic fidelity of the regenerants was also investigated and compared with their mother plant. This study is the first report on micropropagation protocol and assessment of the genetic fidelity for *H. longicornutum*. This protocol can be adopted for germplasm conservation. Moreover, it will provide advantages for commercial cultivation, plant breeding, and molecular biology research of this ornamental plant in the future.

2. Materials and Methods

2.1 Surface sterilization of starting plant materials

Seeds of *H. longicornutum* were obtained from Narathiwat Province, Thailand (Figure 1B). Seeds were carefully washed with running tap water for 15 min, and then their surface were thoroughly scrubbed using a brush and commercial liquid disinfectant 'Teepol' (Sherwood Ltd., Thailand). Seeds were dipped into 70% (v/v) ethanol for 1 min, followed by 15% (v/v) and 10% (v/v) Clorox® (The Clorox Company, USA) for 20 min and 30 min, respectively. A 20 µl/100 ml solution of Tween®-20 (ICI Americas Inc., USA) was added into the surface sterilization solution. Seeds were transferred into a laminar airflow cabinet and rinsed with distilled water 3 times with 5 min of each rinse. The Clorox® contained 7.4% (v/v) sodium hypochlorite (NaOCl). Distilled water was sterilized by autoclaving at 121ºC for 20 min under 15 PSI.

2.2 Initiation of the *in vitro* clean cultures and multiplication of plant materials

Surface-sterilized seeds were cultured onto Murashige and Skoog (MS) medium [51] containing 30 g/l sucrose and solidified with 2 g/l gelrite (Sigma, USA). All media used in this research were adjusted to pH level of 5.7-5.8 and autoclaved at 121ºC for 15 min under 15 PSI. After 1 week of culture, the contamination rate of seeds was observed. Each culture vessel contained an individual seed or explant. Culture conditions throughout this research were 25±2ºC under a photoperiod of 16 h from a cool-white inflorescent light (Philips, Thailand) at 37 μmol/m^2/s intensity and 8 h dark.

When *H. longicornutum* seedlings had grown to 6-7 cm in height, they were excised to obtain leafy-shoot bases at 1.5 cm long. These explants were cultured onto MS medium supplemented with 8 μM BA (Sigma, USA) for 4 weeks, followed by transferring onto MS medium and culturing for 8 weeks (transferred to new medium at 4-week interval). This process was the basic method for multiplying plant materials used in the Plant Tissue Culture Laboratory, Department of Plant Science, Faculty of Science, Mahidol University. Multiplication of *in vitro* plant materials was done for 5 cycles to achieve an adequate quantity of plant materials for performing further experiments.

2.3 Effects of BA and TDZ on shoot proliferation

The 6-7 cm high *in vitro* young leafy-shoots were selected and their bases were excised into 1.5-2 cm long. The leafy-shoot bases were cultured either on MS medium alone (control treatment) or augmented with different concentrations of PGRs including 2, 4, 8, 15, 25, and 35 μM BA (Sigma, USA) or 0.5, 1, 2, 4, 8, 15, 25, and 35 μM TDZ (Sigma, USA) for 8 weeks (transferred to new medium at every 4 weeks).

Each treatment consisted of 20 explants/treatment and all the experiments were repeated 3 times (N = 60 explants/treatment). Data were recorded every 4 weeks in terms of rate of new shoot induction (%), new shoot number per explant, new shoot height (cm), and rate of new root induction (%). The induction rates of new leafy-shoots and roots in each treatment were calculated using the following formula: (Number of explants produced new leafy-shoots or roots/20) X 100. The number of new leafy-shoots and roots were counted from all regenerated leafy-shoots/roots in each culture vessel. Since the leafy-shoot part or aerial stem of the Zingiberaceous plant is a pseudostem (Larsen & Larsen, 2006), the new leafy-shoot height was measured from the base of the leafy-shoot to the longest leaf tip. The height of new leafy-shoot represented the longest new leafy-shoot in each culture vessel.

2.4 Rooting and acclimatization

Clusters of leafy-shoots were cultured on MS medium for rooting and adjusting leafy-shoots to be ready for growing *ex vitro*. Each culture vessel contained one shoot cluster. After culturing on MS medium for 8 weeks, the cluster of plants growing from the best shoot regeneration medium were carefully moved out of the culture vessels and their roots were washed to remove agar residuals. Then, they were planted on plastic nursery bags (7 cm in height × 12 cm in length) containing 3 different sets of planting substrates: 1) 2 garden soil mixture: 1 coconut husk chips, 2) 2 garden soil mixture: 1 smashed charcoal, and 3) 2 garden soil mixture: 1 coconut husk chips: 1 smashed charcoal. Each planting substrate contained 20 plants which was done 3 times (N = 60 plants/treatment). Each nursery bag was covered with a transparent plastic bag, kept in a shaded area (50% of sunshade), and watered once a week. Transparent plastic bags were removed and the survival rate of *in vitro* raised plants was recorded after 4 weeks of acclimatization. The survival rate for each treatment was calculated using this equation: (Number of survived plants/20) X 100.

After acclimatization, the survived *ex vitro* plants were transferred to a greenhouse with full sunlight conditions.

2.5 Assessment of genetic fidelity using RAPD

The genetic fidelity of the micropropagated plants and the field-grown mother plant (seeds donor plant) were checked using RAPD primers. Fresh tender leaves of the mother plant as well as ten randomly selected field-established *in vitro* raised plants were collected and kept in -20ºC prior to extraction of their genomic DNA. Total DNA was extracted from 0.15-0.20 g leaf samples using DNeasy® Plant Mini Kit (QIAGEN, Germany). Qualitative and quantitative assessment of total genomic DNA was verified by NanoDrop micro-UV/VIS spectrophotometer (Thermo Scientific, USA). Each DNA sample was diluted to a concentration of 25 ng/ml with buffer AE (QIAGEN, Germany) and stored at -20ºC. Eighty RAPD primers (OPA, OPC, OPD, and OPN series) were used to evaluate genetic fidelity. PCR analyses were carried out in a volume of 50 µl containing 2.0 µl of 25 ng template DNA, 5.0 µl of 10X Vi Buffer A (Vivantis, Malaysia), 1.5 µl of 1.5 mM MgCl$_2$ (Vivantis, Malaysia), 0.5 µl of 10 mM dNTPs (Vivantis, Malaysia), 1 µl of 200 nM primer, 0.4 µl of *Taq* DNA polymerase (Vivantis, Malaysia), and 39.6 µl of sterile 3B-UV water. Amplification was performed in the GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, USA) using the following temperature profile: 1 cycle of initial DNA denaturation (at 94°C for 5 min), 42 cycles of denaturation (at 94°C for 1 min), 1 cycle of primer annealing (at 37°C for 1 min), 1 cycle of elongation (at 72ºC for 2 min), and 1 cycle of the final extension (at 72ºC for 7 min). All PCR reactions were repeated at least 3 times to check their reproducibility. The amplified products were mixed with 6X ready-to-use loading dye (Vivantis, Malaysia) and analyzed on 1.5% agarose gel with DNA stain G (SERVA, Germany), in 0.5×TAE buffer at pH 8.0 (voltage 100 V, time 40 min). The gels were visualized and photographed using a gel documentation system (Syngene G Box, USA). The sizes of the amplicons were estimated comparing with a VC 100 bp Plus ready-to-use DNA ladder (Vivantis, Malaysia). Only consistently reproducible and clear fragments ranging from 100 to 3,000 bp were scored for analysis. In each gel electrophoresis photo, lane 1 referred to the mother plant, lanes 2-11 referred to micropropagated plants (randomly selected from different seed lines), and lane 12 referred to the negative control (a PCR master mix without template DNA).

