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Establishment of Tissue Culture for selected Medicinal *Curcuma* spp.

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ABSTRACT The effect of cytokinin on shoot multiplication was studied to establish a micropropagation protocol. An average of eight shoots per explant was produced within four weeks on agar solidified Murashige and Skoog medium supplemented with 3.0% sucrose. The optimum concentrations of BAP for *in vitro* multiplications were 3.0 mg/l for all species studied. Plantlets were rooted and successfully acclimatized to field conditions. The field performances between *in vitro* and *in vivo* plants were compared. The result showed very significantly that tissue culture derived plants were better than the ones that were conventionally propagated.

ABSTRAK Kesan sitokinin ke atas multiplikasi pucuk telah dikaji untuk mendapatkan protokol mikropropagasi. Media optima untuk spesies kajian ialah media yang dirawat dengan 3.0 mg/l BAP. Purata lapan pucuk per eksplan telah diperolehi dalam masa empat minggu di atas media Murashige dan Skoog yang mengandungi 3.0% sukrosa. Setelah akar terbentuk plantlet telah berjaya dipindahkan ke rumah hijau. Hasil kajian lapangan di antara tumbuhan *in vitro* dan *in vivo* dibandingkan. Keputusan menunjukkan hasil yang signifikan diperolehi daripada tumbuhan kultur tisu berbanding dengan tumbuhan yang ditanam secara konvensional.

(Shoot multiplication, micropropagation)

INTRODUCTION

Curcuma, belonging to the family Zingiberaceae is a large and important genus in terms of forest biodiversity, economic products and horticulture, and is widely spread over the tropics. *Curcuma* has medicinal property from which rhizomes are commercially exploited. Natural products from this genus are widely used in perfumes, in the food industry as condiments and dyes, and medicine. It has been recently studied for its anti tumor [8, 11], hepatoprotective [12], anti inflammatory [10], antioxidant [19], antimicrobial activities [6] and analgesic effects [5]. Several reports of *in vitro* culture from the Zingiberaceae family have been published [9, 17, 18, 13, and 16]. However, the planting material has a very low multiplication rate. Moreover, many of the *Curcuma* species are susceptible to soft rot disease [1]. Hence, huge amounts of planting material are required every year for plantation purposes [2]. In this study, we report an efficient protocol for *in vitro* micropropagation of six selected medicinal *Curcuma* species through shoot bud.

MATERIALS AND METHODS

Plant material

Rhizomes of *Curcuma aeruginosa* Roxb., *Curcuma mangga* Val., *Curcuma xanthorrhiza* Roxb. and *Curcuma zedoaria* Rosc. were purchased from the local market while rhizomes of *Curcuma rubescens* Roxb. and *Curcuma inodora* aff. were collected from the Malaysian Agricultural Research And Development Institute (MARDI), Hilir Perak. Young sprouting shoots excised from rhizomes were used as the source of explants and were washed with tap water. Under aseptic conditions, shoots were surface sterilized with 20% (v/v) chlorox for 15 minutes followed by a 0.5% (w/v) $HgCl_2$ solution for five minutes and rinsed three times with sterile distilled water. Shoots had their external leaves removed and finally the shoot buds were cut into two different

sizes (a] above 1.0 cm, b] below 1.0 cm) and inoculated on the culture medium.

Culture Media

Explants were inoculated on sterile Murashige and Skoog (MS) [13] medium containing 3.0% (w/v) sucrose and 0.2% (w/v) phytagel as solidifying agent. Basal medium was supplemented with cytokinin (Benzylaminopurine [BAP]) alone or in combinations with auxin (Naphthalene acetic acid [NAA]). Several concentrations of BAP and NAA were attempted (Table 1). The effect of BAP and NAA were studied by adding different levels of the hormone to the medium. The pH of the medium was adjusted to 5.8 before the addition of agar. Jam jars were used as culture vessels. A volume of 30 ml of media was dispensed respectively to the jam jars and closed, and the media was sterilized at a pressure of 1.06 kg cm^2 for 20 minutes. All the cultures were incubated at 25°C with a photoperiod of 16/8 hours (light/dark) conditions. Transfers to fresh medium and subculturing were carried out every 30 days. Well-grown shoots from the shoot multiplication medium were then rooted on optimum medium supplemented with 0.05g/l activated charcoal and solidified with 0.2% (w/v) phytagel before acclimatization.

