



Institute of Biodiversity and Environmental Conservation

**MICROPROPAGATION AND MONITORING OF  
GENETIC STABILITY OF MICROPAGATED  
PLANTLETS OF *Hornstedtia reticulata* K.SCHUM**

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**MICROPROPAGATION AND GENETIC MONITORING OF  
MICROPROPAGATED PLANTLETS OF *HORNSTEDTIA*  
*RETICULATA* K. SCHUM**

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## TABLE OF CONTENTS

	<b>Page</b>
Abstract	iv
Abstrak	vi
Acknowledgement	viii
List of tables	xi
List of figures	x
List of abbreviations	xvi
Chapter 1	
Introduction	1
Chapter 2	
Literature review	6
2.1 The taxonomy and morphology of <i>Hornstedtia</i> species	6
2.2 The potential and commercial importance of Zingiberaceae as ornamental plant	7
2.3 Plant tissue culture in Zingiberaceae	8
2.3.1 Organogenesis	9
2.3.2 Somatic embryogenesis	15
2.4 The use of RAPD-PCR in genetic fidelity assessment	17
Chapter 3	
Materials and methods	22
3.1 Preparation of culture medium	22
3.2 Collection and establishment of axenic mother plants	22
3.3 Direct regeneration of <i>Hornstedtia reticulata</i> K. Schum	23
3.3.1 The effects of TDZ treatments on multiple shoot formation	23
3.3.2 The effects of BAP treatments on multiple shoot formation	23
3.3.3 The efficiency of 3mg/L BAP as best treatment to micropropagate true-to-type plantlets	24
3.3.4 <i>Ex vitro</i> establishment	24
3.3.5 Statistical analyses for direct regeneration	27
3.4 Indirect regeneration of <i>Hornstedtia reticulata</i> K. Schum	27

	<b>Page</b>
3.4.1 Callus induction	27
3.4.2 Induction of plantlets from callus	28
3.4.3 Statistical analyses for indirect regeneration	28
3.5 Genetic monitoring of micropropagated plantlets using RAPD-PCR analysis	29
3.5.1 DNA extraction	29
3.5.2 Quantification of DNA	30
3.5.3 Agarose gel electrophoresis	30
3.5.4 RAPD-PCR analysis	31
3.5.5 Data analyses for genetic fidelity assessment	32
<b>Chapter 4</b>	
<b>Results and discussions</b>	<b>34</b>
4.1 Establishment of axenic mother plant	34
4.2 Direct regeneration of <i>Hornstedtia reticulata</i> K. Schum	34
4.2.1 The effects of TDZ treatments on multiple shoot formation	34
4.2.2 The effects of BAP treatments on multiple shoot formation	40
4.2.3 The efficiency of 3mg/L BAP as best treatment to micropropagate true-to-type plantlets	49
4.2.4 <i>Ex Vitro</i> Establishment	51
4.3 Indirect regeneration of <i>Hornstedtia reticulata</i> K. Schum	53
4.3.1 Callus induction using leaf as explants	53
4.3.2 Callus induction using leaf sheaths as explants	55
4.3.3 Induction of plantlets from callus	61
4.4 RAPD-PCR analysis	68
4.4.1 DNA extraction	68
4.4.2 Primers screening and selection	72
4.4.3 Genetic fidelity assessment	74
<b>Chapter 5</b>	
<b>Conclusion</b>	<b>87</b>
<b>References</b>	<b>89</b>
<b>Appendix 1</b>	<b>96</b>