2.6 Statistical analysis

Data were analyzed using analysis of variance (ANOVA) for a completely randomized design (CRD). Means values for significant effects were separated using Duncan's multiple range test (DMRT). Statistical analyzes in this study were performed on PASW Statistics 18.0 at a significance level of $p < 0.05$. Data were presented as mean±standard deviation (S.D.).

3. Results and Discussion

3.1 Surface sterilization of starting plant materials and initiation of the *in vitro* clean cultures

Various *Hedychium* parts/organs such as aerial stems [13, 25], immature filaments [29], leaf sheaths, and leaves [21] were used as starting plant materials during surface sterilization process. Plant parts from rhizomes including rhizome buds [13, 15, 27], young shoots [6, 30, 32], sprouted shoots [7, 8, 24], rhizome meristems [34] or rhizomes themselves [13, 26, 28, 37] were commonly used as explants for initiating the clean cultures in *Hedychium* because these explants showed high response

and regeneration rates. However, it is extremely difficult to disinfect rhizomes, and a high contamination rate of these explants after surface sterilization process was usually found [13]. This might be because the rhizomes had been in contact with soil that contained many soil-borne pathogens. Therefore, many surface sterilization reports of *Hedychium* rhizomes used mercuric chloride (HgCl$_2$) as a disinfectant. However, HgCl$_2$ is extremely toxic, mutagenic, and must be disposed of with care. Some plant materials of *Hedychium*, such as seeds, were disinfected by NaOCl or commercial disinfectants containing NaOCl [17, 18, 23, 33]. For vulnerable *H. longicornutum*, microbial infection made it hard to use a large number of rhizomes as plant materials, so its seeds were used in this study. One week after surface sterilization, no contamination was found, which was in agreement with Chuengpanya *et al*. [18]. This might be due to *Hedychium* seeds being more hygienic than rhizome because they had been in less contact with soil. Moreover, the hard seed coat of *Hedychium* can protect its embryo from high concentrations of disinfectants for a certain period of time. The seeds of *H. longicornutum* germinated after culturing in *in vitro* for 8 weeks. Several reports revealed that *Hedychium* seeds need time for germination. Therefore, some researchers used sulfuric acid (H$_2$SO$_4$) for seed scarification [17, 33] or added gibberellic acid (GA$_3$) into culture medium [16] to improve seed germination. In addition, other studies reported that plant extracts can act as bio-stimulants which improve seedling emergence in various plants [52, 53], and some plants have their seeds treated with plant extract to increase seed germination. For example, Beyaz *et al*. [54] found that treating *in vitro* seeds of *Brassica napus* with fruit juice from *Ecballium elaterium* increased its germination rate from 9.60% to 87.20%. As a consequence, further study on the effects of GA$_3$, seed scarification, and the effects of natural compounds from plant extracts on seed germination of *H. longicornutum* should be done to improve seed germination rate of this valuable ginger. Though seed germination procedure can be time-consuming, the method can generate a sufficient number of plants that can be easily applied for a large-scale cultivation, as described by Behera *et al*. [16].

In this study, there was a limitation in the number of starting seeds. Therefore, the numbers of seedlings were insufficient for use as explants in further study. Therefore, the multiplication of axenic plants was necessary. In fact, the aerial stems of *Hedychium* are pseudostems [3] which contain nodes and axillary buds that can generate into new shoots [13, 37]. The development of buds and nodes and bud break frequency of some *Hedychium* also depended on the maturity of pseudostems [13]. Therefore, the 12-week-old seedlings of *H. longicornutum* at the height of 6-7 cm were firstly selected as starting materials for multiplication. Their vigorous bases were excised to the size of 1.5-2 cm in height. New leafy-shoots regenerated directly from these areas. Thus, the height of pseudostems and leafy-shoot bases were used to determine the size of starting materials and explants throughout this study. The leafy-shoot base explants were considered as one of the most efficient explants in *in vitro* culture of some *Hedychium* [18, 23]. On the other hand, aerial stem segments [6, 7, 19, 25, 32], cotyledonary nodes [16, 36], explants originated from rhizomes [8, 15, 28, 34], protocorm-like bodies (PLBs) [13], shoot tips [20, 30], somatic embryos [17, 26, 33], whole young shoots [24, 27, 35, 37], and zygotic embryos [31] were used in the other reports as explants. The advantage of leafy-shoot base explants is that they can be repeatedly sub-cultured for upscaling shoot number, and they are easy and fast to excise, resulting in the achievement of an efficient micropropagation protocol for *H. longicornutum* with less complications.

3.2 Effects of BA and TDZ on shoot proliferation

Every explant in all treatments generated new leafy-shoots after 4 weeks of culture, and each treatment showed different leafy-shoot formation. In the control treatment (0 μM PGRs), newly leafy-shoots had the number and height of 1.95 shoots/explant and 3.09 cm, respectively. This treatment generated more shoots (3.10 shoots/explant) and greater height (5.04 cm) of leafy-shoots

after 8 weeks of culture (Table 1, Figures 2A and 2B). These results were in agreement with several reports on *Hedychium* micropropagation in which culturing explants onto basal medium without PGRs could generate new shoots [6, 8, 16, 18, 19, 23, 30]. However, *H. ellipticum* [18] and *H. spicatum* [35] failed to generate new shoots on the control treatment. These different responses might be due to differences in the genetic background of each plant species. Possible reasons for shoot regeneration from *H. longicornutum* leafy-shoot bases on the control treatment might be: (a) the axillary buds at the leafy-shoot base broken from dormancy due to the influence of the shoot apex, and (b) the explants contained sufficient level of nutrients, endogenous plant hormones, and biochemical compounds for developing axillary buds into new shoots.

Explants cultured on BA supplemented MS medium produced new leafy-shoots at 2.05-3.90 shoots/explant with heights of 3.13-4.88 cm after 4 weeks of culture. The maximum number and height of regenerated leafy-shoots among the BA treatments at the 4th week of culture were obtained from MS + 15 µM and 25 µM BA (Table 1, Figure 2A). At the 8th week of culture, BA treatments produced new leafy-shoots number at 3.35-9.75 shoots/explant with 4.94-9.54 cm of new leafy-shoot height. BA at 15 µM showed the greatest leafy-shoot regeneration (9.75 shoots/explant, 9.54 cm) (Table 1, Figure 2B). Outcomes from the entire 8 weeks of culture indicated that BA treatments resulted in significantly higher number and greater length of *H. longicornutum* new leafy-shoots (except shoot height from 2 µM BA) than the control treatment. These outcomes related to the findings of some researchers on *Hedychium* micropropagation who found that using BA alone sufficiently induced multiple shoots [6, 18, 24]. This may be because BA provides cytokinin activities for increasing shoot formation by involving in enhancement of endogenous cytokinin accumulation [39], withdrawal from bud dormancy, breaking of apical dominance, stimulation of cell division, control of morphogenesis and stem elongation [14], and improvement of photosynthesis [40]. However, not all concentrations of BA produce such effects. BA concentrations higher than 15 µM reduced leafy-shoot formation (Table 1, Figure 2B). Several studies also reported that using BA level higher than its optimum level resulted in lower number and height of regenerated shoots [7, 16, 18, 20, 23, 26, 28, 30, 32, 35]. Therefore, instead of stimulating cell division, BA blocked cell proliferation at high concentrations [55] which caused a reduction of shoot regeneration [14]. Moreover, cytokinin elevates ethylene biosynthesis by upregulating 1-aminocyclopropane-1-carboxylate (ACC) synthase [55]. Saha *et al.* [56] noted that a higher amount of ethylene was released in BA supplemented medium of *Lagenaria siceraria* culture, so it is possible that if the concentration of BA in culture medium increases, more ethylene will be produced. Endogenous ethylene production by explants in culture vessels led to less shoot elongation [57].

