LAMPIRAN

LAMPIRAN

Lampiran 1

Bahan dan reagen-reagen yang digunakan dalam pengekstrakan DNA

- Larutan tampan pengekstrakan [2% (b/i) CTAB (cetyltrimethylammonium bromide), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH8.0), 0.2% (i/i) β-merkaptoetanol (ditambah ketika hendak digunakan), klorofom:isoamilalkohol (24:1),
- RNase A [10 mg/ml RNase A dalam Tris-HCl, 15 mM NaCl (pH 7.5) yang dipanaskan selama 15 minit dan disejukkan sehingga ke suhu bilik dan simpan pada suhu -20°C],
- 100% (i/i) isopropanol,
- larutan tampan pencuci (76% (i/i) etanol, 10 mM ammonium asetat) dan
- larutan tampan TE [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)].

Lampiran 2

Bahan-bahan yang digunakan dalam RAPD-PCR

Bahan-bahan yang digunakan dalam proses PCR:

- Templat DNA yang berkepekatan 50-100 ng/μl,
- 50 pmol primer (10mer oligonucleotid daripada Operon Technologies),
- 2X PCR Master Mix (Fermentas) yang mengandungi campuran Taq DNA polimerase (rekombinan) di dalam larutan tampan tindakbalas (0.05 units/μl),
- $MgCl_2$ (4 mM) dan dNTPs (dATP,dCTP, dGTP, dTTP) (0.4 mM masing-masing) dan
- air nyahion yang steril.

Manakala untuk menjalankan elektroforesis pula, bahan-bahan yang digunakan ialah seperti :

- penanda GeneRuler 100 bp 'DNA Ladder Plus' (Fermentas),
- 6x 'Loading Dye solution',
- larutan tampan TAE (larutan tampan elektroforesis),
- 1.5% (i/i) gel agaros di dalam larutan tampan TAE dan
- stok etidium bromida (10 mg/ml-cecair).

Lampiran 3

Penyediaan bahan-bahan untuk pengekstrakan DNA

A. Penyediaan larutan tampan pengekstrakan DNA (CTAB buffer) (untuk penyediaan 1 L)

- 100 mM Tris-HCl (pH 8.0) 12.114 g
- 1.4 M NaCl 81.816 g
- 3.0% CTAB 30.0 g
- 20 mM EDTA 7.444 g
- 0.5% PVP 5.0 g
- Air suling 1000 ml

Campuran bahan-bahan di atas diautoklaf selama 20 minit (121° C)

- 0.2% β-merkaptoetanol sebelum digunakan 2.0 ml

B. Larutan tampan pencuci (Penyediaan 1 L)

- 76.0% etanol 760 ml
- 10 mM ammonium asetat 0.7708 g
- Air suling 240 ml

C. Larutan tampan TE (Penyediaan 50 ml)

- 1 M Tris-HCl (pH 8.0) 2.5 ml
- 0.5 M EDTA 1.0 ml
- Air suling 46.5 ml

D. Larutan RNase (Penyediaan stok 10 mg/ml)

- 2 M Tris-HCl (pH 7.5) 5 µl
- 5 M NaCl 3 µl
- dH_2O 992 µl
- RNase A 10 mg

Larutan di atas dipanaskan dalam 100°C untuk selama 15 minit untuk menyahaktifkan DNase. Kemudian dilarutkan dengan larutan tampan TE dan disimpan dalam -20°C sehingga digunakan.

Lampiran 4

Elektroforesis Gel Agarose.

A. Larutan tampan TAE

Komposisi 50x TAE (penyediaan untuk 1 L):

- 2 M Tris asetat 242.0 g
- Asid asetik 57.1 ml
- 0.5 M EDTA (pH 8.0) 100.0 ml
- Air suling ditambah sehingga 1000 ml.

Untuk mendapatkan kepekatan 10x dan 1x, pencairan dibuat dengan nisbah 1:5 dan 1:50 masing-masing dari larutan stok 50x.

B. Gel agaros

- 1.0% dan 1.5% agar ditambahkan bagi setiap penyediaan .

1.0% untuk pengekstrakan DNA

1.5% untuk produk PCR.

C. 'Loading buffer' (untuk 10 ml)

Komposisi

Serbuk bromofenol biru	10 mg
Gliserol	2.5 mg
Tampan Tris	7 ml

Lampiran 5

Penyediaan bahan-bahan untuk elektroforesis.

1) 0.5 M EDTA (pH 8.0)

EDTA dilarutkan dalam dH_2O, pH dilaraskan sehingga pH 8.0 menggunakan NaOH. Larutan diautoklafkan dan disimpan dalam suhu bilik.

*EDTA tidak larut pada pH yang rendah dan mula melarut sekitar pH 7.6.

Jumlah	100 ml	500 ml
EDTA	18.6 g	93.1 g
NaOH	2.0 g	10.0 g

2) 1 M Tris-HCl (pH 8.0)

Tris dilarutkan dalam dH_2O. pH dilaraskan sehingga pH 8.0. Larutan diautoklafkan dan disimpan dalam suhu bilik.

Jumlah	100 ml	500 ml
Tris	12.1 g	60.6 g
HCl	4.0 ml	20.0 ml

3) TE (10 mM Tris HCl, pH 8.0 ; 1 mM EDTA, pH 8.0)
Autoklaf dan disimpan dalam suhu bilik.