Appendix 2

**Page**  
97

## ABSTRACT

Direct and indirect regeneration pathways were compared for *Hornstedtia reticulata* K. Schum. Seeds of *H. reticulata* collected from the wild were double surface sterilised with 30% Clorox™ followed by 15% Clorox™ each for 20 minutes. The sterilised seeds then, were sown on Gamborg B5 medium. The axenic seedlings were kept *in vitro* as the source of axenic explants. Shoot-tip explants derived from axenic off shoots were cultured in Gamborg B5 media incorporated with 30% sucrose, 3 g/L gelrite and different concentrations of cytokinins i.e. TDZ, BAP alone or in combination with NAA in the direct regeneration pathway. PPM at 1 mg/L was added into the media to prevent fungus contamination. BAP at 3 mg/L could induce the highest multiplication rate hence; it is considered the best cytokinin for micropropagation of *H. reticulata*. The three-month old micropropagated plantlets were acclimatised with the success rate of 89%. The reliability of the developed protocol to produce genetically true-to-type plantlets was assessed at molecular level. The leaf samples were extracted using slightly modified 2x CTAB method. Seven RAPD primers i.e. OPB 7, OPB 12, OPB 15, OPC 2, OPC 5, OPC 11 and OPC 16 generated total of 32, 33 and 32 bands on mother plant M<sub>1</sub>, M<sub>2</sub> and M<sub>3</sub> respectively. The primers produced amplification products which were monomorphic across the mother plants and the micropropagated plantlets, confirming the reliability of developed micropropagation protocol to produce true-to-type plantlets. Both leaves and leaf sheaths were used for callus induction in the indirect regeneration pathway. Result showed leaf-sheath explants were more responsive when compared to leaf explants for callus induction. Two types of callus were induced on B5 medium incorporated with 2,4-D either alone or in combination with of BAP. Four months observations showed that B5 medium incorporated with 2.5 mg/L 2,4-D and 0.5mg/L BAP

was the best for callus induction with the percentage of  $26.8 \pm 0.9\%$  of total leaf explants producing callus. When transferred to regeneration medium, the callus differentiated into globular, heart and oblong shapes of embryos. However, regeneration of plantlets was incomplete as the differentiation only resulted in rhizogenesis.

**Keywords:** Zingiberaceae, direct multiple shoots, RAPD-PCR

## **ABSTRAK**

*Laluan regenerasi langsung dan laluan regenerasi tak langsung telah dikaji untuk *Hornstedtia reticulata* K. Schum. Biji benih yang diperolehi daripada *H. reticulata*. di kawasan hutan akan disterilkan dua kali dengan menggunakan kepekatan Clorox™ 30% diikuti dengan kepekatan Clorox™ 15%. Setiap langkah sterilisasi dilakukan selama 20 minit. Biji benih yang telah disterilkan dibiar bercambah pada medium Gamborg B5 tanpa pengawal atur pertumbuhan. Benih yang telah bercambah disimpan dalam keadaan in vitro dan digunakan sebagai sumber explant. Hujung pucuk yang berasal daripada pucuk sisi yang steril digunakan sebagai explant untuk eksperimen laluan regenerasi langsung. Explan dikultur pada media Gamborg B5 yang mengandungi 30% sukrosa, 3g/L gelrite dan pelbagai kepekatan sitokinin seperti TDZ, BAP atau BAP yang digabung dengan NAA. PPM pada kepekatan 1mg/L ditambah ke dalam media untuk mengelakkan kontaminasi fungus. Berdasarkan keputusan eksperimen, BAP pada kepekatan 3mg/L berjaya mempergiatkan pertumbuhan pucuk pada kadar yang tertinggi dan ini bererti BAP merupakan sitokinin yang terbaik untuk micropropagasi *H. reticulata*. Plantlet yang berumur tiga bulan berjaya diaklimatisasi dengan kadar kejayaan 89%. Seterusnya, keberkesanan protokol regenerasi ini untuk menghasilkan klon-klon yang seratus peratus sama diuji pada tahap molekul. Sampel daun diekstrak menggunakan kaedah 2x CTAB yang diubah suai. Primer RAPD iaitu OPB 7, OPB 12, OPB 15, OPC 2, OPC 5, OPC 11 dan OPC 16 berjaya menamplikasikan sejumlah 32 jalur pada pokok induk M<sub>1</sub>, 33 jalur pada pokok induk M<sub>2</sub> dan 32 jalur pada pokok induk M<sub>3</sub>. Produk PCR yang diamplifikasikan dengan menggunakan primer-primer tersebut menghasilkan profil yang monomorfik antara pokok induk dan klon-klonnya. Keputusan ujian ini membuktikan protokol laluan regenerasi langsung ini adalah ternyata berkesan untuk menghasilkan klon-klon yang seratus peratus seiras. Bagi laluan regenerasi tak langsung,*