Since TDZ, a phenylurea cytokinin, is biologically active even at low concentrations [44]; the studied concentrations of TDZ in this study ranged from 0.5 µM to 35 µM. Data from the 4th and 8th weeks of culture found that TDZ treatments provided new leafy-shoots at 3.35-4.50 and 4.80-17.90 shoots/explant, respectively. TDZ at 8 µM produced leafy-shoots number of 4.50 after 4 weeks of culture and 17.90 shoots/explant at the 8th week, which were the highest shoot numbers among all treatments. These new leafy-shoots had the heights of 1.42-2.28 cm after 4 weeks of culture and 1.89-4.33 cm after 8 weeks of culture. TDZ at 0.5 µM provided the highest height of new leafy-shoots among the TDZ treatments (Table 1, Figures 3A and 3B). As with BA, using TDZ at high concentrations decreased both shoot number and height of *H. longicornutum* shoots, a result that was similar to previous *Hedychium* micropropagation reports [7, 16, 23, 28, 30, 35]. Comparing the regeneration shoot ability of BA and TDZ at the same concentration, TDZ provided leafy-shoots in higher number than BA at all concentrations, i.e. 17.90 shoots/explant from 8 µM TDZ compared to 3.85 shoots/explant with 8 µM BA. Higher numbers of new leafy-shoots by TDZ in this study possibly resulted from TDZ having higher cytokinin activities than adenine derivatives [42]. Besides the effect of TDZ on endogenous cytokinins accumulation [43], TDZ acts as an inhibitor of various cytokinin oxidases, causing extremely slow metabolism of TDZ [58], and therefore, TDZ is more

Table 1. *In vitro* shoot regeneration from leafy-shoot bases of *H. longicornutum* on MS medium alone or supplemented with various concentrations of BA and TDZ

PGRs (µM)	New shoots numbers/explant		New shoot height (cm)	
	4 weeks	8 weeks	4 weeks	8 weeks
0 (control)	1.95±0.51[F]	3.10±0.45[H]	3.09±0.68[D]	5.04±0.66[F]
BA 2	2.05±0.60[F]	3.35±0.93[H]	3.13±1.33[D]	4.94±1.07[F]
BA 4	2.35±0.75[F]	3.45±0.51[H]	3.57±0.75[C]	5.57±0.81[E]
BA 8	3.10±0.97[E]	3.85±0.75[GH]	3.98±1.06[B]	6.19±0.90[D]
BA 15	3.90±0.79[BC]	9.75±1.71[D]	4.03±0.52[B]	9.54±1.01[A]
BA 25	3.50±0.5[CDE]	8.35±1.27[E]	4.88±0.75[A]	8.99±0.90[B]
BA 35	3.10±0.45[E]	8.05±0.69[E]	4.60±0.90[A]	7.39±0.72[C]
TDZ 0.5	4.25±0.91[AB]	4.80±1.28[G]	2.28±0.36[E]	4.33±0.83[G]
TDZ 1	3.35±0.75[DE]	6.25±1.33[F]	2.25±0.20[E]	4.17±0.63[G]
TDZ 2	3.85±0.99[BCD]	6.85±1.84[F]	2.00±0.24[E]	2.46±0.33[H]
TDZ 4	4.45±0.60[A]	14.35±2.03[B]	1.57±0.17[F]	2.12±0.20[HI]
TDZ 8	4.50±0.95[A]	17.90±3.68[A]	1.49±0.21[FG]	2.03±0.37[HIJ]
TDZ 15	3.85±0.75[BCD]	11.95±1.43[C]	1.46±0.26[FG]	1.96±0.22[IJ]
TDZ 25	3.75±0.79[BCD]	11.40±1.93[C]	1.42±0.28[FG]	1.89±0.28[IJ]
TDZ 35	3.60±0.50[CDE]	9.25±3.70[DE]	1.09±0.21[G]	1.62±0.32[J]

Values represent means ± S.D. In a column, different letters in superscripts indicate statistically significant differences between the means (DMRT at $p<0.05$).

stable [44] in plant tissue. Furthermore, the main metabolite products of TDZ are O-glucosides which resist cytokinin oxidases and can be converted back to active cytokinins [58]. Conversely, 9-glucoside, a main metabolites product of BA, is converted into inactive or irreversible forms of cytokinins [59]. Various micropropagation studies of *Hedychium* have also shown that TDZ showed superior effect on multiple shoot induction over BA [7, 16, 23, 28, 30, 35]. However, the regenerated leafy-shoots of *H. longicornutum* from TDZ were shorter than BA in all treatments (Table 1, Figures 3A and 3B), which also related to several *in vitro* propagation reports on *Hedychium* [28, 30, 35]. TDZ causing reduction in shoot elongation may be due to: (a) its high cytokinin activity, (b) the presence of a phenyl group in TDZ being a possible cause of shoot bud fasciation, and (c) its relationship with gibberellin metabolism [49]. In addition, the morphological characters of regenerated leafy-shoots of *H. longicornutum* from TDZ treatments were different from the control and BA treatments. New leafy-shoots from the control and BA treatments showed similar morphology whereas shoots from 0.5-1 µM TDZ showed less elongation of shoots, and shoots from 2-35 µM TDZ were stunted and had swollen bases (Figure 4A). This was the first *Hedychium* micropropagation report that revealed abnormal morphology of shoots (swollen base) caused by TDZ. TDZ at higher concentrations than 2.0 µM induced swollen shoot bases in various plant species, as described by Dewir *et al*. [49]. Possible reasons for swollen morphology may be because TDZ led to disorganized vascular bundles and TDZ induced expression of 1-aminocyclopropane-1-carboxylate synthase (ACS), resulting in enhanced ethylene accumulation that later affected shoot morphology [49].

Figure 2. Shoot and root production by culturing leafy-shoot bases of *H. longicornutum* onto MS medium alone or MS medium supplemented with various concentrations of BA (µM) for 4 (A) and 8 weeks (B), followed by transferring regenerated shoots onto MS medium for another 8 weeks (C)

Figure 3. Shoot and root production by culturing leafy-shoot bases of *H. longicornutum* onto MS medium supplemented with various concentrations of TDZ (µM) for 4 (A) and 8 weeks (B), followed by transferring regenerated shoots onto MS medium for another 8 weeks (C)
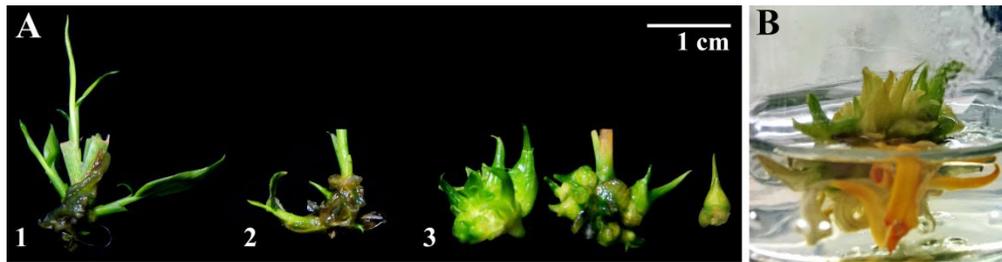
Figure 4. Effects of BA and TDZ on the shoots of *H. lomgicornutum*: morphological comparison of regenerated leafy-shoots grown from control/BA treatments (A1), 0.5-1 µM TDZ (A2), and 2-35 µM TDZ (A3), *in vitro* flowering from 25 or 35 µM TDZ (B)