Jumlah	100 ml	500 ml
1 M Tris-HCl (pH 8.0)	1.0 ml	5.0 ml
0.5 M EDTA (pH 8.0)	0.2 ml	1.0 ml

Lampiran 6

Pengukuran DNA

- Menggunakan Biofotometer (eppendorf).
- Mengukur penyerapan cahaya UV oleh sampel DNA
- Pembacaan ukuran ketumpatan optik (O.D) pada 260 dan 280 nm
- Sampel DNA dicairkan dan diemparkan sekejap (2µl DNA + 998µl ddH_20) sebelum penentuukuran.
- Larutan campuran ini dimasukkan ke dalam tiub 'cuvet'.
- 1 ml dH_20 dimasukkan ke dalam tiub 'cuvet' yang lain sebagai 'blank'.
- Kedua-dua 'cuvet' tersebut dimasukkan ke dalam slot biofotometer dan ukuran O.D dibaca. Kepekatan dan ketulenan ($O.D_{260/280}$) juga diperolehi daripada bacaan.

Lampiran 7

Kaedah penyediaan mini gel agarose

1. Untuk penyediaan 1.0% gel agarose, 0.4 g agaros ditambahkan ke dalam 40 ml larutan tampan 0.5X TAE.
2. Larutan dipanaskan larutan tersebut dan dibiarkan sehingga 60°C dan ditambahkan 1.0 µl etidium bromida.
3. Secara perlahan gel tersebut dimasukkan ke dalam acuan yang berada di dalam tangki elektroforesis yang telah disediakan (gelembung-gelembung buih dibuang menggunakan pipet).
4. Selepas gel habis dituang, ia dibiarkan sejuk supaya gel membeku. Seterusnya sikat dicabut dengan perlahan daripada gel tersebut.
5. Larutan tampan 0.5X TAE dimasukkan ke dalam tangki elektroforesis sehingga menenggelamkan sedikit gel kira-kira 4.0 mm.

Lampiran 8

Penyediaan 'master mix' untuk kajian RAPD

Kandungan untuk 1 tindakbalas (jumlah isipadu = 25 µl) :

- 12.5 µl PCR Master Mix
- 2.0 µl primer
- 2.5 µl DNA
- 8.0 µl ddH_20

"Master mix" untuk 8 tindakbalas ;

- 100.0 µl PCR Master Mix
- 16.0 µl primer
- 64.0 µl ddH_2O

Jumlah "master mix" = 180 µl (÷8 tiub) dan kemudiannya ditambahkan dengan 2.5 µl DNA untuk setiap tiub PCR.

Lampiran 9

Elektroforesis sampel DNA di dalam gel agarose

1. Sampel DNA ditambah sebanyak 1/10 hingga 5/10 di dalam 'loading dye'. Campuran diemparkan selama 2-3 saat di dalam alat pengempar.
2. Sampel DNA dituangkan secara perlahan-lahan menggunakan mikropipet. Penanda molekul (Marker 100 bp ladder plus) dimasukkan untuk analisa RAPD.
3. Setelah memasukkan ke semua sample DNA, litar disambungkan dan aliran elektrik dihidupkan (80 V selama 1-2 jam).
4. Arus elektrik dimatikan setelah 'loading dye' menghampiri lebih kurang 2 sm daripada terminal positif.

Lampiran 10

Komposisi media Murashige & Skoog (MS) (1962)

Komposisi	Kepekatan (mg/L)
Makronutrien	
$CaCl_2.2H_2O$	440.0
NH_4NO_3	1650.0
KNO_3	1900.0
KH_2PO_4	170.0
$MgSO_4.7H_2O$	370.0
Mikronutrien	
KI	0.83
$CoCl_2.6H_2O$	0.025
H_2BO_3	6.3
Na_2MNO_4	0.25
$MnSO_4.5H_2O$	0.025
$ZnSO_4.7H_20$	8.6
Garam Ferum	
$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25
Vitamin	
Glisin	2.0
Asid nikotinik	0.5
Piridoksin HCl	0.5
Tiamin HCl	0.1
Inositol	100.0
Tambahan :	
Agar (Fitagel)	2000 (0.2%)
Sukrosa	30000 (3%)

Air suling ditambah sehingga isipadu menjadi 1000 ml

Lampiran 11

Teknik-teknik pensterilan eksplan yang digunakan dalam kajian kultur tisu

Teknik steril 1 (TS1) :

1. 100% NaOCl + Tween 20 selama 1-2 minit
2. 70% NaOCl + Tween 20 selama 5 minit
3. 50% NaOCl + Tween 20 selama 5 minit
4. Air suling steril (1x) selama 3 minit
5. 70% etanol (1 minit)
6. Air suling steril (3x) selama 1 minit setiapnya.
7. Eksplan dipotong kepada saiz yang sesuai di mana bahagian kulit luar dibuang serta bahagian atas dan pangkal eksplan dipotong (untuk eksplan tunas pucuk)
8. Eksplan dimasukkan ke dalam media MS tanpa hormon (MSO) (3.0 % sukrosa, 0.2 % phytagel).

Kaedah 1-6 adalah diubahsuai untuk teknik seterusnya.

Kaedah 7-8 adalah sama untuk teknik-teknik pensterilan yang lain

Teknik steril 2 (TS2) :

1. 50% NaOCl dan Tween 20 selama 30 minit
2. Air suling steril (3x) selama 3 minit.
3. 70% etanol selama 3 minit.
4. Air suling steril (1x) selama 3 minit.
5. Diikuti kaedah 7-8 dari TS1.