Table 1. MS medium supplemented with various concentrations of BAP and NAA

MSO
MS + 0.5 mg/l BAP
MS + 1.0 mg/l BAP
MS + 3.0 mg/l BAP
MS + 5.0 mg/l BAP
MS + 10.0 mg/l BAP
MS + 1.0 mg/l BAP + 1.0 mg/l NAA
MS + 1.0 mg/l BAP + 0.5 mg/l NAA

Acclimatization

The *in vitro* micropropagated plants were individually transferred to 200ml plastic pots, filled with a mixture of garden soil and sand (1:1 w/w) as substrate for acclimatization.

Small rhizome segments with single axillary shoot and similar height with *in vitro* plantlet were chosen as the control (*in vivo*). It was then individually transferred to 200ml plastic pots in the same condition with *in vitro* plantlet. After twelve months in the green house, various measurements were carried out to compare between *in vitro* and *in vivo* plant (Table 2).

RESULTS AND DISCUSSION

Since the explants were taken from underground rhizomes, establishment of contamination free culture was a major task [9]. In this study, rhizomes were sprouted in soil free condition until shoot buds appear. These shoot buds without any rhizomes were used as explants. About 70% of the explants remained free of contamination after four weeks in MS medium. Hosaki and Sagawa [5] also reported a high degree of contamination in *in vitro* culture of *Curcuma* after sterilizing with 0.5% (v/v) $HgCl_2$. Once the contamination-free cultures of the shoot buds were established, these were easily maintained by sub culturing on fresh medium. Axillary buds developed from the base of the main shoot grew vigorously (Figure 1c).

The best results were obtained using rhizome shoot buds of less than 1.0 cm as explants for micropropagation of *Curcuma*. The explants which responded to BAP and NAA, individually and in combination, were evaluated after 30 days of inoculation (Figure 1). Explants transferred to MS with BAP and NAA resumed growth, producing shoot and root simultaneously (Figure 1e). Of the different concentrations of BAP, 3.0 mg/l was very effective, which induced 6.0 shoots per explant (Figure 1c). Increased concentrations of BAP reduced the number of shoots (Figure 1d). This result was in agreement with previous researchers [4]. The cytokinin BAP promotes cell division, shoot multiplication and axillary bud formation while inhibiting root development [5]. Higher or lower levels of BAP decreased the number of shoots (Figure 1b and 1d). Of the different type of hormone, BAP was superior, which developed 6.0 shoots per explant (Figure 1c).

The occurrence of diverse morphogenetic responses were observed, and could basically be grouped into a) explants presenting different levels of development with high micropropagation frequency (more than five shoots per explant) and variable number of leaves, occurring in the presence of low BAP concentrations (3.0 mg/l) and absence of NAA; b) explants with low level of development and low micropropagation frequency (on the average less than three shoots per explant), occurring in the presence of high concentration of both BAP (5.0 and 10.0 mg/l) and NAA (1.0 mg/l); c) explants presenting root formation and low

micropropagation frequency (on the average less than two shoots per explant or absent), occurring in the presence of NAA (1.0 mg/l) and absence of BAP. The two morphogenetic types (b and c) which did not show good developmental stages were discarded. Culture media containing high BAP and NAA concentration or absence of BAP were not recommended for *Curcuma* micropropagation.

Micropropagation quantified by the number of shoots/explant was higher in the absence of NAA but was BAP-dependent. Multiplication frequency was increased until BAP concentration reached 3.0 mg/l. The frequency of plants with well-formed shoots was BAP-dependent and was inhibited by NAA.