3.3 Rooting and acclimatization

During the 4th week of culture, some treatments spontaneously rooted. Shoots from the control treatment rooted at 90%. Meanwhile, shoots from all BA treatments rooted from 73.33% in 35 µM to 100% in 2 µM. In contrast to BA treatments, explants cultured on 0.5-2 µM TDZ developed roots with the rates of 15-46.67%. Other TDZ treatments failed to root (Table 2, Figures 2B and 3A). Roots became more developed after the 4th week. Every treatment generated new roots but at different regeneration rates. Shoots from the control and all BA treatments exhibited rooting at 100%, while TDZ treatments produced rooting rates of 21.67% in 35 µM to 78.33% in 0.5 µM (Table 2, Figures 2B and 3B). Prior *in vitro* propagation reports also found roots developed simultaneously during shoot multiplication in culture medium presence with cytokinin [7, 8, 15, 16, 18, 23, 30, 32]. Generally, cytokinin acts as an auxin antagonist, so it can block or delay root initiation [14]. This study found that roots spontaneously formed from all treatment media over 8 weeks. So, it is possible that the concentrations of BA and TDZ in this research were not intense enough to completely inhibit root germination. However, high cytokinin concentration can reduce root formation [14] as was found in this study when TDZ concentration were increased. The effect of increasing cytokinin concentrations on reducing or inhibiting root formation was found in several micropropagation reports of this genus [7, 16, 18, 32]. Since BA and TDZ at 2-35 µM did not inhibit root formation of *H. longicornutum*, the PGRs-free MS medium was used to enhance root development in order to obtain the complete plantlets ready for transplantation. George *et al*. [14] indicated that cytokinin is usually omitted from culture media when shoots are enabled to root and complete plantlets are subsequently achieved. Then, subculture of shoots in a cytokinin-free medium to sufficiently reduce cytokinin levels is another method for *in vitro* rooting. Some *in vitro* propagation reports of *Hedychium* also used PGRs-free MS medium for promoting more root development [23, 37]. More stem elongation along with more rooting and complete plantlets were obtained after transferring the regenerated leafy-shoots from BA treatments onto PGRs-free MS medium for 8 weeks (Figure 2C). Moreover, these plantlets were ready to be transplanted *ex vitro*. These occurrences indicated that the effects of BA supplementation on leafy-shoots of *H. longicornutum* were sufficiently reduced.

The regenerated leafy-shoots of *H. longicornutum* from TDZ treatments were also cultured onto PGRs-free MS medium for promoting shoot elongation and more root regeneration. However, all TDZ treatments showed poor root development. Less shoot elongation was observed on 0.5-1 µM TDZ, nevertheless, most of the shoots from 2-35 µM did not elongate and their bases were still swollen (Figure 3C). These incidents indicated that culturing regenerated shoots from TDZ onto PGRs-free MS medium for 8 weeks may not sufficiently decrease TDZ content inside *H. longicornutum* shoots due to TDZ being stable [44] and degrading slowly [58]. Therefore,

regenerated shoots from TDZ may require prolonged culturing on PGRs-free MS medium for more than 8 weeks. Similar to this study, some *Hedychium* micropropagation reports indicated problems in shoot elongation [7, 28] and rooting [30] after multiple shoots induction on basal medium with TDZ. Stunted shoots are not desired for micropropagation due to the difficulty in isolating individual shoots from multiple shoots, which increases the labor, time, and the extra subcultures or processes needed for stimulating shoot elongation and growth [49]. Previous *Hedychium* micropropagation studies showed different strategies to overcome or prevent the TDZ-induced inhibition of shoot elongation, such as addition of shoot elongation process after large-scale propagation by GA₃ [7], or the inclusion of other cytokinins with TDZ during *in vitro* propagation [13, 15, 22]. The carryover effects of TDZ on the low rooting ability when using TDZ for shoot multiplication can be overcome by adjusting the appropriate type and concentrations of auxins during rooting process [49]. Verma and Bansal [28] obtained normally-developed roots when the regenerated shoots of *H. coronarium* from TDZ were transferred to a rooting medium containing 1.0 mg/l NAA. With the abnormalities resulting from TDZ treatments and the requirement of additional steps or PGRs to overcome the problems that increase cost, labor, and time when compared to BA, TDZ is not suitable for use as PGR during micropropagation of *H. longicornutum*. It can be noted that TDZ may cause abnormalities in *H. longicornutum* even at very low concentrations (0.5 µM). Regenerated shoots from 25 µM and 35 µM TDZ treatments had flowering at rates of 3.33% and 8.33% after culturing their shoots onto PGR-free MS medium for 6 weeks (Figure 4B). The effects of TDZ on flower induction *in vitro* were also found in various plant species [49]. Exogenous cytokinins alter the level of endogenous PGRs in shoot apical meristems (SAM), resulting in the transition of SAM from the vegetative to the floral state [60], and these processes might have occurred when TDZ was applied to the explants of *H. longicornutum*.

Though BA treatments produced new leafy-shoot number of *H. longicornutum* lower than TDZ treatments, shoots regenerated from BA did not have the same abnormalities as TDZ. Therefore, a reliable micropropagation method for this valuable plant in a short time with low cost and labor be obtained by BA. Moreover, shoots cultured in BA treatments spontaneously rooted, so the inclusion of auxin during the rooting step was unnecessary in this study, and the cost of micropropagation protocol could be saved [7, 8, 16]. MS medium supplemented with 15 µM BA was considered as the suitable medium for *in vitro* propagation of *H. longicornutum* because this medium exhibited the highest number of new shoots among all BA treatments with low adverse effects on shoot height and root regeneration (Tables 1, 2 and Figure 2). Grzegorczyk-Karolak *et al*. [61] also reported that BA was more suitable than TDZ during micropropagation of *Scutellaria alpina*. BA alone induced a sufficient number of multiple shoots of *H. longicornutum*; however, some micropropagation studies with *Hedychium* showed the production of multiple shoots using BA with added auxins [8, 13, 20, 25, 31, 32, 37] or organic additives [27].

