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Improved *in vitro* propagation of *Curcuma caesia*, a valuable medicinal plant

(Penambahbaikan dalam pembiakan *in vitro Curcuma caesia*, tumbuhan ubatan berharga)

A.R. Zuraida*

Keywords: BAP, NAA, micropropagation, *kunyit hitam*, medicinal plant, tissue culture

Abstract

Curcuma caesia is a perennial herb with high economic importance owing to its putative medicinal properties. An efficient protocol for the micropropagation of *C. caesia* followed by successful acclimatization to soil was developed. Sterilised rhizome buds were cultured on Murashige and Skoog's (MS) medium supplemented with or without BAP (5 mg/litre). The optimum length of rhizomes to produce microshoots at initial stage cultured on medium supplemented with 5 mg/litre BAP was more than 2 cm. For microshoot propagation, explants were cultured on medium supplemented with different concentrations and combinations of BAP (6-benzyl-amino-purine) and NAA (α-naphthalene acetic acid). Explants cultured on MS basal medium supplemented with 3 mg/litre BAP showed highest proliferation rate (95%) while average number of microshoots produced was 9.5. *In vitro* microshoots that were subcultured on full salt strength of MS media supplemented with 3 mg/litre BAP and 0.5 mg/litre NAA performed better in terms of the number of shoots produced as well as rooting. Rooted shoots transplanted in the glasshouse for hardening showed 95% survival rate.

Introduction

Curcuma caesia Roxb. is a member of the family Zingiberaceae and in Malaysia, it is popularly known as *kunyit hitam* (black turmeric). It is a perennial herb with high economical importance owing to its putative medicinal properties. Besides being a food, it is used to treat many medical conditions, including rheumatoid arthritis, diabetes, heart diseases and other conditions related to inflammation (Baker 1894). This herb is also believed to provide protection against Alzheimer's disease and inflammatory bowel diseases such as ulcerative colitis and Crohn's disease (Behar et al. 2013).

The rhizomes are also claimed to have some element that could act against leucoderma, epilepsy, cancer and HIV/AIDS (Hui et al. 2007). In India, the rhizomes are used as medicine for sprains and bruises and also employed in the preparation of cosmetics (Nadkarni 1976). The plant is regarded as very auspicious and it is often used in India for various magic remedies. The rhizomes are often used for treatment of pneumonia, cough and cold in children, and for fever and asthma in adults. The powdered *C. caesia* is used by tribal women as a face-pack during their engagement and marriage period (Kirtikar and Basu 1987).

*Biotechnology Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
Author's full name: Zuraida Ab. Rahman
E-mail: azuraida@mardi.gov.my

The endangered and rare medicinal plants can be conserved *ex situ* by the invention of advanced biotechnological approach of culturing plant cells and tissues (Nakano et al. 2005; Anisuzzaman et al. 2008). The vegetative propagation of this rare species through conventional method is considered not efficient due to low propagation rate and also takes a long time to mature. Natural propagation of *C. caesia* is very slow and even in the controlled conditions they do not yield good results. Furthermore, flowering is very rare, thus seed formation hardly occurs (Hutami and Purnamaningsih 2003). For these reasons, tissue culture technique is found to be the best alternative way to overcome such problems.

The micropropagation technique provides an alternative method of propagation which is very essential for adequate supply of planting materials. Thus, the aim of this study was to establish an efficient micropropagation protocol for plant tissue culture of *C. caesia* which can be used for rapid mass propagation, *in vitro* biomass production and conservation of *C. caesia* to meet the pharmaceutical demand. Attempts were made to evolve a low cost rapid micropropagation technology to conserve this valuable medicinal herb.

Materials and methods

Establishment of initial cultures

Underground rhizomes of *C. caesia* (*Plate 1a*) were obtained from Ladang Mak Yah nursery in Temerloh, Pahang. The rhizomes were cultivated in the glasshouse at the Malaysian Agricultural Research and Development Institute (MARDI) to allow sprouting of immature buds. The sprouted immature buds with different lengths of explants (0.5, 1.0, 2.0 and 3 – 4 cm) (*Plate 1b*) were collected and used as the source of explants. They were cleaned under running tap water for an hour, then washed with commercial laboratory detergent (Decon 5%, v/v) and rinsed thoroughly with water. The explants were then immersed in 1% (v/v) fungicide for one hour and subsequently rinsed thoroughly under running tap water for 5 min.