Teknik steril 3 (TS3) :

1. 20% NaOCl + Tween 20 selama 15 minit
2. Air suling steril (1x) selama 3 minit
3. 0.1% $HgCl_2$ + Tween 20 selama 5 minit
4. Air suling steril (3x) selama 1 minit setiapnya.
5. Diikuti kaedah 7-8 dari TS1

Teknik steril 4 (TS4) :

1. 15% NaOCl + Tween 20 selama 15 minit
2. Air suling steril (1x) selama 3 minit
3. 0.5% $HgCl_2$+ Tween 20 selama 5minit
4. Air suling steril (3x) selama 1 minit setiapnya
5. Diikuti oleh kaedah 7-8 dari TS1

Teknik steril 5 (TS5) :

1. 15% NaOCl + Tween 20 selama 15 minit
2. Air suling steril (1x) selama 3 minit
3. 0.5% $HgCl_2$+ Tween 20 selama 5 minit
4. 70% etanol selama 1 minit
5. Air suling steril (3x) selama 1 minit setiapnya.
6. Diikuti kaedah 7-8 dari TS1

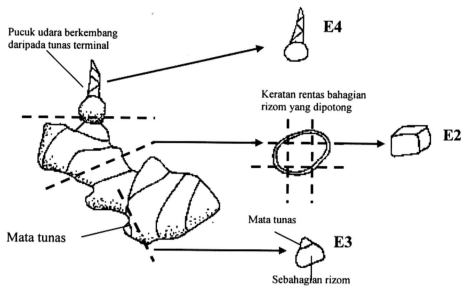
Teknik steril 6 (TS6)

1. 15% NaOCl + Tween 20 selama 15 minit
2. Air suling steril (1x) selama 3 minit
3. 0.1% $HgCl_2$+ Tween 20 selama 5 minit
4. 70% etanol selama 1 minit
5. Air suling steril (3x) selama 1 minit setiapnya.

Diikuti kaedah 7-8 dari TS1.

Lampiran 12

Gambarajah sumber-sumber eksplan yang digunakan pada awal eksperimen.



Bahagian rizom *Z. officinale*

Bahagian daun *Z. officinale*

Petunjuk :

– – – – garisan menunjukkan bahagian yang dipotong untuk dijadikan eksplan

E1- Eksplan daun yang dipotong di antara 1.0-2.0 sm^2

E2- Eksplan bahagian dalam rizom yang dipotong di antara 0.5-1.0 sm^3

E3- Eksplan mata tunas pucuk termasuk sebahagian rizom

E4- Eksplan tunas pucuk yang aktif memanjang

Lampiran 13

Bilangan pucuk terminal dan pucuk aksilari yang dihasilkan pada permulaan kultur mengikut umur dan saiz eksplan bagi setiap spesies kajian pada akhir lapan minggu kultur. Jumlah pucuk yang dihasilkan adalah daripada sebanyak lapan hingga sepuluh replikat eksplan yang digunakan.

Spesies	Umur dan Panjang eksplan (sm)	Jumlah pucuk terminal	Jumlah pucuk aksilari	Jumlah pucuk terminal dan aksilari	Purata bil. pucuk per eksplan yang dihasilkan
Z. officinale var. *officinale* (halia)	A1	5	6	11	1.38±0.52
	A2	8	3	11	1.38±0.52
	B1	5	6	11	1.38±0.52
	B2	9	7	**16**	**1.60±0.70**
Z.officinale var. *rubrum* (halia bara)	A1	5	3	8	1.00±0.00
	A2	7	2	**9**	**1.13±0.35**
	B1	4	4	8	1.00±0.00
	B2	7	1	8	1.00±0.00
Z.officinale var. *rubrum* (halia padi)	A1	7	4	11	1.38±0.52
	A2	8	5	13	1.63±0.74
	B1	6	11	17	2.13±0.64
	B2	8	15	**23**	**2.88±0.83**
Z. zerumbet	A1	4	5	9	1.13±0.35
	A2	8	3	11	1.38±0.52
	B1	**5**	**8**	**13**	**1.63±0.52**
	B2	**5**	**8**	**13**	**1.63±0.52**
Z. ottensii	A1	7	3	10	1.25±0.46
	A2	7	3	10	1.25±0.46
	B1	5	10	**15**	**1.88±0.64**
	B2	4	9	13	1.63±0.52
Z. montanum	A1	8	2	10	1.25±2.95
	A2	7	2	9	1.13±0.35
	B1	3	10	13	1.63±0.52
	B2	5	10	**15**	**1.67±0.50**
K. galanga	A1	8	5	13	1.63±0.74
	A2	8	7	15	1.88±0.64
	B1	8	10	18	2.25±0.71
	B2	9	13	**22**	**2.44±0.88**

A1 = Umur seminggu ; Saiz <0.8 sm
A2 = Umur seminggu ; Saiz >1.2 sm
B1 = Umur empat minggu ; Saiz <0.8sm
B2 = Umur empat minggu ; Saiz >1.2sm

*Tulisan tebal menunjukkan sumber eksplan yang dipilih berdasarkan jumlah bilangan pucuk yang terbanyak dihasilkan.

Lampiran 14

Purata bilangan pucuk dan akar setiap eksplan serta nisbah bilangan akar:pucuk yang dihasilkan setelah 8 minggu di dalam media permulaan (MSO).

Spesies	Purata bilangan pucuk*	Purata bilangan akar*	Nisbah akar:pucuk
Z. officinale var. *officinale* (halia)	1.20 ± 0.41	2.40 ± 0.99	2.00
Z. officinale var. *rubrum* (halia bara)	1.13 ± 0.36	2.27 ± 0.99	2.01
Z. officinale var. *rubrum* (halia padi)	1.80 ± 0.56	3.53 ± 1.46	1.96
Z. zerumbet	1.40 ± 0.63	2.67 ± 1.18	1.91
Z. ottensii	1.47 ± 0.52	2.67 ± 1.11	1.82
Z. montanum	1.33 ± 0.49	2.73 ± 1.53	2.05
K. galanga	1.93 ± 0.59	3.93 ± 1.28	2.04

*purata daripada 15 replikat bacaan.

Lampiran 15

Purata bilangan pucuk yang dihasilkan mengikut umur dan saiz eksplan yang digunakan.