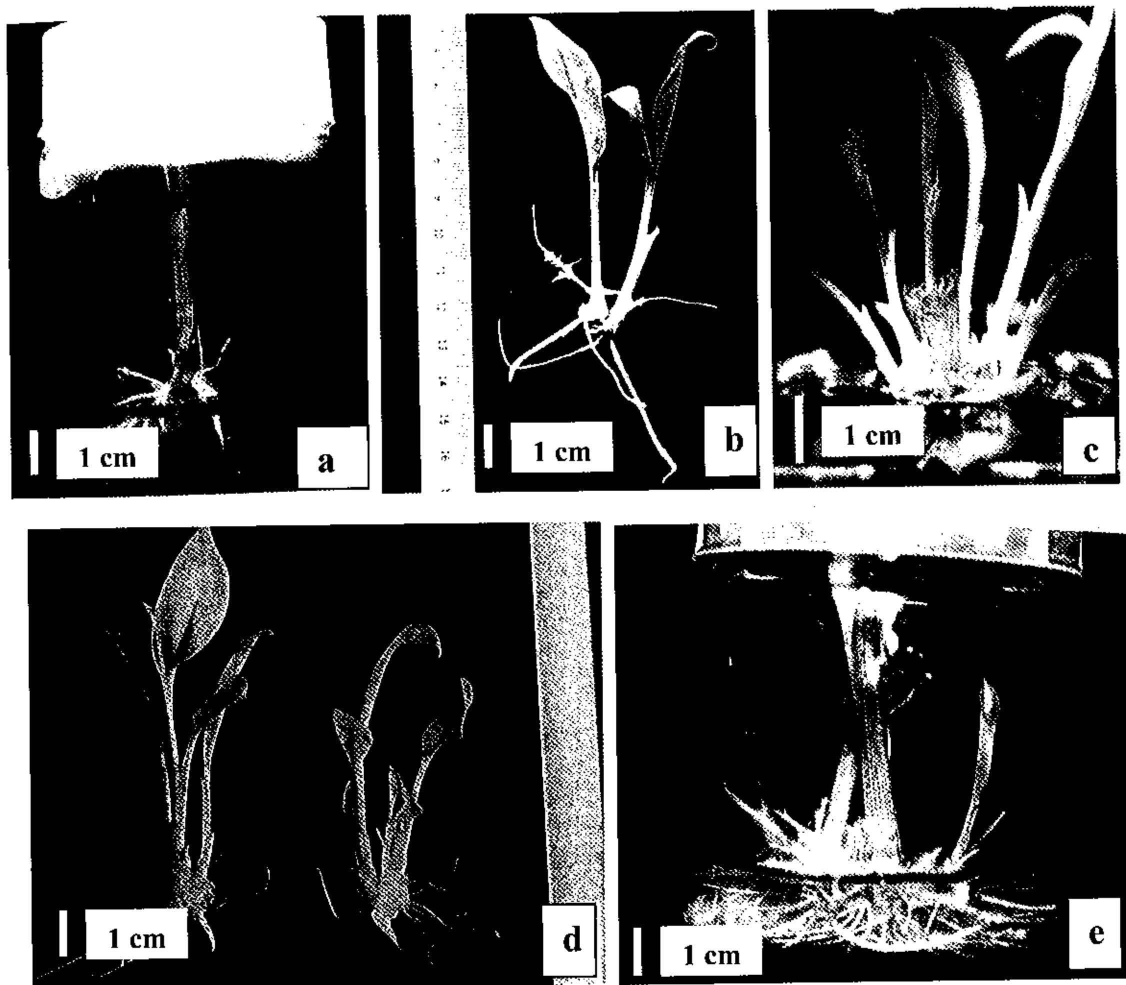


Figure 1. Effect of using BAP hormone after 4 weeks.
a) Explant in MS medium did not induce any multiple shoots.
b) Explant in MS medium + 1.0 mg/l BAP produced 2 multiple shoots.
c) Explant in MS medium + 3.0 mg/l BAP produced 6 multiple shoots.
d) Explant in MS medium + 5.0 mg/l BAP (left) produced 3 multiple shoots and explants in MS medium + 10.0 mg/l BAP (right) produced 3 multiple shoots but abnormal.
e) Explant in MS medium + 1.0 mg/l NAA produced 2 multiple shoots and more roots (absence of BAP).