*explant upih daun dan explant daun dibandingkan keberkesanan dalam induksi kalus. Keputusan eksperimen menunjukkan upih daun ternyata lebih berkesan dalam induksi kalus. Dua jenis kalus diperolehi apabila explant dikultur pada media yang mengandungi pelbagai kepekatan 2,4-D sahaja atau 2,4-D yang digabungkan dengan BAP pada kepekatan 0.5 mg/L. Pemerhatian selama empat bulan menunjukkan B5 media yang mengandungi 2.5mg/L 2,4-D + 0.5mg/L BAP adalah terbaik dalam induksi kalus di mana  $26.8 \pm 0.9\%$  daripada sejumlah explant upih daun menghasilkan kalus. Apabila kalus dipindah ke medium regenerasi, kalus berbeza kepada embrio yang berbentuk bulat, hati dan bujur. Walau bagaimanapun, perbentukan plantlet adalah tidak lengkap kerana akhir pemerhatian menunjukkan proses pembezaan hanya mengakibatkan rhizogenesis sahaja.*

**Kata Kunci:** Zingiberaceae, Pucuk berbilang langsung, RAPD-PCR

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## LIST OF TABLES

	<b>Page</b>	
<b>Table 1</b>	PCR condition for primers with 39.5°C melting temperature	33
<b>Table 2</b>	PCR condition for primers with 43.6°C melting temperature	33
<b>Table 3</b>	Regeneration response of shoot tip cultures in B5 media supplemented with low concentration of TDZ	35
<b>Table 4</b>	Regeneration response of shoot tip cultures in B5 media supplemented with high concentration of TDZ	35
<b>Table 5</b>	Regeneration responses of shoot tip cultures in B5 media supplemented with low concentration of BAP	44
<b>Table 6</b>	Regeneration responses of shoot tip cultures in B5 media supplemented with high concentration of BAP	44
<b>Table 7</b>	Shoot multiplication on B5 media supplemented with BAP at 3.0 mg/L	50
<b>Table 8</b>	Induction of callus using lamina as explants	54
<b>Table 9</b>	Effects of 2,4-D on callus induction in leaf sheath explants cultured on B5 medium	59
<b>Table 10</b>	Effects of 2,4 -D + BAP on callus induction in leaf sheath explants cultured on B5 medium	59
<b>Table 11</b>	Spectrophotometer reading for DNA extracted by CTAB Method	70
<b>Table 12</b>	Preparation for master mix	70
<b>Table 13</b>	Sequences of 10-mer primers (1 <sup>st</sup> BASE Laboratories Sdn Bhd) used in RAPD-PCR analysis	72
<b>Table 14</b>	Number of RAPD amplicons produced from mother plants samples of <i>Hornstedtia reticulata</i> K. Schum according to banding patterns	77

## LIST OF FIGURES

		<b>Page</b>
Fig. 1	Inflorescence of <i>Hornstedtia reticulata</i> K. Schum in the wild	5
Fig. 2	Seedlings kept in <i>in vitro</i> condition as source of explants	25
Fig. 3	Shoot-tip explant used in the study of direct regeneration pathway	25
Fig. 4	Flow chart illustrating the protocol for direct regeneration of micropropagated plantlets and its fidelity assessment	26
Fig. 5	Leaf slurry in CTAB buffer after incubated for 30 minutes in 65°C water bath	33
Fig. 6	DNA pellets after precipitated overnight in ice-cold isopropanol	33
Fig. 7	Induction of multiple shoot formation in <i>Hornstedtia reticulata</i> K. Schum by TDZ	
	(a) Explant in 0.5mg/L TDZ after one month of sub-culturing	39
	(b) Explant in 1.0mg/L TDZ after one month of sub-culturing	39
	(c) Explant in 1.5mg/L TDZ after one month of sub-culturing	39
	(d) Stunted multiple shoots induced on shoot tip in medium supplemented with 0.5mg/L TDZ after four months of sub-culturing	39
	(e) Shoot induction from shoot tip in medium supplemented with 1.5 mg/L TDZ after two months of sub-culturing	39
	(f) Multiple shoots induced on medium with 1.5 mg/L TDZ were stunted even after seven subcultures	39
Fig. 8	Regeneration responses of shoot tip cultures from seedlings in B5 media supplemented with various concentrations of BAP and 0.5 mg/L NAA	41
Fig. 9	Induction of multiple shoot formation using shoot-tip explants derived from seedling of <i>Hornstedtia reticulata</i> K. Schum by BAP alone and BAP with 0.5 mg/L NAA	
	(a) Tiny multiple shoots proliferated from the basal of shoot tip after three weeks in B5 medium supplemented with 0.5 mg/L BAP	42