In vitro raised plants grown from different culture media exhibited different survival rates after planting *ex vitro* [62]. Therefore, only *in vitro* plantlets from the best shoot multiplication medium of this study, 15 µM BA, were used for acclimatization process. Environments of *in vitro* and *ex vitro* culture are dramatically different, especially in terms of humidity and light intensity. *In vitro* culture normally involves high humidity. This condition slows down the development of leaf cuticles and the functioning of stomata. These retardations cause high water loss when *in vitro* raised plants are taken out of the culture vessels and later leads to high mortality rate during acclimatization. Plant tissue culture usually uses low light intensity (1,200-3,000 lux); therefore, the transfer of *in vitro* plantlets to *ex vitro* conditions under direct sunlight (4,000-12,000 lux) can cause photoinhibition and chlorophyll photobleaching [63]. As a consequence, nursery bags for acclimatizing *in vitro* plantlets of *H. longicornutum* were covered with transparent plastic bags to ensure high humidity and kept in a shaded area (50% of sunshade) during acclimatization. These conditions allow the development of leaf cuticles and gradually restore stomatal function and photosynthesis. Various reports on *Hedychium* micropropagation also used similar methods to this

study during transplantation of *in vitro* raised plants [7, 8, 16, 19, 35, 37]. The substratum is another crucial parameter that affects the survival rate of *in vitro* plants during acclimatization [63]. The substratum used during transplantation of *Hedychium* varied greatly across the research and each substratum exhibited different survival rates of acclimatized plants. Parida *et al*. [15] grew *in vitro* *H. coronarium* plants on soil, cow dung, and sand mixture in a 1:1:1 ratio and obtained a rate of survival of 80% while Mohanty *et al*. [8] achieved a survival rate of *H. coronarium* at 100% when they used 2 garden soil mixture: 1 sand as substratum. In this study, three sets of planting substrates were used for transplantation of *H. longicornutum* and survival rates of 85-100% were obtained (Table 3, Figure 5). The high survival rate of acclimatized plants in this study might be due to the *in vitro* plants had their shoots and roots generated in the same medium at the same time, leading to the establishment of a proper anatomical connection between the shoots and roots of the *in vitro* plants before acclimatization [8]. However, only the substratum containing 2 garden soil mixture: 1 smashed charcoal provided a survival rate of acclimatized plants at 100%. It is possible that this planting substrate supplies plenty of organic substances for plant growth, allows roots to spread, and provides good water and air drainage. The addition of coconut husk chips into the substratum reduced the survival rate of acclimatized *H. longicornutum*, which might be related to the higher moisture-holding capacity and lower water drainage ability of the coconut husk chips when compared to smashed charcoal.

Table 2. *In vitro* root regeneration rate (%) using leafy-shoot bases of *H. longicornutum* on MS medium alone or supplemented with various concentrations of BA and TDZ

PGRs (µM)	4 weeks	8 weeks
0 (control)	90.00±10.00^A	100^A
BA 2	100^A	100^A
BA 4	96.67±5.77^A	100^A
BA 8	95.00±5.00^A	100^A
BA 15	93.33±11.55^A	100^A
BA 25	76.67±7.64^B	100^A
BA 35	73.33±17.56^B	100^A
TDZ 0.5	46.67±12.58^C	78.33±2.89^B
TDZ 1	28.33±2.89^D	70.00±5.00^C
TDZ 2	15.00±5.00^E	55.00±5.00^D
TDZ 4	0^F	48.33±2.89^E
TDZ 8	0^F	45.00^E
TDZ 15	0^F	36.67±2.89^F
TDZ 25	0^F	26.67±2.89^G
TDZ 35	0^F	21.67±5.77^H

Values represent means ± S.D. In a column, different letters in superscripts indicate statistically significant differences between the means (DMRT at *p*<0.05).

Table 3. Effect of different planting substrates on survival rate of *H. longicornutum* after 4 weeks of acclimatization

Planting substrates	Survival rate (%)
2 garden soil mixture: 1 coconut husk chips	85.00±5.00[C]
2 garden soil mixture: 1 smashed charcoal	100[A]
2 garden soil mixture: 1 coconut husk chips: 1 smashed charcoal	91.67±2.89[B]

Values represent means ± S.D. In a column, different letters in superscripts indicate statistically significant differences between the means (DMRT at $p<0.05$).

Figure 5. Transplantation processes of *H. longicornutum*: *in vitro* raised plants were cultivated on plastic bags containing different planting substrates before being covered with transparent plastics bags and kept in a shaded area (A), acclimatized plants (B).

3.4 Assessment of genetic fidelity using RAPD

In vitro new shoots of *Hedychium* could be induced through direct organogenesis [6-8, 15, 16, 18, 19, 20, 22-25, 27, 28, 30-32, 34, 35, 37], indirect somatic embryogenesis [17, 21, 26, 29, 33], direct somatic embryogenesis [36], and protocorm like bodies (PLBs) [13]. Each regeneration pathway depended on explant source or type and concentration of PGRs that were used during *in vitro* culture. Plant regeneration through callus mediated somatic embryogenesis or direct somatic embryogenesis could produce somaclonal variation [46]. Wang *et al.* [29] noted that some regenerated shoots of *H. coronarium via* indirect somatic embryogenesis had ploidy levels as triploid, tetraploid, and hexaploid whereas most of the regenerated plants were diploid. To avoid the genetic variation, this study used leafy-shoot base that were organized meristematic explants, with the appropriate concentrations of cytokinins to induce new shoots of *H. longicornutum* directly from the explants. After the acclimatization process, every micropropagated plantlets of *H. longicornutum* showed similar morphology and was also similar to the mother plant. However, *in vitro* culture environments can be mutagenic [46-48] and causes changes to the genetic materials of *in vitro* shoots without revealing any morphological variations [7]. Therefore, genetic fidelity confirmation of micropropagated plants is necessary to assure that the established protocol is reliable.

Genetic fidelity assessment between *in vitro* raised plants and field grown mother plants of *H. longicornutum* was done by RAPD primers. Out of 80 RAPD primers, 31 primers generated reproducible and clear bands, with the band number for each primer varying from 2 to 8 and an average of 4.61 bands per RAPD primer ranging from 250 to 2,500 bp (Table 4). The number of bands were the highest, i.e. 8 in primer OPN-09 and OPN-10, and lowest, i.e. 2 in primer OPA-10, OPA-18, OPC-15, OPD-1. A total of 4,719 bands [(number of plantlets analyzed = 11) x (number of bands with all primers = 143) x (times repeated of PCR = 3)] were generated, and all bands showed monomorphic patterns across all *H. longicornutum* comparing between the mother plant and micropropagated plants (Figure 6). These outcomes confirmed the genetic stability of the *in vitro* raised plants. Moreover, the micropropagation protocol for *H. longicornutum* established in this study is reliable even though the plants were cultured under *in vitro* conditions for 96 weeks (20 weeks of seed germination, 60 weeks of plant material multiplication, 8 weeks of multiple shoot induction, and 8 weeks of rooting). Behera *et al*. [19] and Parida *et al*. [15] reported the monomorphic patterns evaluated by RAPD primers of *in vitro* regenerated plants *via* direct organogenesis of *H. coronarium*. Mohanty *et al*. [22] revealed that shoots of *H. coronarium* regenerated through direct organogenesis were genetically uniform according to RAPD results even though the plants were preserved under *in vitro* conditions for 30 months. Regeneration of shoot through seed may result in genetic variation in progenies [13]. However, Behera *et al*. [16] used inter simple sequence repeats (ISSR) primers to check genetic uniformity of the plants, and no genetic variation in the progenies of *H. coronarium* micropropagated from different seeds was found. Furthermore, micropropagated plants through seeds also showed genetic and biochemically fidelity to the mother plant. With a similar RAPD banding profile of the mother and micropropagated plants, this study succeeded in establishing an efficient *in vitro* true-to-true type propagation protocol for *H. longicornutum*.