ACCLIMATIZATION

The highest acclimatization frequency occurred after 20 days in the greenhouse. Plants with well developed roots were transferred to polybags containing sand and soil (1:1 w/w) for acclimatization for three weeks before transplanting to the field. Microrhizomes

sprouted and were planted in sand. Sprouted microrhizomes were easily field transferred [16].

After six months, field evaluation was carried out by determining the number of leaves per plant, height of plant, length and width of leaves and weight of rhizomes and tubers (data not shown). The second field evaluation was undertaken after twelve months at the time of harvest (Figure 2, Figure 3). All parameters measured during the second evaluation showed an increase over the first one.

Various measurements were compared between *in vitro* (12 months) and *in vivo* (12 months) plants so as to evaluate the growth and yield from both sources. The result showed very significantly that tissue culture derived plants were better than the ones that were conventionally propagated (Table 2).

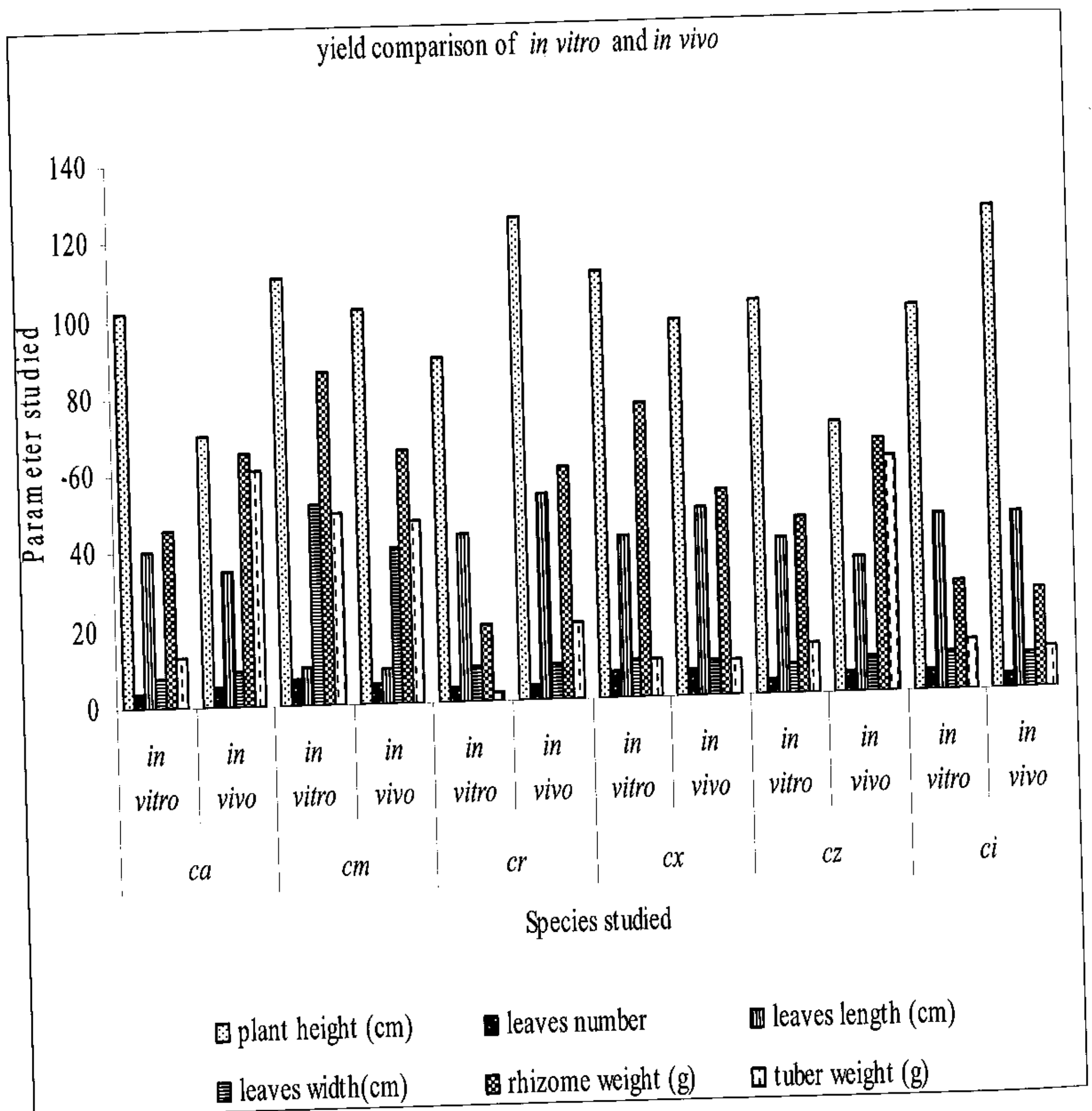
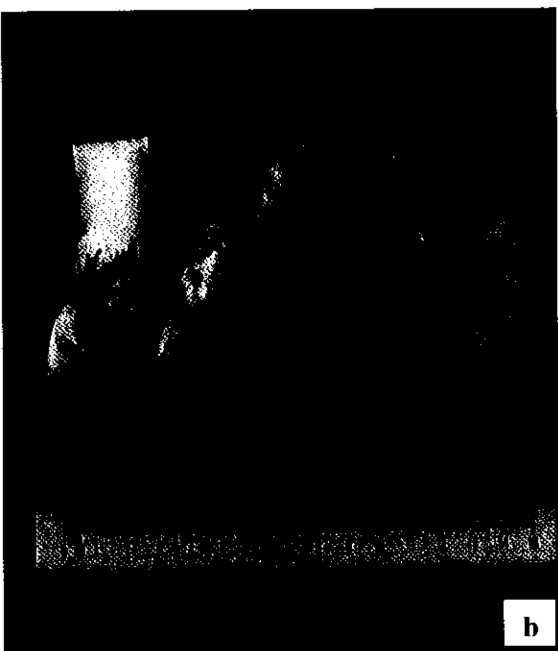


Figure 2. After twelve months, field evaluation was carried out where parameters such as number of leaves per plant, height of plant, length and width of leaves and weight of rhizomes and tubers were measured.
ca: *Curcuma aeruginosa; cm*: *Curcuma mangga; cr*: *Curcuma rubescens; cx*: *Curcuma xanthorrhiza; cz*: *Curcuma zedoaria* and *ci*: *Curcuma inodora* aff.