	<b>Page</b>
(b) Four clearly visible shoots proliferated from the shoot tip after four weeks in B5 medium supplemented with 1.0 mg/L BAP	42
(c) A tiny bud induced from the basal part of shoot tip after four weeks in B5 medium supplemented with 1.0 mg/L BAP + 0.5 mg/L NAA	42
(d) Induction of shoot was inhibited when explant was cultured in B5 medium supplemented with 2.0mg/L BAP + 0.5 mg/L NAA	42
<b>Fig. 10</b> Induction of multiple shoot formation using shoot-tip explants derived from lateral shoots of <i>Hornstedtia reticulata</i> K. Schum by BAP alone	
(a) Axillary buds protruded from shoot-tip explant	47
(b) Buds induced from explant in B5 medium supplemented with BAP at 0.5 mg/L after three subcultures	47
(c) Buds induced from explant in B5 medium supplemented with BAP at 1.0 mg/L after three subcultures	47
(d) Tiny shoots induced from explant in B5 medium supplemented with BAP at 1.5 mg/L after three subcultures	47
(e) Shoots induced from explant in B5 medium supplemented with BAP at 2.5 mg/L after one month on culture	47
(f) Shoots induced from explant in B5 medium supplemented with BAP at 3.0 mg/L after one month on culture	47
(g) The explant from B5 medium supplemented with BAP at 3.5 mg/L turned brownish after two subcultures	47
(h) A clump of well rooted multiple shoots derived from explant cultured on 3 mg/L BAP after subcultures to B5 medium	47
<b>Fig. 11</b> Induction of multiple shoot formation in B5 medium supplemented with BAP at 3 mg/L	50
<b>Fig. 12</b> <i>Ex vitro</i> establishment of micropropagated plantlets	
(a) Plantlets after two months in <i>in vitro</i> condition	52
(b) Plantlets after four months in <i>in vitro</i> condition	52

	<b>Page</b>
(c) The plantlets successfully grown under ambient environment	52
<b>Fig. 13</b> Induction of callus using leaves of <i>Hornstedtia reticulata</i> K. Schum	
(a) The basal part of leaf started to show sign of callusing after two months of subcultures on B5 medium with 5 mg/L 2,4-D	54
(b) Creamy white and fluffy callus on leaf explant after sub-cultured in B5 medium with 2 mg/L 2,4-D + 0.5 mg/L BAP	54
(c) The fluffy callus were gradually shrunk and turning brown after five months subcultures on B5 medium with 2 mg/L 2,4-D + 0.5 mg/L BAP	54
<b>Fig. 14</b> Induction of callus using leaf sheaths of <i>Hornstedtia reticulata</i> K. Schum	
(a) Callus in B5 medium supplemented with 1.0 mg/L 2,4-D after four subcultures	56
(b) Callus in B5 medium supplemented with 2.0 mg/L 2,4-D after four subcultures	56
(c) Callus in B5 medium supplemented with 2.5 mg/L 2,4-D after four subcultures	56
(d) Callus in B5 medium supplemented with 1.0 mg/L 2,4-D + 0.5 mg/L BAP after four subcultures	56
(e) Callus in B5 medium supplemented with 1.5 mg/L 2,4-D + 0.5 mg/L BAP after four subcultures	56
(f) Callus in B5 medium supplemented with 2.0 mg/L 2,4-D + 0.5 mg/L BAP after four subcultures	56
(g) Two types of calli were clearly seen at leaf sheath explant on B5 medium supplemented with 1.5 mg/L 2,4-D + 0.5 mg/L BAP	56
(h) Type I and type II callus at basal part of leaf sheath explant on B5 medium supplemented with 2.0 mg/L 2,4-D + 0.5 mg/L BAP	56
(i) Type II callus on B5 medium supplemented with 2.5 mg/L 2,4-D + 0.5 mg/L BAP	56
<b>Fig. 15</b> Induction of somatic embryogenesis through callus	