Umur dan saiz sumber eksplan		Purata bilangan pucuk baru yang dihasilkan							
		Ha	HB	HP	Le	LH	Bo	Ce	Semua spesies
A.	A1	1.38	1.00	1.38	1.13	1.25	1.25	1.63	1.29
	A2	1.38	1.13	1.63	1.38	1.25	1.13	1.88	1.40
	nilai ujian-t	0n-s	1n-s	0.78n-s	1.13n-s	0n-s	0.61n-s	0.72n-s	1.07n-s
	P / DF	1 / 14	0.33 / 14	0.45 / 14	0.28 / 14	1 / 14	0.55 / 14	0.48 / 14	0.29 / 110
B.	B1	1.38	1.00	2.13	1.63	1.88	1.63	2.25	1.70
	B2	1.60	1.00	2.88	1.63	1.63	1.67	2.44	1.83
	nilai ujian-t	0.78n-s	n-s	2.02n-s	0n-s	0.86n-s	0.17n-s	0.40n-s	0.97n-s
	P / DF	0.46 / 16	-	0.06 / 14	1 / 14	0.41 / 14	0.87 / 15	0.69 / 16	0.34 / 114
C.	A1	1.38	1.00	1.38	1.13	1.25	1.25	1.63	1.29
	B1	1.38	1.00	2.13	1.63	1.88	1.63	2.25	1.70
	nilai ujian-t	0n-s	n-s	2.58*	1.72n-s	2.24*	1.53n-s	1.73n-s	3.73*
	P / DF	1 / 14	-	0.02 / 14	0.11 / 14	0.04 / 14	0.15 / 14	0.11 / 14	0.30E-03 / 110
D.	A2	1.38	1.13	1.63	1.38	1.25	1.13	1.88	1.40
	B2	1.60	1.00	2.88	1.63	1.63	1.67	2.44	1.83
	nilai ujian-t	0.78n-s	1n-s	3.16*	0.78n-s	1.53n-s	2.55*	1.50n-s	3.28*
	P / DF	0.46 / 16	0.33 / 14	0.01 / 14	0.45 / 14	0.15 / 14	0.02 / 15	0.15 / 15	0.14E-02 / 114
E.	A1	1.38	1	1.38	1.13	1.25	1.25	1.63	1.29
	B2	1.60	1	2.88	1.63	1.63	1.67	2.44	1.83
	nilai ujian-t	0.78n-s	n-s	4.32*	1.72n-s	1.53n-s	1.78n-s	2.05n-s	4.21*
	P / DF	0.46 / 16	-	0.71E-03 / 14	0.11 / 14	0.15 / 14	0.10 / 15	0.06 / 15	5.04E-05 / 114
F.	A2	1.38	1.13	1.63	1.38	1.25	1.13	1.88	1.39
	B1	1.38	1.00	2.13	1.63	1.88	1.63	2.25	1.70
	nilai ujian-t	0n-s	1n-s	1.44n-s	0.97n-s	2.24*	2.26*	1.11n-s	2.62*
	P / DF	1 / 14	0.33 / 14	0.17 / 14	0.35 / 14	0.04 / 14	0.04 / 14	0.29 / 14	0.01 / 110
G.	A1+A2	1.38	1.06	1.50	1.25	1.25	1.19	1.75	1.35
	B1+B2	1.50	1.00	2.50	1.63	1.75	1.65	2.35	1.79
	nilai ujian-t	0.64n-s	1n-s	3.87*	1.96n-s	2.74*	2.92*	2.35*	4.48*
	P / DF	0.52 / 32	0.33 / 30	0.54E-03 / 30	0.06 / 30	0.01 / 30	0.01 / 31	0.03 / 31	1.26E-05 / 196
H.	A1+B1	1.38	1	1.75	1.38	1.56	1.44	1.94	1.49
	A2+B2	1.50	1	2.25	1.50	1.44	1.41	2.18	1.66
	nilai ujian-t	0.64n-s	n-s	1.65n-s	0.62n-s	0.62n-s	0.15n-s	0.88n-s	1.70n-s
	P / DF	0.52 / 32	-	0.11 / 30	0.52 / 30	0.54 / 30	0.89 / 31	0.39 / 31	0.09 / 196

P ='level of significance' ; DF=Darjah kebebasan ; n-s=tidak signifikan ; *=signifikan ; (purata bilangan pucuk adalah berbeza secara signifikan pada p<0.05 di dalam ujian t)

Ha=*Z. officinale* var. *officinale* ; HB=*Z. officinale* var. *rubrum* ; HP=*Z. officinale* var. *rubrum* (halia padi) ; Le=*Z. zerumbet* ; LH=*Z. ottensii* ; Bo=*Z. montanum* ; Ce=*K. galanga*

A1 = Umur seminggu ; Saiz <0.8 sm ; A2 = Umur seminggu ; Saiz >1.2 sm ; B1 = Umur empat minggu ; Saiz <0.8sm ; B2 = Umur empat minggu ; Saiz >1.2sm

PENERBITAN

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Micropropagation Study on Three Varieties of *Zingiber officinale* Rosc.

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ABSTRACT The *in vitro* propagation protocols for three varieties of *Zingiber officinale* were successfully established in this study. Averages of three to five shoots per exnlant were successfully regenerated on MS medium supplemented with 3.0% (w/v) sucrose, 0.2% (w/v) phytagel, and 1.0-3.0 mg/l BAP. All varieties studied have shown that the rhizome yield from *in vitro* plantlets were higher than those from *in vivo* materials. The protocols developed can be adopted for propagation and conservation purposes.