Subsequently, the explants were surface sterilized with 10 – 20% Clorox® and a few drops of Tween-20 under sterile conditions. Then, the explants were rinsed several times with sterile distilled water. They were then dissected with a sterile surgical blade to remove the outer layers of leaf sheaths under aseptic conditions to produce 5 – 8 mm sized pieces having at least one eye. The sterilized explants were inoculated onto Murashige and Skoog's (1962) basal medium supplemented with 3% sucrose with and without BAP (5.0 mg/litre). The pH of the medium was adjusted to 5.8 prior to autoclaving (15 min, 121 °C). The culture bottles were sealed with parafilm and incubated in the culture room under white fluorescent light with a light intensity of 3,000 lux at a photoperiodic period of 16 h at 25 ± 2 °C. The growth of the cultures was observed at weekly intervals.

Proliferation of shoots

For shoot multiplication, all survived explants were transferred on to MS or Woody Plant Medium (WPM) within 6 weeks of culture. The media used was supplemented with various concentratio ns of BAP (1, 3 and 5 mg/litre) or NAA (1, 3 and 5 mg/litre) alone and in combination of BAP (1, 3 and 5 mg/litre) and NAA (0.5 and 1.0 mg/litre) respectively. Results were expressed as percentage of proliferation and number of microshoots after 45 days of culture. To optimize the salt strength of MS medium, the clump of microshoots were subcultured on medium containing BAP (1 and 3 mg/litre) alone or in combination with NAA (0.5 and 1.0 mg/litre) with 2 different strengths: half and full strength. Data were recorded at weekly intervals. Mean number of shoots, shoot length, roots and root length was recorded after 45 days of culture. Standard error of mean was calculated for the degree of response.

Plate 1. In vitro micropropagation of Curcuma caesia. A = cross section of underground rhizomes, B = Different lengths of sprouted immature buds, C = Microshoots initiation, D, E = Development of plantlets, F = Elongation of roots, G = In vitro plantlets and H = In vivo plant after hardening. (Scale = 1 cm)

Acclimatization

Rooted explants with shoots about 4 – 5 cm long were removed from the culture bottles and the roots were washed under running tap water to remove the agar. The plantlets were individually transplanted into polybags containing organic soil and topsoil at a ratio of 1:1 maintained under controlled condition in the glasshouse with 75% shading. To ensure its humidity, the plants were watered periodically. The survival rate of the plantlets was recorded after 4 weeks.

Statistical analysis

The data were subjected to one way analysis of variance (ANOVA) to assess treatment differences and interaction using SPSS version 11. Significance between means was tested by Duncan Multiple Range Test (DMRT) ($p \leq 0.05$). The experiment

was conducted with 25 replications per treatment.

Results and discussion

Establishment of initial culture

The experiment was done to select the optimum length of immature bud to establish culture with better survival rate, minimal contamination and higher number of microshoots per explant produced. The minimal days (31 – 33 days) to first bud initiation was observed from explants with a length ranging from 2 – 4 cm (*Table 1*). The explants with 0.5 cm length required more time (41 days) to produce the first bud when compared to the other sizes. Results also indicated that the survival rate was significantly highest at 45% ($p \leq 0.05$) when the initial length of the explants was 3 – 4 cm. In addition, explants at 2 cm length showed the second highest survival rate at 31 – 35% ($p \leq 0.05$). The least survival pattern was observed in explants at 0.5 cm length as more explants turned brown and died due to injury. The appearance of browning could enhance the contamination rate in the explants. It was observed that explants at 2 – 4 cm length survived well compared to less than 2 cm.