	<b>Page</b>
(a) The competence cells on callus gradually turned greenish after one week in B5 medium supplemented with 1mg/L BAP + 0.5 mg/L NAA	63
(b) Callus after third week on B5 medium supplemented with 3 mg/L BAP + 0.5 mg/L NAA	63
(c) Close up showed the callus turned to globular embryos after second sub-culturing to B5 medium supplemented with 1mg/L BAP + 0.1 mg/L NAA	63
(d) Close up showed different stages of embryos can clearly seen on callus after end of second sub culturing on B5 medium supplemented with 1mg/L BAP + 0.5 mg/L NAA	63
(e) (i) embryo, (ii) heart shape embryo, (iii) oblong embryo, (iv) formation of radicle	63
(f) Rhizogenesis on callus at B5 medium supplemented with 1mg/L BAP + 0.5 mg/L NAA	63
(g) Rhizogenesis on callus at B5 medium supplemented with 3 mg/L BAP + 0.5mg/L NAA	63
(h) Rhizogenesis on callus at B5 medium supplemented with 1 mg/L BAP + 0.1 mg/L NAA	63
(i) The smooth surface of type II callus turned lumpy when transferred to B5 medium supplemented with 1mg/L BAP + 0.5 mg/L NAA	63
(j) The type II callus turned greenish after three week in the regeneration B5 medium supplemented with 1mg/L BAP + 0.5 mg/L NAA	63
(k) Rhizogenesis occur on type II callus after second subcultures to B5 medium supplemented with 3 mg/L BAP + 0.1 mg/L NAA	63

**Fig. 16** Qualification of template DNA of M3.2 in series dilution test

(a) Amplification products of template DNA M3.2 in series dilution tested with primers OPB1, OPB2, OPB14 and OPB11	71
(b) Amplification products of template DNA M3.2 in series dilution tested with primers OPB12, OPB15, OPB16 and OPB17	71

	<b>Page</b>	
Fig. 17	Amplification pattern from <i>Hornstedtia reticulata</i> and <i>Globba atosanguinea</i> using RAPD primers kit OPB and kit OPC	
(a)	Amplification pattern obtained from <i>Hornstedtia reticulata</i> and <i>Globba atosanguinea</i> using primers OPB4, OPB7, OPB8, OPB12 and OPB15	73
(b)	Amplification pattern obtained from <i>Hornstedtia reticulata</i> and <i>Globba atosanguinea</i> using primers OPC2, OPC5, OPC11, OPC13 and OPC 16	73
Fig. 18	Amplification pattern obtained from <i>Hornstedtia reticulata</i>	
(a)	Amplification pattern obtained from <i>Hornstedtia reticulata</i> (M <sub>1</sub> , M <sub>2</sub> and M <sub>3</sub> ) using primer OPB 7	75
(b)	Amplification pattern obtained from <i>Hornstedtia reticulata</i> (M <sub>1</sub> , M <sub>2</sub> and M <sub>3</sub> ) using primer OPB 12	75
(c)	Amplification pattern obtained from <i>Hornstedtia reticulata</i> (M <sub>1</sub> , M <sub>2</sub> and M <sub>3</sub> ) using primer OPB 15	75
(d)	Amplification pattern obtained from <i>Hornstedtia reticulata</i> (M <sub>1</sub> , M <sub>2</sub> and M <sub>3</sub> ) using primer OPC 2	75
(e)	Amplification pattern obtained from <i>Hornstedtia reticulata</i> (M <sub>1</sub> , M <sub>2</sub> and M <sub>3</sub> ) using primer OPC 5	76
(f)	Amplification pattern obtained from <i>Hornstedtia reticulata</i> (M <sub>1</sub> , M <sub>2</sub> and M <sub>3</sub> ) using primer OPC 11	76
(g)	Amplification pattern obtained from <i>Hornstedtia reticulata</i> (M <sub>1</sub> , M <sub>2</sub> and M <sub>3</sub> ) using primer OPC 16	76
Fig. 19 (a) – (c)	RAPD profiles generated by primer OPB7	80
Fig. 20 (a) – (c)	RAPD profiles generated by primer OPB12	81
Fig. 21 (a) – (c)	RAPD profiles generated by primer OPB15	82
Fig. 22 (a) – (c)	RAPD profiles generated by primer OPC2	83
Fig. 23 (a) – (c)	RAPD profiles generated by primer OPC5	84
Fig. 24 (a) – (c)	RAPD profiles generated by primer OPC11	85