ABSTRAK Protokol propagasi secara *in vitro* ke atas tiga varieti *Zingiber officinale* telah berjaya dilakukan di dalam kajian ini. Secara purata antara tiga hingga lima pucuk setiap eksplan berjaya diregenerasikan di dalam media MS yang mengandungi 3.0% (b/i) sukrosa, 0.2% (b/i) fitagel dan BAP pada kepekatan 1.0-3.0 mg/l. Kesemua varieti yang dikaji menunjukkan penghasilan rizom yang lebih baik daripada plantlet *in vitro* berbanding dengan *in vivo* semasa ditanam di lapangan. Dengan itu, kaedah kultur tisu yang telah dirumuskan dalam kajian ini, amat sesuai digunakan untuk tujuan propagasi dan pemuliharaan varieti-varieti yang dikaji.

(Zingiber officinale, micropropagation , shoot buds)

INTRODUCTION

Ginger (*Z. officinale* Rosc.) belonging to the family Zingiberaceae is an important spice crop and is also widely used in traditional medicine and culinary preparations. In practice, it is vegetatively propagated through underground rhizomes, but at a very low multiplication rate. Heavy losses in yield have been reported due to bacterial wilt (*Pseudomonas solanacearum*), soft rot (*Phytium aphanidermatum*) and nematodes (*Meloidogyne* spp.) [1, 2]. Since these diseases are mainly transmitted through rhizomes, the production of disease-free clones with a rapid multiplication rate is necessary for a successful commercial cultivation of this crop.

In the present study, experiments were conducted to establish micropropagation protocols from ginger shoot buds using various concentrations of growth hormone. Three varieties of *Z. officinale* with valuable medicinal properties were used as plant materials which are locally known as '*halia*' (*Z. officinale* Rosc. var. *officinale*), '*halia bara*' (*Z. officinale* Rosc. var. *rubrum* Theilade) and '*halia padi*' (*Z. officinale* Rosc. var. *rubrum* Theilade).

MATERIALS AND METHODS

Four week-old shoot buds from the varieties studied were used as experimental materials. Shoot buds were washed thoroughly and the outer leaves were removed and subsequently explants were cut into 2-3 cm sections. Buds were soaked in 15.0% (v/v) domestic chlorox including 1-2 drops Tween 20 for 15-20 minutes followed by 0.5% (w/v) $HgCl_2$ for five minutes, 70% (v/v) ethanol (one minute) and then rinsed in sterile distilled water (three times). After sterilisation, buds were cut approximately to 1.0 cm and were placed into culture medium.

Murashige and Skoog (MS) [3] were used as the basal medium containing 3.0% (w/v) sucrose and 0.2% (w/v) phytagel. Different concentrations of

BAP (0-10.0 mg/l) were added to the basal medium. The pH of the medium was adjusted to 5.8 before autoclaving. The cultures were incubated at 25±2°C and 16 hours photoperiod.

Contaminant free explants grown on the MS medium without hormones for eight weeks were then transferred to fresh MS media supplemented with different concentrations of BAP for shoot multiplication. Repeated subcultures were carried out until four times at four week intervals before a suitable and optimum medium was determined. The number and length of shoots and roots obtained per explant were recorded for each subculture. Explants that produced the highest number of new shoots in the optimum medium were selected for further subcultures.

Plantlets with well developed shoots and roots were transferred to soil mixed with 'cocopeat' (1:1) for field evaluation. Micro propagated plants were compared with conventional propagated plants for various morphological and rhizome characters. The control plants were initially taken from one well rooted shoot, excluding the rhizome and were planted in uniform sized pots as the ones used for growing the *in vitro* plantlets. Data on plant height, number of tillers per plant, leaf area, number of leaves and rhizome weight were determined after 8 months of field transplantation.

RESULTS AND DISCUSSION

All varieties of *Z. officinale* best responded on MS medium with 3.0% (w/v) sucrose, 0.2% (w/v) phytagel supplemented with low concentrations of BAP (1.0-3.0 mg/l). Two to eight shoots per explant were produced with simultaneous root formation. Two types of shoots were initiated from one explant i.e single shoots from the main axis and axillary buds at the base of the explants.

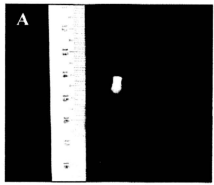
The optimum level of BAP is 1.0 mg/l for '*halia*' and '*halia bara*' and 3.0 mg/l for '*halia padi*'. The optimum media were selected based on the highest number of shoots produced. At higher concentrations of BAP (5.0-10.0 mg/l), less number of shoots was formed.

Shoots produced were compact, folded and recalcitrant to elongation. Very rare or no rooting were observed for all varieties. This showed that propagation was only suitable at low concentrations of BAP for the varieties studied. Similar results were also reported by Sharma and Singh [5] where kinetin was used to propagate *Z. officinale*. At high concentrations of kinetin, less shoot elongation, folded leaves and very rare rooting were observed. Upadhyay *et al.* [6] made an in-depth study on the influence of cytokinins (BA, 2ip and kinetin) individually where each cytokinin was used at high concentrations (3.0-5.0 mg/l) in the media. It was observed that an increase in the concentration led to an increase in the frequency of the incidence of abnormalities such as vitrification and fasciation of the shoots formed.

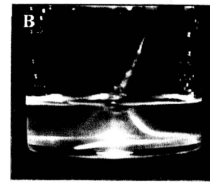
After three subcultures (4 weeks intervals) on optimum medium, no adverse effect was observed either on the rate of proliferation or quality of plantlet. Table 1 showed that 'halia padi' was more productive in terms of shoot multiplication compared to other varieties cultured in the optimum media.