According to Sathyagowri and Seran (2011), the length of ginger explants at 0.5 – 1.0 cm showed low survival rate during the latter part of the establishment due to endogenous microorganisms existing in the cultured explants or due to nutrient depletion in the media. Nutrient depletion of media causes browning of explants and death. This observation was further strengthened by Smith (2000) who explained that the larger explants probably contained more nutrient reserves and endogenous plant growth regulators to sustain the culture. Contamination of explants is related to several factors such as plant species, age, explants source and prevailing weather condition. Despite the best timing and selection efforts, it is almost impossible to eliminate contamination from *in vitro* plants. In fact according to Danby et al. (1993), losses due to contamination *in vitro* average between 3 and 15% at every subculture in the majority of commercial and scientific plant tissue culture laboratories, the majority of which is caused by fungal, yeast and bacterial contaminants (Leifert et al. 1994). Reducing contamination at the whole plant level as described by Matthews and Duncan (1993) is a laborious and drawn-out process.

During the culture initiation, explants with a length of 2 cm that were cultured on medium containing 5 mg/litre BAP produced a maximum mean number of microshoots at 2.4 microshoots per explant. This was followed by lengths of 3 – 4 cm (2.1 microshoots per explant) *(Table 1* and *Plate 1c*). The length of explants ranging

Table 1. The effect of various lengths of explants cultured on MS medium supplemented with or without BAP after 45 days

Length of explants (cm)	BAP (mg/litre)	Mean days to first bud initiation	Survival (%) after 45 days	Number of microshoots/explant
0.5	0	41.3 ± 6.3ab	15 ± 2.3d	0.6 ± 0.01c
	5	42.6 ± 8.1a	13 ± 4.4d	0.9 ± 0.01cb
1.0	0	36.1 ± 3.1cb	23 ± 6.7c	0.5 ± 0.01c
	5	35.3 ± 4.2cb	26 ± 3.1c	1.3 ± 0.23b
2.0	0	32.6 ± 3.4c	31 ± 5.6b	0.8 ± 0.01cb
	5	33.1 ± 2.5c	35 ± 1.1b	2.4 ± 0.24a
3 – 4	0	31.2 ± 3.5c	45 ± 6.8a	0.9 ± 0.03cb
	5	33.4 ± 4.5h	45 ± 3.4a	2.1 ± 0.04a

Data are given as means ± SD of results from 25 replicates
Means with the same letters in the same column are not significantly different at $p \leq 0.05$

from 2 – 4 cm produced significantly higher microshoots per explant ($p \leq 0.05$) compared to lengths less than 2 cm. The mean number of microshoots per explant obtained was less than one in media without BAP. This is in agreement with the findings of Da Silva et al. (2005) and Ghiorghita et al. (2005) where addition of BAP could enhance multiple shooting. The effect of BAP as the most reliable, useful and effective cytokinin for shoot proliferation in several plant species has been documented (Fracaro and Echeverrigary 2001; Balogun et al. 2007; Bohidar et al. 2008). George (2003) stated that the ability of BAP to induce microshoots is also well documented. Therefore, in the present study, the application of 5 mg/litre BAP into the media was effective to produce more shoots per explant. This result was in agreement with the findings of Nayak (2000) in turmeric and Sharma and Singh (1995) in ginger.

Micropropagation of shoot cultures

Microshoot tips about 1 – 2 cm were used as explants and cultured on MS or WPM medium consisting of varying concentrations of BAP alone or in combination with 1 mg/litre NAA. After 40 days of treatment, the explants were able to produce multiple microshoots. In general, explants cultured using MS medium produced better percentage of proliferation and mean number of microshoots per explant (*Table 2*) compared to WPM medium. Percentage of proliferation was highest in MS medium supplemented with 3 mg/litre BAP (95%). In addition, MS medium supplemented with 3 and 5 mg/litre BAP alone had significantly higher number of microshoots produced (9.5 and 9.7 respectively; $p \leq 0.05$).