	<b>Page</b>
Fig. 25 (a) – (c) RAPD profiles generated by primer OPC16	86
Fig. 26 Degree of callusing among the leaf-sheath explants	
(a) non callusing	96
(b) slight callusing	96
(c) moderate callusing	96
(d) heavy callusing	96

## LIST OF ABBREVIATIONS

<b>2ip-R</b>	6-( $\gamma,\gamma$ -Dimethylallylamino) purine ribolusa
<b>2,4-D</b>	2,4-Dichlorophenoxyacetic acid
<b>BA</b>	6-Benzylamino
<b>BAP</b>	6-Benzylaminopurine
<b>CTAB</b>	Cetyl trimethyl ammonium bromide
<b>DNA</b>	Deoxyribonucleic acid
<b>dNTP</b>	Deoxynucleotide triphosphates
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>HCl</b>	Hydrochloric acid
<b>IMA</b>	Imazalil
<b>Kin</b>	Kinetin
<b>MgCl<sub>2</sub></b>	Magnesium chloride
<b>mRNA</b>	Messenger ribonucleic acid
<b>NAA</b>	Naphthalene acetic acid
<b>NaCl</b>	Sodium chloride
<b>NaOH</b>	Sodium hydroxide
<b>PEM</b>	Pre-embryo mass
<b>PCR</b>	Polymerase chain reaction
<b>PGR</b>	Plant growth regulator
<b>PPM</b>	Biocide plant preservative mixture
<b>PVP</b>	Polyvinylpyrrolidone
<b>RAPD</b>	Random amplified polymorphic DNA polymerase
<b>TBE</b>	Tris-borate EDTA buffer
<b>TDZ</b>	Thidiazuron
<b>Tris-HCl</b>	Tris-hydrochloric acid

# CHAPTER 1

## INTRODUCTION

Zingiberaceae is comprised of approximately 1200 species of which about 1000 occur in tropical Asia. The richest area is in the Malesia, a floristically distinct region that include Malaysia, Indonesia, Brunei, Singapore, Philipines and Papua New Guinea with 24 genera and about 600 species (Larsen *et al.*, 1999). However, area such as Sumatra and Borneo are still very insufficiently known and their ginger floras are still largely under explored. Activities such as logging, deforest and rural regions development have caused the forests destroyed at an increasing rate. At the pace the natural habitats are being destroyed or altered, it is feared that many plant species will perish without any identification as well as investigation for exploitation. Hence, conservation of underexploited gingers is crucial not only for basic research, on which a lot of the basic information is still lacking, but also for applied research with the aim of exploiting and utilizing species with economic potential.

Sarawak, the largest state in Malaysia is located in the island of Borneo. Geographically, Sarawak lies within the equatorial wet tropics with a highly divided and mountainous topography providing a huge range of habitats for numerous herbaceous terrestrial monocotyledons. Boyce (2006) has affirmed that the state is the centre of diversity for both families of Araceae and Zingiberaceae in the Asian tropics and is arguably the richest and most diverse area for the gingers globally. At present, there are 18 indigenous genera of Zingiberaceae recorded for Sarawak and a lot more are waiting to be explored and exploited for their valuable usages. At least 20 or more ginger species have been cultivated for their use as spices, condiments, flavours, fresh vegetables, medicine, and recently is popular for ornamentals and as cut flowers.

*Hornstedtia* is a well defined genus characterized by the rigid involucre of sterile bracts which enclose the entire inflorescence from the uppermost part of the open flowers. Valetton (1921, cited by Smith, 1985) had subdivided *Hornstedtia* into three subgenera, *Hornstedtia* (*scyphifera* Val.), *Elettariostemon* and *Rosianthus*. The Bornean plants all fall within the first two groups. *Hornstedtia reticulata* is very distinctive. The cyathiform inflorescence is borne on stilt roots and the sterile bracts, which are the most strongly reticulated of all Bornean *Hornstedtia* are scabrid to the touch. Traditionally, the dried culm and petiole sheath of *Hornstedtia reticulata* are used by local in Sarawak as weave mats (kasah), small basket and trays. Recently, this species has been exploited for ornamental purpose due to its unique and exotic clusters of scarlet candle-like flower heads as shown in Fig.1. When these flower heads reach the flowering size, the terminal bracts will re-curve to form a liquid-filled pool with orange flowers rising on the pool for several weeks making this plant ideally to be promoted as indoor ornamental plant or cut flowers. This species has been online introduced to flower lovers as “robust ginger with attractive honey-comb stems bearing richly aromatic leaves” by Malesiana Tropical Sdn Bhd, Sarawak, Malaysia.