In vitro derived plantlets with well-developed root and shoot system were successfully transferred to pots in potting mixture containing soil and cocopeat (1:1), and healthy rhizomes were obtained after harvesting. The performance of micro propagated plants for five quantitative traits under field conditions is given in Table 2. Average plant height, number of tillers per plant, number of leaves, leaf area and rhizome weight per plant for all varieties revealed that the micro propagated plants were superior compared to the control plants. The higher number of tillers and leaves suggested a residual effect of the cytokinins used in the culture medium [4].

The present experiment has demonstrated a very simple protocol for the rapid propagation of a medicinally important herb such as *Z. officinale*. This protocol may also be adapted as a part of the *in vitro* conservation for germplasm collection.



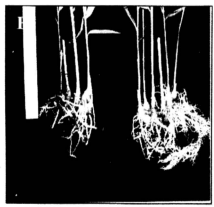
Explant



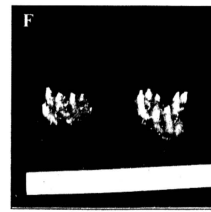
Initiation stage
(Establishment of contaminant-free explant)

Shoot multiplication (2 weeks in culture)

Field planting



in vivo plant (left) and *in vitro* plant (right)



in vivo rhizome (left) and *in vitro* rhizome (right)

Figure 1. Stages of micro propagation on '*Halia bara*'

Table 1. Number, average number and maximum length of shoots and roots per explant produced on optimum medium after 3 subcultures.

Species	Optimum medium	Number of shoots per explant	Average number of shoots per explant	Number of roots per explant	Average number of roots per explant	Max. length of shoot cm)	Max. length of root (cm)
Halia	MS + 1.0 mg/l BAP	2-6	4.2 ± 0.2	7-18	11.5 ± 0.3	5.0	7.5
Halia bara	MS + 1.0 mg/l BAP	2-5	3.3 ± 0.2	5-17	9.9 ± 0.4	4.5	7.0
Halia padi	MS + 3.0 mg/l BAP	3-8	4.8 ± 0.5	8-17	11.8 ± 0.9	4.5	8.0

Table 2. Field performance of micro propagated and control plants for five quantitative traits

Parameter study	*Halia*		*Halia bara*		*Halia padi*	
	in vitro	control	*in vitro*	control	*in vitro*	control
No. of tillers	6.0 ± 1.0	4.6 ± 0.9	4.8 ± 0.8	3.4 ± 0.6	6.6 ± 0.6	4.4 ± 1.1
Plant height (cm)	56.3 ± 5.2	57.2 ± 9.6	26.3 ± 2.3	24.5 ± 3.9	24.3 ± 6.1	24.8 ± 4.7
No. of leaves	64.8 ± 16.3	54.8 ± 7.4	35.0 ± 6.4	31.4 ± 3.1	52.0 ± 15.8	33.0 ± 4.7
Leaf area (cm^2)	27.6 ± 1.2	25.9 ± 1.4	20.5 ± 0.4	18.2 ± 0.6	17.4 ± 0.7	17.2 ± 0.3
Fresh weight of rhizome (g)	50.7 ± 23.3	38.4 ± 20.0	19.3 ± 3.2	12.0 ± 5.0	21.8 ± 4.5	11.3 ± 4.9

Acknowledgements We wish to thank the Ministry of Science, Technology and Environment (NSF scholarship and IRPA grant: 08-02-03-0229.) and University of Malaya (Vote F (2000&2001) for the financial support.

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Differentiation of Three Varieties of *Zingiber officinale* Rosc. by RAPD Fingerprinting

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ABSTRACT The genetic variation between three varieties of *Zingiber officinale* Rosc. was investigated using Random Amplified Polymorphic DNA (RAPD) analysis. The varieties are reported to be useful in traditional medicine and food flavours in Peninsular Malaysia. Analysis was carried out on the RAPD profiles generated by 16 arbitrary primers to determine genetic differences between the varieties. The clearest polymorphic bands were obtained from OPA1, OPA8, OPA9, OPA10, OPA13, OPA16 and OPA20 primers. A total of 104 bands were scored and analysed.

ABSTRAK Perbezaan genetik di antara tiga varieti *Zingiber officinale* Rosc. dikaji dengan menggunakan teknik 'Random Amplified Polymorphic DNA' (RAPD). Varieti-varieti tersebut merupakan tumbuhan yang banyak digunakan di dalam perubatan tradisional dan sebagai perasa masakan di Semenanjung Malaysia. Analisis dilakukan berdasarkan profil-profil RAPD yang dihasilkan dengan menggunakan 16 jenis primer rambang untuk menentukan perbezaan genetik di antara varieti tersebut. Jalur-jalur polimorfik yang jelas telah diperolehi daripada primer-primer OPA1, OPA8, OPA9, OPA10, OPA13, OPA16 dan OPA20. Sejumlah 104 jaluran telah diskor dan dianalisa.

(*Zingiber officinale*, RAPD, primer, ginger, fingerprint)

INTRODUCTION

Holttum [1] recorded 13 species of *Zingiber* for Peninsular Malaysia and of these approximately 5 to 6 species are widely cultivated. In Malaysia, the cultivated species of *Zingiber* such as *Z. officinale*, *Z. montamum* provide an important source for spices, flavour and traditional medicine.

Holttum [1] also reported on three local races for *Zingiber officinale* Rosc., but only two races were discovered and these are not common. These races differ in size and colour of their rhizomes. Mature plants of *Z. officinale* Rosc. var. *officinale* (local name - halia), *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) may be confused in their general appearance in the absence of their inflorescences or flowers. Theilade [2] reported that *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) can only be differentiated from *Z. officinale* Rosc. var. *officinale* by its smaller, red coloured rhizomes which have stronger and more pungent smell.