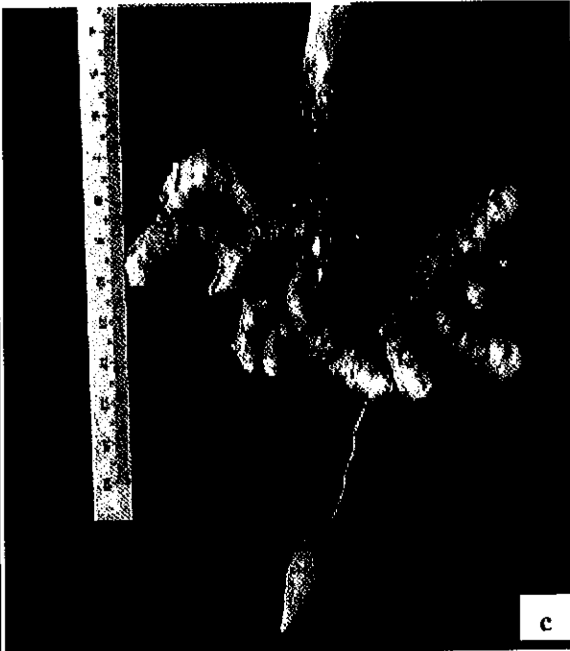


Figure 3. Rhizomes of *in vitro and in vivo*

a) *In vitro C. xanthorrhiza* in green house after 10 months
b) *In vivo* rhizome of *C. xanthorrhiza*
c) *In vitro* rhizome of *C. xanthorrhiza*

Table 2. Result of various measurements in comparison between *in vitro* and *in vivo* after twelve months of transfer to the greenhouse.

SPECIES/MEASUREMENT	*In vitro*	*In vivo* (CONTROL)
C. aeruginosa		
Plant height (cm)	100.0 ± 0.3	90.2 ± 1.1
Number of leaves	6.5 ± 0.2	5.3 ± 0.5
Leaves length (cm)	8.7 ± 0.3	8.2 ± 1.0
Leaves width (cm)	43.5 ± 0.4	40.3 ± 1.7
Weight of rhizome (g)	77.9 ± 2.3	65.8 ± 2.1
Weight of tuber (g)	39.3 ± 2.0	37.9 ± 1.5
C. mangga		
Plant height (cm)	110.0 ± 0.3	102.2 ± 2.1
Number of leaves	7.0 ± 0.4	5.3 ± 0.2
Leaves length (cm)	9.8 ± 0.3	8.9 ± 1.0
Leaves width (cm)	51.5 ± 0.9	40.3 ± 1.7
Weight of rhizome (g)	85.9 ± 2.3	65.8 ± 3.1
Weight of tuber (g)	49.3 ± 2.0	46.9 ± 2.6
C. rubescens		
Plant height (cm)	89.0 ± 5.7	125.0 ± 5.2
Number of leaves	4.1 ± 2.5	4.0 ± 0.2
Leaves length (cm)	43.0 ± 2.2	53.0 ± 1.5
Leaves width (cm)	9.0 ± 0.6	9.5 ± 2.1
Weight of rhizome (g)	19.6 ± 1.0	60.4 ± 5.2
Weight of tuber (g)	2.1 ± 0.4	20.1 ± 3.0
C. xanthorrhiza		
Plant height (cm)	110.0 ± 3.1	97.6 ± 2.8
Number of leaves	7.0 ± 0.5	6.7 ± 0.5
Leaves length (cm)	42.0 ± 3.1	49.0 ± 3.4
Leaves width (cm)	10.0 ± 1.0	9.5 ± 2.1
Weight of rhizome (g)	76.0 ± 3.2	52.9 ± 2.3
Weight of tuber (g)	10.3 ± 2.0	9.1 ± 0.7
C. zedoaria		
Plant height (cm)	102.0 ± 4.1	70.0 ± 2.7
Number of leaves	4.0 ± 0.2	5.0 ± 1.0
Leaves length (cm)	40.2 ± 2.3	35.0 ± 1.8
Leaves width (cm)	7.6 ± 1.1	8.8 ± 1.2
Weight of rhizome (g)	45.7 ± 4.2	65.1± 3.3
Weight of tuber (g)	13.0 ± 2.1	61.1 ± 2.6
***C. inodora* aff.**		
Plant height (cm)	100.0 ± 4.2	125.0 ± 3.2
Number of leaves	5.0 ± 0.3	4.0 ± 0.2
Leaves length (cm)	46.0 ± 0.1	45.5 ± 4.1
Leaves width (cm)	10.0 ± 0.3	9.5 ± 2.5
Weight of rhizome (g)	28.2 ± 2.1	26.2 ± 0.9
Weight of tuber (g)	13.3 ± 1.3	10.5 ± 0.6

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