Owing to its potential in horticulture industry, finding an alternative propagation protocol to produce large number of planting material becomes important. For this reason, micropropagation using tissue culture technique is considered a good approach. Plant micropropagation is a combination of the arts and sciences of plant multiplication *in-vitro* and plant acclimatization to obtain clones in large quantity. It is the true-to-type propagation of a genotype which includes steps such as stock plant care, explants selection and sterilization, media manipulation to attain proliferation, rooting, acclimatization, growing of explants and it is usually associated with commercial production.

Even though micropropagation is an expensive technique compared to conventional macropropagation, it is the most promising technique to attain rapid plant propagation of desired genotypes. Nowadays, about 150 plant species are commercially micropropagated. Many of these have reached the limits of their improvement by traditional methods. The emphasis on sustainable agriculture, increasing world population and the loss of prime land to housing and industry make this method of propagation indispensable.

Although micropropagation is considered as a method of propagating genetically true-to-type offsprings however, somaclonal variation has been detected to occur in plantlets produced via tissue culture techniques (Larkin and Scowcroft, 1981). This variation can be epigenetic but often heritable (Breiman *et al.*, 1987). In ornamentals such variation may be a source of novel plants whereas, for agricultural and horticultural plants somaclonal variation may be undesirable. For mass production of planting material, it is desired that the progenies are identical to the mother plants. Morphological mutations can be visualized by just looking at the individual. Nevertheless, certain plant characteristics such as flower colour, shape, size and fruits can be only assessed after the plantlets have been transplanted and grown till flowering.

Thus, it is desirable that the genetic stability of micropropagated plants derive from an efficient regeneration protocol to be examined as early as possible to save overall time and cost. Ideally, the selected marker should be able to generate reproducible results and require small amounts of plant tissue. Due to its simplicity with no radioactivity is needed, RAPD marker is a suitable tool to assess genetic stability of tissue culture derived plants. Apart from taxonomy, other information especially biotechnology of *Hornstedtia* sp. is scanty. For this

reason, the objectives of this research are to develop a micropropagation protocol which can be used to mass produce plantlets at low cost and shorter time as compared with the conventional propagation method. Furthermore, the genetic stability of the produced plantlets is to be assessed by RAPD-PCR analysis. As a result, a reliable protocol of producing genetically true-to-type plantlets can be established to conserve this wild species and mass produce this species for use as ornamental plants. To the best of my knowledge and from the available literature, this is the first report on direct shoots differentiation and regeneration followed by genetic fidelity assessment for micropropagated plantlets using RAPD markers in *Hornstedtia reticulata*.



Fig.1 Inflorescence of *Hornstedtia reticulata* K. Schum in the wild

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 The taxonomy and morphology of *Hornstedtia* species.

The species of interest i.e. *Hornstedtia reticulata* belongs to Zingiberaceae which comes under the order Zingiberales that form an isolated group among the monocotyledons. The genus *Hornstedtia* is named after Swedish naturalist Claes F. Hornstedt (1758-1809). It is commonly very tall gingers of which some species could reach a height of 7 m or more (Smith, 1985). The leafy shoot is coarse, often swollen at the base, with a diameter of up to 6 cm. The underground rhizome is coarse, in some species just below the soil surface, while others are deep in the ground. The inflorescence, arising on a separate ~~side~~ shoot from the rhizome, is somewhat spindle-shaped on a very short peduncle. The involucres bracts are closely overlapping, stiff, often ribbed in dark red. The flowers are red, in some species with cream or yellow margins, emerging a few at a time from tip of the spindle-shaped inflorescence. There are no lateral staminodes, the corolla lobes and the labellum are about the same length. The most common species are recognized by the spindle-shaped inflorescence with striate or reticulate nervation, a character that remains even in the dried state (Larsen *et al.*, 1999).

*Hornstedtia reticulata* is a rigid plant that reaches 3 m tall. The plant is often on silt roots and the bracts are very reticulate. This is how the species got its name. In both the roots and rhizomes, oil cells containing aromatic compounds give a spicy smell when bruised. In the field, the flowering event happens twice a year and it takes a period of three years from one