At present, there is limited information on the RAPD markers of Zingiberaceae species. RAPD markers have been found to be useful in characterizing and studying the diversity of crop plants [3, 4]. RAPD markers have been found to be useful in differentiating species, varieties, cultivars and races especially in the absence of flower parts. The aim of this study is to analyse the genetic variation between the varieties investigated and to evaluate the significance of these molecular fingerprint.

MATERIALS AND METHODS

Plant Material and DNA extraction

Leaves of *Z. officinale* Rosc. var. *officinale* (halia) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) were collected from plants that were grown at Rimba Ilmu, University

var. *rubrum* Theilade (halia padi) which were collected at Muar, Johor and were planted at Rimba Ilmu. DNA was extracted from very young leaves using modified CTAB method [5] with additional RNase treatment.

RAPD Amplification Condition

The RAPD reaction mixture consisted of the following ingredients in a final volume of 25µl, autoclaved water, 12.5µl RAPD 'Master Mix' (Fermentas) containing Taq DNA polymerase (0.05units/µl), $MgCl_2$ (4mM) and dNTPs (0.4mM), 50 picomoles primer and 50 ng template DNA. Random primers (10-mer kits) (Operon Technologies,Inc., CA.) were used as sequences of Kit labeled OPA1 to OPA20. All the primer sequences given are in the 5' to 3' direction. RAPD was carried out using PCR machine (Eppendorf-Mastercycler gradient). The amplification conditions were as follows: 94ºC for 90 sec and 45 sec (DNA denaturation), 40ºC for 30 sec (DNA annealing), and 72ºC for 120 seconds (DNA synthesis) for 40 cycles. After the completion of 40 cycles, the reactions were kept at 4ºC. The RAPD products were separated by agarose (1.7%) gel electrophoresis in 0.5X TAE buffer at 80 volts for about two hours. The gels were viewed and photographed under UV light. The bands were visually scored as present (1) or absent (0). The band sizes were estimated by comparing them to bands of GeneRuler 100bp DNA Ladder Plus (Fermentas). To compare and calculate genetic distance between pairs of variety studied, a formula called similarity index (S) was used. S was calculated from band sharing data for each pair of bands according to the formula:

$$S_{xy} = 2n_{xy}/n_x + n_y$$

Where n_x and n_y represent the number of scorable fragments from individual x and y, respectively. The number of fragments shared by both individuals is represented by n_{xy}. S will give the value of similarity index between 0 and 1, where values near to 0, signify less similarity or not similar and values near to 1, indicate high similarity.

RESULTS AND DISCUSSION

RAPD technique is often described as not reliable because of the inability to reproduce the results. Hence in this work, RAPD analysis was repeated to ensure the reproducibility of the banding pattern and concomitantly the validity of the results.

Out of 20 primers examined, only 16 primers produced scorable bands. The remaining four primers either did not produce any bands or the bands were not clear enough to be evaluated. In evaluating the number of bands, only those bands with clear intensities were used. The size of RAPD bands produced was between 2333 bp to about 135 bp (Table 1). The clearest banding patterns differentiating the three varieties studied were produced by seven primers (43.8% of all primers tested) i.e. OPA1, OPA8, OPA9, OPA10, OPA13, OPA16 and OPA20 where a total of 62 scorable bands were evaluated (Table 1). No polymorphic bands were observed from primers OPA2, OPA3, OPA4, OPA5, OPA6, OPA7, OPA11, OPA15 and OPA18. Bands of equal sizes (monomorphic patterns) obtained in the three varieties studied are 27 (65.9% genetic similarity) out of a total of 41 bands produced. The highest number of RAPD bands observed for *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) was 6 (OPA20) and 5 for *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) (OPA20).

Figure 1 shows polymorphic patterns in primers OPA1, OPA8 and OPA20. In primer OPA1 and OPA8, one specific band was obtained at 1500 bp for *Z. officinale* Rosc. var. *officinale* and 2000 bp for *Z. officinale* Rosc. var. *rubrum* Thelaide (halia padi) respectively. Monomorphic bands were visualised at 1031 bp and 500 bp (OPA1), 1500 bp and 550 bp (OPA8) and 1750 bp, 1200 bp and 700 bp (OPA20).

The binary matrix of polymorphic primers is presented in Table 2. The number of rare RAPD markers was very low that is only five variety specific markers were found (OPA1 – 1500 bp marker and OPA20 – 1350 bp marker, specific for *Z. officinale* Rosc. var. *officinale*; OPA20 – 800 bp marker, specific for *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara); OPA8 – 2000 bp marker and OPA16 – 1031 bp marker, specific for *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi). Table 2 showed that all 41 patterns were identified by as few as seven of the 16 informative primers and from a total of 14 bands (OPA1 – 1500 bp; OPA8 – 2000 bp; OPA9 – 400 bp; OPA10 – 1200 bp, 600 bp, 150 bp; OPA13 – 200 bp, 1200 bp, 900 bp; OPA16 – 1031 bp; OPA20 – 1350 bp, 900 bp, 800 bp, 550 bp). The results also showed that *Z. officinale* Rosc. var.

officinale (halia) shared 32 bands (82.1%) with *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) higher than 27 bands (67.5%) shared with *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi). Whilst, *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) shared 31 bands (77.5%) with *Z . officinale* Rosc. var. *rubrum* Theilade (halia padi).

From the values of the similarity index calculated, the three varieties of the *Z. officinale* were shown to be very similar (Table 3). However, in comparison, *Z. officinale* Rosc. var. *officinale* (halia) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) showed the highest S value (0.94), followed by the latter and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) (0.91) and the most dissimilar being, *Z. officinale* Rosc. var. *officinale* (halia) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) at 0.84.