The results indicated that MS medium supplemented with 3 – 5 mg/litre BAP was the most effective in promoting microshoots production of *C. caesia* through *in vitro* technique. The ability of high concentrations of BAP for producing multiple microshoots was conspicuous. Hiremath (2006) also reported that the highest number of multiple shoots of ginger was found in 2 mg/litre BAP supplemented media. Earlier, Dipti et al. (2005) stated that the highest number of multiple shoots in rhizome bud of turmeric was cultured in MS medium supplemented

Table 2. Effect of different types of media and plant growth regulators (BAP and NAA) after 45 days of culture

Media	BAP (mg/litre)	NAA (mg/litre)	Percentage of microshoots propagation (%)	Mean number of microshoots/explant
Control	0	0	45	2.2 ± 0.23d
MS	1	0	78	5.3 ± 0.34bc
	3	0	95	9.5 ± 1.11a
	5	0	68	9.7 ± 2.21a
	1	1	87	6.3 ± 0.41bc
	3	1	68	7.5 ± 1.32ab
	5	1	65	5.8 ± 0.25bc
WPM	1	0	70	6.2 ± 0.14bc
	3	0	86	7.3 ± 1.35ab
	5	0	67	7.1 ± 1.23ab
	1	1	75	5.7 ± 0.56bc
	3	1	68	7.8 ± 0.89ab
	5	1	64	4.1 ± 0.23cd

Data are given as means ± SD of results from 25 replicates
Means with the same letters in the same column are not significantly different at $p \leq 0.05$

with 3 mg/litre BAP. In contrast, Nasiruzzaman et al. (2005) found that young shoots of *C. longa* spp. cultured on WPM medium supplemented with 4 mg/litre BAP showed the best medium for regeneration of new shoots.

When clumps of microshoots were subcultured on MS medium containing 2 different salt strengths of BAP and NAA, the number and length of shoots/roots produced were better in MS medium containing full salt strength compared to media with half salt strength. The data in *Table 3* show that media with full salt strength produced more shoots (*Plates 1d* and *1e*) especially when cultured on MS medium supplemented with 3 mg/litre BAP and 0.5 mg/litre NAA. The number of shoots at 14.6 ($p \leq 0.05$) was significantly higher than the other treatments. This treatment also produced shoot lengths between 3 – 5 cm with optimum number of roots (11.1 per explant) with root lengths between 3 – 5 cm *(Plate 1f* and *Table 3*). On the other hand, MS medium containing half salt strength produced lower number of shoots and roots.

In the present study, MS medium containing combination of BAP (3.0 mg/litre) and NAA (0.5 mg/litre) produced the highest number of shoots of *C. caesia* (*Table 3*). The number of shoots and roots produced and their growth was better in this combination of plant growth regulators compared to single application. A comparatively lower response was recorded when BAP was applied alone in the medium. It has been reported that the addition of NAA in the culture medium improved the shoot growth in a number of species (Kambaska et al. 2010). They found that *C. longa* cultured on MS medium containing growth regulator (BAP and NAA) supplements produced better results in terms of percentage of explants, shoots/explant, average shoot length and average number of roots and length of roots. Raihana et al. (2011) also observed that explants of *C. mangga* that were inoculated in the combination media had better shoot and root formation compared with single media. Sathyagowri and Seran (2011) also found that ginger plantlets that were transferred to MS medium fortified with 5.0 mg/litre BAP and 0.5 mg/litre NAA produced multiple shoots which were thereafter

Table 3. The effect of MS medium salt strength (half and full strength) and plant growth regulators (BAP and NAA) after 45 days of subculture

MS	BAP (mg/litre)	NAA (mg/litre)	No. of shoots	Shoot length (cm)	No. of roots	Root length (cm)
Full	1	–	8.0 ± 0.93cde	6 – 8	9.9 ± 2.13abc	4 – 5
	3	–	12.3 ± 1.34ab	6 – 8	10.8 ± 3.31ab	3 – 6
	1	0.5	11.0 ± 2.11bc	4 – 5	12.1 ± 3.40a	4 – 6
	3	0.5	14.6 ± 3.14a	3 – 5	11.1 ± 1.20ab	3 – 5
	1	1	9.3 ± 0.67dc	5 – 6	9.4 ± 50.91abc	4 – 6
	3	1	8.1 ± 0.56cde	3 – 5	7.5 ± 0.67cd	3 – 5
Half	1	–	7.4 ± 0.89de	5 – 6	7.5 ± 0.89cd	4 – 5
	3	–	8.3 ± 2.34cde	5 – 6	5.3 ± 1.11d	3 – 6
	1	0.5	7.0 ± 1.56de	4 – 5	7.1 ± 0.98cd	4 – 6
	3	0.5	7.9 ± 2.22de	3 – 5	7.2 ± 1.35cd	3 – 5
	1	1	8.3 ± 1.56cde	5 – 6	8.6 ± 2.34bc	4 – 6
	3	1	6.3 ± 1.45e	3 – 5	5.5 ± 1.45d	3 – 5