4. Conclusions

An efficient true-to-true type micropropagation protocol for *H. longicornutum* was investigated. New leafy-shoots of this valuable plant were generated through a direct organogenesis pathway. This protocol required at least 40 weeks (20 weeks for seed germination, 8 weeks of multiple shoot induction, 8 weeks for rooting, 4 weeks for acclimatization) to promote the vigorous plants. Optimum result of shoot regeneration with root simultaneously was observed when *in vitro* leafy-shoot bases were cultured on MS medium supplemented with 15 µM BA. Shoots developed more roots after translocating the shoot clusters onto PGR-free MS medium. The regenerated plants grew well in a substratum containing 2 soil mixture: 1 smashed charcoal, and showed 100% survival rate after acclimatization. All of the plants were the same genetically clones as the mother plant as ascertained by RAPD analysis. This micropropagation protocol of *H. longicornutum* may be beneficial for mass-rapid production of clonal plants to be used for conservation, commercial use in the ornamental plant industry, and can be applied for further studies in plant breeding, genetic engineering, and molecular biology.

5. Acknowledgements

Table 4. List of primers and their sequences, total number of bands and size of amplified products generated by RAPD primers in both field grown mother plants and micropropagated plants of *H. longicornutum*

| Primer	Sequence (5' to 3')	Total bands	Range of amplicons (bp)
OPA-09	GGGTAACGCC	5	450–1800
OPA-10	GTGATCGCAG	2	500–1500
OPA-13	CAGCACCCAC	4	700–1600
OPA-15	TTCCGAACCC	5	600–1600
OPA-17	GACCGCTTGT	5	500–1600
OPA-18	AGGTGACCGT	2	500–650
OPC-04	CCGCATCTAC	7	350–1800
OPC-08	TGGACCGGTG	4	400–1800
OPC-09	CTCACCGTCC	6	1000–2400
OPC-11	AAAGCTGCGG	7	300–2500
OPC-13	AAGCCTCGTC	6	650–2250
OPC-15	GACGGATCAG	2	650–900
OPC-19	GTTGCCAGCC	6	450–1100
OPD-01	ACCGCGAAGG	2	900–2300
OPD-02	GGACCCAACC	7	750–1900
OPD-15	CATCCGTGCT	3	800–1300
OPD-16	AGGGCGTAAG	2	500–800
OPD-18	GAGAGCCAAC	3	650–1250
OPD-20	ACCGGTCAC	4	600–1900
OPN-02	ACCAGGGGCA	4	350–1800
OPN-03	GGTACTCCCC	4	350–1300
OPN-04	GACCGACCCA	4	650–1300
OPN-05	ACTGAACGCC	6	300–2000
OPN-06	GAGACGCACA	6	650–2000
OPN-07	CAGCCCAGAG	3	900–1600
OPN-08	ACCTCAGCTC	5	500–1900
OPN-09	TGCCGGCTTG	8	300–1900
OPN-10	ACAACTGGGG	8	250–2000
OPN-11	TCGCCGCAAA	5	350–2750
OPN-16	AAGCGACCTG	5	320–1500
OPN-20	GGTGCTCCGT	3	700–1250
Total		143	
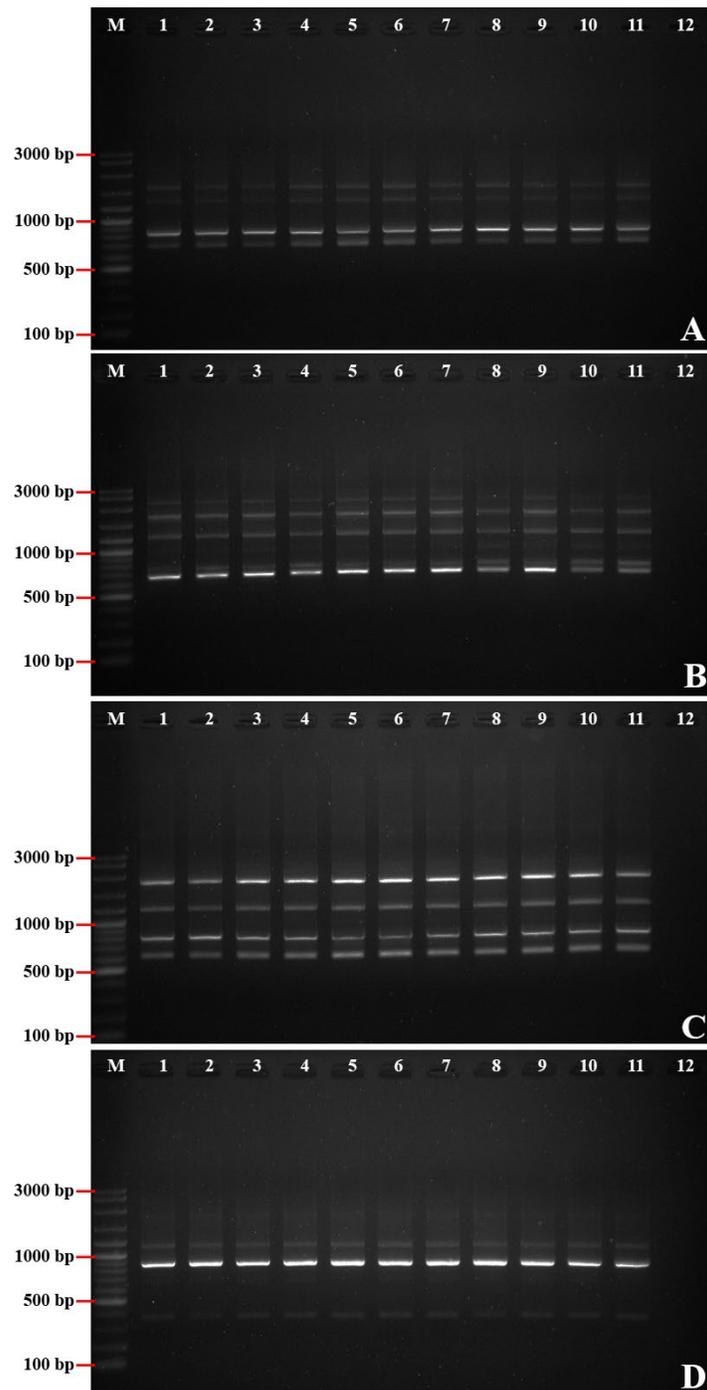
Figure 6. RAPD banding profiles of mother plant and micropropagated plants of *H. longicornutum* using RAPD primers: OPA 13 (A), OPC 13 (B), OPD 20 (C) and, OPN 2 (D). M is the marker (3 k bp), lane 1 is mother plant, lanes 2 -11 are micropropagated plants, and lane 12 is a negative control.

References

[1]   Royal Botanic Gardens Kew, 2018. *Hedychium J.Koenig*. [online]. Available at: http://plantsoftheworldonline.org/taxon/urn:lsid:ipni.org:names:30080711-2.

[2]   Sakhanokho, H.F. and Rajasekaranb, K., 2010. Pollen biology of ornamental ginger (*Hedychium* spp. J. Koenig). *Scientia Horticulturae*, 125, 129-135.

[3]   Larsen, K. and Larsen, S., 2006. *Ginger of Thailand*. Chiang Mai: Queen Sirikit Botanic Garden, the Botanical Garden Organization.

[4]   Eusebio, J.E. and Umali, B.E., 2004. Inventory, documentation and status of medicinal plants research in Philippines. In: P.A. Batugal, J. Kanniah, L.S. Young and J.T. Oliver, eds., *Medicinal Plants Research in Asia, Vol. 1: The Framework and Project Workplans*, Selangor: International Plant Genetic Resource Institute-Regional office for Asia, the Pacific and Oceania (IPGRI-APO), pp.147-183.

[5]   Tavares, W.R., Barreto, M.D.C. and Seca, A.M.L., 2020. Uncharted source of medicinal products: the case of the *Hedychium* genus. *Medicines*, 7(23), https://doi.org/10.3390/medicines7050023.