These results implicate that there is low genetic variation within *Z. officinale* varieties, which is in agreement with their close morphological affinities. With the observed similarities and dissimilarities in RAPD profiles and morphology, we may conclude that *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) should not be treated as synonyms. Earlier report [6] has regarded 'halia bara' as synonymous to 'halia padi'. This result may not be conclusive but a very good indicator of such differentiation among varieties and other DNA finger printing methods could be employed to further strengthen these results.

Table 1. RAPD bands of *Z. officinale* Rosc. var. *officinale*, *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) for selected OPA primers and estimated size of the bands (bp) distinguishing the varieties.

Primers	H	HB	HP	No. of shared bands by 3 varieties	Estimated size (bp) of the most distinguishable bands for the 3 varieties studied		
					H	HB	HP
OPA1	3	2	2	2	1436,1039,491	1019,485	1012,479
OPA2	1	1	1	1	312	310	310
OPA3	2	2	2	2	911,782	890,782	886,777
OPA4	2	2	2	2	2278,575	2333,571	2333,571
OPA5	2	2	2	2	1519,668	1519,668	1556,664
OPA6	1	1	1	1	975	984	966
OPA7	1	1	1	1	1750	1788	1750
OPA8	2	2	3	2	1586,548	1586,548	1931,1500,544
OPA9	2	3	3	2	1200,857	1162,850,448	1125,864,439
OPA10	2	3	1	0	1192,613	1175,608,135	135
OPA11	1	1	1	1	753	753	753
OPA13	4	4	1	1	1963,1137,955,632	1925,1119,960,626	639
OPA15	2	2	2	2	1893,733	1929,740	1893,727
OPA16	3	3	4	3	1330,727,486	1322,727,486	1314,1056,736,497
OPA18	2	2	2	2	1183,717	1183,706	1183,711
OPA20	4	6	5	3	1740,1325,1130,671	1720,1120,939,763,676,548	1720,1130,939,676,548

*The most prominent bands are underlined

H = *Z. officinale* Rosc. var. *officinale* (halia), HB = *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara), HP = *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi).

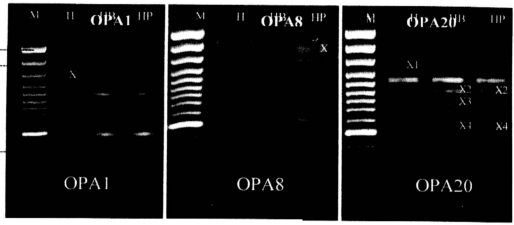

Figure 1. RAPD markers produced by primers OPA1, OPA8 and OPA20.

'X' shows the polymorphic banding patterns distinguishing all varieties studied;
(OPA1; X=1500bp, OPA8; X=2000bp, OPA20; X1=1350bp, X2=900bp, X3=800bp, X4=550bp)
H = *Z. officinale* Rosc var.*officinale* (halia), HB = *Z. officinale* Rosc var. *rubrum* Theilade (halia bara),
HP = *Z. officinale* Rosc var. *rubrum* Theilade (halia padi), M = Marker 100bp Ladder Plus.

Table 2. RAPD markers of three varieties of *Z. officinale*

Primer	bp	*Z. officinale* var. *officinale*	*Z. officinale* var. *rubrum*	*Z. officinale* var. *officinale* (halia padi).
OPA1	1500	1*	0	0
	1031	1	1	1
	500	1	1	1
OPA2	300	1	1	1
OPA3	900	1	1	1
	800	1	1	1
OPA4	2000	1	1	1
	600	1	1	1
OPA5	1500	1	1	1
	700	1	1	1
OPA6	1031	1	1	1
OPA7	1250	1	1	1
OPA8	2000	0	0	1*
	1500	1	1	1
	550	1	1	1
OPA9	1200	1	1	1
	900	1	1	1
	400	0	1	1
OPA10	1200	1	1	0
	600	1	1	0
	150	0	1	1
OPA11	800	1	1	1
OPA13	2000	1	1	0
	1200	1	1	0
	900	1	1	0
	600	1	1	1
OPA15	2000	1	1	1
	700	1	1	1
OPA16	1300	1	1	1
	1031	0	0	1*
	700	1	1	1
	500	1	1	1
OPA18	1200	1	1	1
	700	1	1	1
OPA20	1750	1	1	1
	1350	1*	0	0
	1200	1	1	1
	900	0	1	1
	800	0	1*	0
	700	1	1	1
	550	0	1	1

1- bands present, 0- bands absent, * - variety specific band

Table 3. Values of Similarity Index between varieties of *Zingiber officinale*.

Primer	H + HB	H + HP	HB + HP
OPA1	0.8	0.8	1.0
OPA2	1.0	1.0	1.0
OPA3	1.0	1.0	1.0
OPA4	1.0	1.0	1.0
OPA5	1.0	1.0	1.0
OPA6	1.0	1.0	1.0
OPA7	1.0	1.0	1.0
OPA8	1.0	0.8	0.8
OPA9	0.8	0.8	1.0
OPA10	0.8	0.0	0.5
OPA11	1.0	1.0	1.0
OPA13	1.0	0.4	0.4
OPA15	1.0	1.0	1.0
OPA16	1.0	0.9	0.9
OPA18	1.0	1.0	1.0
OPA20	0.6	0.7	0.9
	0.94	**0.84**	**0.91**
	82.1%	**67.5%**	**77.5%**

H = Z. *officinale* Rosc. var. *officinale* (halia), HB = Z. *officinale* Rosc. var. *rubrum* Theilade (halia bara), HP = Z. *officinale* Rosc. var. *rubrum* Theilade (halia padi).

CONCLUSION

Polymorphic RAPD patterns distinguishing *Z. officinale* Rosc. var. *officinale*, *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) were generated using 16 primers. Based on these results, it can be concluded that RAPD technique is useful for the differentiation of varieties of *Z. officinale* using the primers used in this work.

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