

Full Length Research Paper

***In vitro* culture of *Curcuma mangga* from rhizome bud**

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Accepted 8 July, 2011

***Curcuma mangga* is one of the important and valuable medicinal crops of the family Zingiberaceae. This species also has a high potential for essential oil. An efficient protocol for the micropropagation of *C. mangga* followed by successful acclimatization to soil was developed. The aseptic rhizome buds were cultured onto Murashige and Skoog (MS) medium supplemented with different concentrations of BAP, IAA and NAA for 10 weeks. MS medium supplemented with 9 mg/L BAP was found to be the optimum for shoot multiplication while MS medium supplemented with 1 mg/L NAA gave the highest root number. The explants were then subcultured on to MS medium supplemented with different concentrations of BAP and NAA for 4 weeks. The MS medium supplemented with a combination of 3 mg/L BAP and 1 mg/L NAA gave the highest number of shoot multiplication. The plantlets were successfully acclimatized with 75% survival rate.**

Key words: *Curcuma mangga*, micropropagation, explants, acclimatization, Zingiberaceae.

INTRODUCTION

Curcuma mangga belongs to the family Zingiberaceae. It is also known as temu pauh in Malaysia, temu mangga in Indonesia and 'Khamin Khao' in Thailand. It also has a generic name, "mango turmeric" or "mango ginger" because when the fresh rhizome is cut, it produces a mango-like smell (Kaewkroek et al., 2009). *C. mangga* is mainly used for cooking purposes, food decoration as well as vegetable and traditional medicine (Wong et al., 1999). The young rhizomes and shoots are consumed raw with rice and these plants are well known especially among people in the villages. For medicinal purposes, the rhizomes are used to treat illnesses such as stomachache, chest pain, and also in postpartum care, specifically to aid in womb healing (Abas et al., 2005). It is also used to treat skin diseases such as red spot which cause itching and can reduce body heat caused by continuous fever (Hutami and Purnamaningsih, 2003).

The micropropagation technique provides an alternative method of propagation which is very essential for adequate supply of planting materials. 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. Furthermore, the flowering usually is very rare, thus the 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. Moreover, there are very limited reports on regeneration protocol of *C. mangga* in Malaysia. Thus, the aim of this research is to establish an efficient micropropagation protocol for plant tissue culture of *C. mangga*.

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Abbreviations: MS, Murashige and Skoog; BAP, 6-benzalaminopurine; NAA, naphthaleneacetic acid; IAA, indole acetic acid.

MATERIALS AND METHODS

Plant material

The rhizomes of *C. mangga* were collected during the rainy season in October and November 2009 from a nursery named Ladang Mak

Yah in Temerloh, Pahang. The species was identified by Mr. Shamsul Khamis and the voucher specimen has been deposited at the Herbarium of the Biodiversity Unit, Institute of Bioscience (IBS) with voucher number SK 1736/10.

Surface sterilization

About 2.0 to 3.0 cm apical buds from the *C. mangga* rhizomes were soaked in a few drops of commercial detergent and washed thoroughly in running tap water for a few minutes to remove all the mud. The explants were surface sterilized with clorox and a few drops of Tween-20 to reduce the chances of bacterial contamination. Then, the explants were rinsed several times in distilled water. Sterilized explants were then dissected with a sterile surgical blade to remove the outer layers of leaf sheaths under aseptic conditions to produce 5 to 8 mm sized pieces having at least one eye (Chirangini and Sharma, 2005).

Culture medium and incubation conditions

The explants were inoculated on to Murashige and Skoog (1962) medium supplemented with BAP, IAA and NAA, each with concentrations of 0, 1, 3, 5, 7 and 11 mg/L. The pH of the medium was adjusted to 5.8 prior to autoclaving. The culture bottles were sealed with parafilm and were incubated in the culture room under white fluorescent light with light intensity of 3000 lux at a photoperiodic 16 h at 25±2°C. The growth of the cultures was observed after 10 weeks.

Subculturing technique

The *in vitro* shoots of *C. mangga* were cut to 1 to 2 cm from the base and were transferred onto MS medium supplemented with combination of BAP and NAA (3 mg/L BAP + 0.5 mg/L NAA, 3 mg/L BAP + 1.0 NAA, 5 mg/L BAP + 0.5 mg/L NAA, 5 mg/L BAP + 1.0 mg/L NAA). MS medium without any supplemented plant growth regulator was used as the control. The subculture technique was carried out in laminar air-flow cabinet. The cultures were incubated under the same conditions as the single media. The following parameters were recorded after four weeks.

Acclimatization

Acclimatization was established under a controlled condition in the net house with 75% shading. The explants were transferred to small pots containing a mixture of husk and peat moss with ratio of 1:3 (v/v), covered with a rigid plastic cover to maintain a high relative humidity environment (Faria and Ilg, 1995). As to ensure its humidity, the plants were sprayed periodically with water. The explants were left to grow. The survival rate percentage of the explants was observed after four weeks.

Experimental design and data analysis

All the cultures were examined periodically and the morphological changes were recorded. The effect of different treatments was determined with respect to number of shoots, roots, leaves and the length of shoots and roots. The experimental design used was Randomized complete block design (RCBD). Data were analyzed using the Statistical package for the social science (SPSS) software version 18.0 (Chicago, USA). Analysis of variance (ANOVA) was used to investigate if there is any significant difference. Mean separation test was conducted using Duncan's new multiple range

test (DNMRT).