Data are given as means ± SD of results from 25 replicates
Means with the same letters in the same column are not significantly different at $p \leq 0.05$

subcultured to MS containing 3.0 mg/litre BAP and 0.5 mg/litre NAA for further shoot elongation. Previous study by Bharalee et al. (2005) on *C. caesia* cultured in MS medium supplemented with 4 mg/litre BAP and 0.5 mg/litre NAA, only managed to produce 3.5 shoots/explant. However, findings by Yusuf et al. (2007) showed that the optimum concentration for BAP for *in vitro* multiplications of *Curcuma* spp. was 3.0 mg/litre. BAP, a common type of cytokinin, promotes cell division, cell proliferation and shoot elongation (Raihana et al. 2011).

In the present study, *in vitro* plantlets (*Plate 1g*) with shoots and roots were successfully acclimatized with a survival rate of 95%. The well rooted plants were transferred to polybags containing organic soil and topsoil for hardening and kept under controlled conditions with 75% shading in the glasshouse. The plants produced fresh shoots and roots after 4 weeks of transplanting (*Plate 1h*).

Conclusion

The present experiment concluded that the appropriate size of the explants for initiation of cultures is 3 – 4 cm length for initial culture establishment in which 45% of the explants survived. The optimum medium to produce multiple microshoots is MS medium supplemented with 3 – 5 mg/litre BAP. For further shoot development and root elongation, the plantlets were transferred to full strength MS supplemented with 3 mg/litre BAP and 0.5 mg/litre NAA. Subsequently, the *in vitro* plantlets were successfully acclimatized in the glasshouse with 75% shading. This method will be useful for rapid clonal propagation of *C. caesia* under *in vitro* conditions. Moreover, the present protocol is a step forward towards an efficient and productive commercial propagation and production system of stem cells of *C. caesia*.

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Abstrak

Curcuma caesia merupakan tumbuhan herba saka yang mempunyai nilai ekonomi yang tinggi dalam bidang perubatan. Satu protokol berkesan untuk mikropropagasi tanaman *C. caesia* diikuti dengan penyesuaian iklim kepada tanah telah dibangunkan. Tunas rizom disteril dan dikultur dalam medium Murashige dan Skoog (MS) yang ditambah dengan atau tanpa BAP (5 mg/liter). Panjang rizom paling optimum yang dikultur di atas medium yang ditambah dengan 5 mg/liter BAP ialah melebihi 2 cm untuk menghasilkan pucuk pada peringkat awal. Untuk pembiakan pucuk, eksplan dikultur di atas medium yang mengandungi pengawal atur pertumbuhan BAP (6-benzil-amino-purina) dan NAA (asid asetik α-naftalena) pada kepekatan dan kombinasi yang berbeza. Eksplan yang dikultur di atas medium MS yang ditambah dengan 3 mg/liter BAP menunjukkan kadar percambahan yang tertinggi (95%) dengan purata 9.5 bilangan pucuk dihasilkan. Pucuk yang diperoleh secara *in vitro*, kemudian disubkultur di atas medium MS dengan kepekatan garam penuh yang ditambah dengan 3 mg/liter BAP dan 0.5 mg/liter NAA menunjukkan penghasilan pucuk dan akar yang baik. Pucuk berakar yang dipindahkan ke rumah kaca untuk pengerasan menpunyai kadar kemandirian 95%.

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