[6]   Rodpradit, S., Songnun, K. and Shusuwanaruk, K., 2017. *In vitro* microrhizome induction in *Hedychium stenopetalum* Lodd. *Acta Horticulturae*, 1167, 163-168.

[7]   Behera, S., Kamila, P.K., Rout, K.K., Barik, D.P., Panda, P.C. and Naik, S.K., 2018. An efficient plant regeneration protocol of an industrially important plant, *Hedychium coronarium* J. Koenig and establishment of genetic and biochemical fidelity of the regenerants. *Industrial Crops and Products*, 126, 58-68.

[8]   Mohanty, P., Behera, S., Swain, S.S., Barik, D.P. and Naik, S.K., 2013. Micropropagation of *Hedychium coronarium* J. Koenig through rhizome bud. *Physiology and Molecular Biology of Plants*, 19(4), 605-610.

[9]   Chamchumroon, V., 2017. *Threatened Plants in Thailand*. Bangkok: Department of National Parks, Wildlife and Plant Conservation.

[10]   Sirirugsa, P., 1998. Thai Zingiberaceae: species diversity and their uses. *Pure and Applied Chemistry*, 70, 2111-2118.

[11]   Hodgkison, R., Balding, S.T., Akbar, Z. and Kunz, T., 2003. Roosting ecology and social organization of the spotted-winged fruit bat, *Balionycteris maculata* (Chiroptera: Pteropodidae), in a Malaysian lowland dipterocarp forest. *Journal of Tropical Ecology*, 19, 667-676.

[12]   Olander, S.B., 2020. *Hedychium longicornutum. The IUCN Red List of Threatened Species 2020*. [online] Available at: https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T117356150 A1242 83162.en.

[13]   Hu, X., Tan, J., Chen, J., Li, Y. and Huang, J., 2020. Efficient regeneration of *Hedychium coronarium* through protocorm-Like Bodies. *Agronomy*, 10(8), 1068, https://doi.org/10.3390/agronomy10081068.

[14]   George, E.F., Hall, M.A. and De-Klerk, G.J., 2008. *Plant Propagation by Tissue Culture*. 3rd ed. Dordrecht: Springer.

[15]   Parida, R., Mohanty, S. and Nayak, S., 2013. *In vitro* propagation of *Hedychium coronarium* Koen. through axillary bud proliferation. *Plant Biosystems*, 147(4), 905-912.

[16]   Behera, S., Kar, S.K., Rout, K.K., Barik, D.P., Panda, P.C. and Naik, S.K., 2019. Assessment of genetic and biochemical fidelity of field-established *Hedychium coronarium* J. Koenig regenerated from axenic cotyledonary node on meta-topolin supplemented medium. *Industrial Crops and Products*, 134, 206-215.

[17]   Sakhanokho, H.F., Kelly, R.Y. and Rajasekaran, K. 2009. Somatic embryogenesis in *Hedychium bousigonianum*. *HortScience*, 44, 1487-1490.

[18] Chuengpanya, R., Karoojee, S., Lekbamroong, M., Muangkroot, A. and Chuenboonngarm, N., 2020. Effect of N^6-benzyladenine on *in vitro* propagation of *Hedychium coccineum* Buch.-Ham. ex Sm. and *Hedychium ellipticum* Buch.-Ham. ex Sm. *Srinakharinwirot Science Journal*, 36(2), 91-107.

[19] Behera, S., Rout, K.K., Panda, P.C. and Naik, S.K., 2020. Production of non-embryogenic synthetic seeds for propagation and germplasm transfer of *Hedychium coronarium* J. Koenig. *Journal of Applied Research on Medicinal and Aromatic Plants*, 19, https://doi.org/10.1016/ j.jarmap.2020.100271.

[20] Bisht, S., Bisht, N.S. and Bhandari, S., 2012. *In vitro* plant regeneration from seedling explants of *Hedychium coronarium* J. Koenig. *Journal of Medicinal Plants Research*, 6(43), 5546-5551.

[21] Huang, P.L. and Tsai, C.C., 2002. Micropropagation of *Hedychium coronarium* Koenig. *via* somatic embryogenesis. *Journal of the Chinese Society for Horticultural Science*, 48, 239-246.

[22] Mohanty, S., Parida, R., Sahoo, S. and Nayak, S., 2014. *In vitro* conservation of nine medicinally and economically important species of Zingiberaceae from Eastern India. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, 84(3), 799-803.

[23] Rungjindamai, C., Thammasiri, K., Chuenboonngarm, N. and Jenjittikul, T., 2014. Micropropagation of *Hedychium coronarium*. *Acta Horticulturae*, 1025, 223-229.

[24] El-Sadat, N., Soliman, A. and El-Zeiny, O., 2020. The effect of plant growth regulators on shoot formation from corm explant of *Hedychium coronarium*. *Scientific Journal of Flowers and Ornamental Plants*, 7(4), 401-407.

[25] Tu, H.Y., Zhang, A.L., Xiao, W. Lin, Y.R., Shi, J.H., Wu, Y.W., Wu, S.T., Zhong, C.H. and Mo, S.X., 2018. Induction and identification of tetraploid *Hedychium coronarium* through thin cell layer culture. *Plant Cell, Tissue and Organ Culture*, 135, 395-406.

[26] Verma, M. and Bansal, Y.K., 2012. Induction of somatic embryogenesis in endangered butterfly ginger *Hedychium coronarium* J. Koenig. *Indian Journal of Experimental Biology*, 50, 904-909.

[27] Verma, M. and Bansal, Y.K., 2013. Effect of additives on plant regeneration in *Hedychium coronarium* J. Koenig an endangered aromatic and medicinal herb. *International Journal of Pharmaceutical Sciences Review and Research*, 23, 105-110.

[28] Verma, M. and Bansal, Y.K., 2014. Effect of a potent cytokinin thidiazuron (TDZ) on *in vitro* regeneration of *Hedychium coronarium* J. Koenig - a valuable medicinal plant. *International Journal of Recent Biotechnology*, 2(1), 38-44.

[29] Wang, X., Hongyan, T. and Ailing, Z., 2016. Embryogenic callus induction and plant regeneration from *Hedychium coronarium via* somatic embryogenesis. *Acta Horticulturae Sinica*, 43, 1605-1612.

[30] Klaharn, P., Pumisutapon, P., Songnun, K. and Rodpradit, S., 2019. Effects of BA and TDZ for *in vitro* shoot multiplication of three *Hedychium* species. *Acta Horticulturae*, 1298, 353-358.

[31] Xiong, Y.H., Ma, G.H., Liu, N. and Huang, B.H., 2005. Tissue culture and rapid propagation of zygotic embryo of *Hedychium forrestii*. *Guihaia*, 25(3), 24l-244.

[32] Dohling, S., 2012. *In vitro* propagation of *Hedychium gardnerianum* Sheppard ex Ker Gawl., an important ornamental plant. *Keanean Journal of Science*, 1, 31-35.

[33] Sakhanokho, H.F., Kelly, R.Y. and Rajasekaran, K., 2008. First report of plant regeneration *via* somatic embryogenesis from shoot apex derived callus of *Hedychium muluense*. *Journal of Crop Improvement*, 21, 191-200.

[34] Tripathi, B.K. and Bitaillon, C., 1985. *In vitro* plant regeneration of *Hedychium roxburghii* blume through rhizome-meristem culture. *Plant Cell, Tissue and Organ Culture*, 4(1), 11-17.

[35] Giri, D. and Tamta, S., 2011. Effect of plant growth regulators (PGRs) on micropropagation of a vulnerable and high value medicinal plant *Hedychium spicatum*. *African Journal of Biotechnology*, 10(20), 4040-4045.

[36] Giri, D. and Tamta, S., 2013. Induction of somatic embryogenesis in vulnerable medicinal plant *Hedychium spicatum* Buch-Ham ex Smith. *Plant Tissue Culture and Biotechnology*, 23(2), 147-155.

[37] Koul, S., Raina, V. and Sharma, S.K., 2005. Conservation and propagation of high altitude medicinal and aromatic plant: *Hedychium spicatum*. *Journal of Plant Biochemistry and Biotechnology*, 14(1), 57-59.

[38] Gordon, S.P., Chickarmane, V.S., Ohno, C. and Meyerowitz, E.M., 2009. Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem. *Proceedings of the National Academy of Sciences of the United States of America*, 106(38), 16529-16534.

[39] Auer, C.A., Motyka, V., Brezinová, A. and Kamínek, M., 1999. Endogenous cytokinin accumulation and cytokinin oxidase activity during shoot organogenesis of *Petunia hybrida*. *Physiologia Plantarum*, 105(1), 141-147.

[40] Ren, B., Zhu, Y., Zhang, J., Dong, S., Liu, P. and Zhao, B., 2016. Effects of spraying exogenous hormone 6-benzyladenine (6-BA) after waterlogging on grain yield and growth of summer maize. *Field Crops Research*, 188, 96-104.

[41] Gavinlertvatana, P. and Prutpongse, P., 1991. Commercial micropropagation in Asia. In: P.C. Debergh and R.H. Zimmerman, eds. *Micropropagation: Technology and Application*. Dordrecht: Kluwer Academic Publishers, pp. 181-189.

[42] Mok, M.C., Mok, D.W.S., Armstrong, D.J., Shudo, K., Isogai, Y. and Okamoto, T., 1982. Cytokinin activity of N-phenyl-N-1,2,3-thiadiazol-5-ylurea (thidiazuron). *Phytochemistry*, 21, 1509-1511.

[43] Victor, J.M.R., Murthy, B.N.S., Murch, S.J., Krishnaraj, S. and Saxena, P.K., 1999. Role of endogenous purine metabolism in thidiazuron induced somatic embryogenesis of peanut (*Arachis hypogea* L). *Plant Growth Regulation*, 28, 41-47.

[44] Genkov, T. and Ivanova, I., 1995. Effect of cytokinin-active phenyl urea derivatives on shoot multiplication, peroxidase and superoxide dismutase activities of *in-vitro* cultured carnation. *Bulgarian Journal of Plant Physiology*, 21(1), 73-83.

[45] Mok, M.C., Mok, D., Turner, J. and Mujer, C., 1987. Biological and biochemical effects of cytokinins-active phenylurea derivatives in tissue culture systems. *HortScience*, 22(6), 1194-1197.

[46] Bairu, M.W., Aremu, A.O. and Van Staden, J., 2011. Somaclonal variation in plants: causes and detection methods. *Plant Growth Regulation*, 63(2), 147-173.

[47] Saha, M.M.R., Liang, Y., Cheng, J.J., Daroch, M., 2016. Astaxanthin-producing green microalga *Haematococcus pluvialis*: from single cell to high value commercial products. *Frontiers in Plant Science*, 7, https://doi.org/10.3389/fpls.2016.00531.

[48] Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M. and Sadh, R.K., 2016. Somaclonal variations and their applications in horticultural crops improvement. *3 Biotech*, 6(1), https://doi.org/10.1007/s13205-016-0389-7.

[49] Dewir, Y.H., Naidoo, Y. and da Silva, J.A.T., 2018. Thidiazuron-induced abnormalities in plant tissue cultures. *Plant Cell Reports*, 37(11), 1451-1470.

[50] Saha, S., Kader, A., Sengupta, C. and Ghosh, P., 2012. *In vitro* propagation of *Ocimum gratissimum* L. (Lamiaceae) and its evaluation of genetic fidelity using RAPD marker. *American Journal of Plant Sciences*, 3, 64-74.

[51] Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15(3), 473-497.

[52] Arzoo, K. and Biswas, S.K., 2013. Effect of plant extracts as seed treatments on growth parameters, seedlings mortality and biochemical changes in tomato. *International Journal of Bio-resource and Stress Management*, 4, 47-53.

[53] Nwachukwu, E.O. and Umechuruba, C.I., 2001. Antifungal activities of some leaf extracts on seed-borne fungi of African yam, bean seed germination and seedling emergence. *Journal of Applied Sciences and Environmental Management*, 5(1), 29-32.

[54] Beyaz, R., Ozgen, Y., Poyraz, I., Cavdar, A. and Yildiz, M., 2021. Effects of squirting cucumber fruit juice on seed germination and seedling growth in rapeseed. *Fresenius Environmental Bulletin*, 30(1), 48-53.

[55] Carimi, F., Zottini, M., Formentin, E., Terzi, M. and Schiavo, F.L., 2003. Cytokinins: new apoptotic inducers in plants. *Planta*, 216(3), 413-421.

[56] Saha, S., Mori, H. and Hattori, K., 2007. Synergistic effect of kinenin and benzyl adenine plays a vital role in high frequency regeneration from cotyledon explants of bottle gourd (*Lagenaria siceraria*) in relation to ethylene production. *Breeding Science*, 57, 197-202.

[57] Gaba, V.P., 2005. Plant growth regulators in plant tissue culture and development. In R.N. Trigiano and D.J. Gray, eds. *Plant Development and Biotechnology*. Florida: CRC Press, pp. 87-99.

[58] Mok, M.C., Martin, R.C. and Mok, D.W., 2000. Cytokinins: biosynthesis metabolism and perception. *In Vitro Cellular and Developmental Biology – Plant*, 36(2), 102-107.

[59] Bairu, M.W., Novák, O., Doležal, K. and Staden, J.V., 2011. Changes in endogenous cytokinin profiles in micropropagated *Harpagophytum procumbens* in relation to shoot-tip necrosis and cytokinin treatments. *Plant Growth Regulation*, 63(2), 105-114.

[60] Sim, G.E., Loh, C.S. and Goh, C.J., 2007. High frequency early *in vitro* flowering of *Dendrobium* Madame Thong-In (Orchidaceae). *Plant Cell Reports*, 26, 383-393.

[61] Grzegorczyk-Karolak, I., Kuzma, L. and Wysokinska, H., 2015. The effect of cytokinins on shoot proliferation, secondary metabolite production and antioxidant potential in shoot cultures of *Scutellaria alpina*. *Plant Cell, Tissue and Organ Culture*, 122(3), 699-708.

[62] Chuengpanya, R., Chuenboonngarm, N., Kongtook, N., Longsaward, R., Noppncharoenkul, N., Muangkroot, A., Jenjittikul, T. and Soonthornchainaksaeng, P., 2019. *In vitro* propagation of *Curcuma candida* (Wall.) Techapr. and Škornick., a vulnerable plant of Thailand. *Srinakharinwirot Science Journal*, 35(2), 31-49.

[63] Chandra, S., Bandopadhyay, R., Kumar, V. and Chandra, R., 2010. Acclimatization of tissue cultured plantlets: from laboratory to land. *Biotechnology Letters*, 32(9), 1199-1205.