RESULTS AND DISCUSSION

Establishment of *in vitro* culture

In this first set of experiment, the rhizome buds of *C. mangga* were cultured in MS medium supplemented with four different hormones namely: BAP, IAA and NAA of concentration (1 to 11) mg/L. The establishment of the aseptic bud culture was very difficult because the explants were taken from underground rhizomes. These rhizomes were exposed to different types of soil-borne pathogens that may lead to contamination. Almost 30% of the cultures were found to be contaminated initially. However, once the sterile and healthy cultures were established after 1 month of culture, there was no further contamination.

Among the various hormones used for shoot formation the best response was produced with MS medium containing 9 mg/L of BAP with an average of 3.3 ± 0.9 shoots while MS medium supplemented with 1 mg/L NAA gave the highest root number with 62.5 ± 22.5 roots (Table 1). According to the research by Stanly and Chan (2007), the numerous shoots were induced in *Curcuma zedoaria*, after treatment with a higher concentration of BAP, which was optimally at 10 mg/L. However, Yusuf et al. (2007) reported that the optimum concentrations for BAP for *in vitro* multiplications of *Curcuma* spp was 3.0 mg/L. BAP is a common type of cytokinin which promotes cell division, cell proliferation and shoot elongation. Nasirujjaman et al. (2005) stated that herbaceous plants such as *C. mangga* are commonly and highly responsive to treatment of BAP as it is able to induce auxiliary buds. They also reported that most of cultured herbaceous species in BAP treatment produce robust, well-formed shoots suitable for further shoot proliferation. For root formation, the synthetic auxin, NAA was found to be more efficient in promoting cell expansion and root formation than the natural occurring auxin, IAA. Zapata et al. (2003) stated that the positive effect of auxin for the *in vitro* roots formation is widely reported, specifically NAA, IBA and IAA. According to Loc et al. (2005), MS medium supplemented with 2 mg/L NAA indicated almost the same number of roots which were 18.5 for root induction of *C. zedoaria*.

Induction of shoots and roots

In the second set of experiment, the aseptic explants were cultured in the MS medium supplemented with different combinations of BAP and NAA concentrations. Among all the concentrations, the best response for shoot and root formation was obtained in the MS medium with presence of 3 mg/L BAP and 1.0 mg/L NAA (Table 2). The multiple shoots formation was statistically

Table 1. Effect of different concentrations of BAP, IAA, and NAA on the number of shoots, shoot length, number of roots and root length per shoot of *Curcuma mangga* cultured on MS basal medium after 10 weeks of culture.

PGR (mg/L)		No. of shoot	Shoot length (cm)	No. of root	Root length (cm)	No. of leaves
Control	0	2.3±0.3 ^{ab*}	4.2±0.4 ^{ab}	7.7±1.7 ^b	5.9±1.9 ^a	3.0±0.6 ^a
BAP	1.0	2.3±0.3 ^{ab}	2.6±1.2 ^{bc}	7.5±1.5 ^b	1.3±0.8 ^a	4.5±1.5 ^a
	3.0	2.3±0.3 ^{ab}	2.9±1.0 ^{bc}	7.3±2.4 ^b	3.3±0.9 ^a	5.0±2.3 ^a
	5.0	2.3±0.3 ^{ab}	2.0±0.9 ^{bc}	-	-	3.5±1.5 ^a
	7.0	-	-	-	-	-
	9.0	3.3±0.9 ^a	2.1±0.6 ^{bc}	-	-	1.5±0.7 ^a
	11.0	1.5±0.5 ^b	2.9±1.3 ^{bc}	-	-	3.0±2.0 ^a
IAA	1.0	1.5±0.5 ^b	2.5±0.3 ^{bc}	-	-	1.0±0.0 ^a
	3.0	2.0±0.0 ^{ab}	3.7±0.3 ^{ab}	-	-	2.0±1.0 ^a
	5.0	1.0±0.0 ^b	2.4±0.2 ^{bc}	4.5±3.5 ^b	2.2±0.7 ^a	1.5±0.5 ^a
	7.0	1.3±0.3 ^b	3.1±0.5 ^{bc}	2.3±0.3 ^b	1.5±1.1 ^a	1.3±0.3 ^a
	9.0	1.3±0.3 ^b	3.0±0.8 ^{bc}	4.5±2.5 ^b	3.3±0.6 ^a	2.0±1.0 ^a
	11.0	2.0±0.6 ^{ab}	3.2±1.0 ^{bc}	5.0±3.0 ^b	3.0±2.4 ^a	2.0±1.0 ^a
NAA	1.0	1.5±0.5 ^b	6.1±1.1 ^a	62.5±22.5 ^a	4.9±3.9 ^a	2.0±1.0 ^a
	3.0	1.5±0.5 ^b	2.8±0.5 ^{bc}	6.0±4.0 ^b	0.7±0.3 ^a	-

*Means followed by the same letter(s) within each column are not significantly different at $P \leq 0.05$, according to Duncan's New Multiple Range Test (DNMRT). *PGR: plant growth regulator, *(-): data are not available (NA).

Table 2. Effect of various combinations of BAP and NAA on the number of shoots, shoot length, number of roots, root length and number of leaves per shoot of *Curcuma mangga* cultured on MS basal medium after 4 weeks of subculture.

PGR (mg/L)		No. of shoot	Shoot length (cm)	No. of root	Root length (cm)	No. of leaves
BAP	NAA					
0	0	1.2±0.2 ^{b*}	4.0±0.5 ^a	12.7±2.9 ^a	3.5±0.7 ^a	2.6±0.2 ^a
3.0	0.5	1.9±0.2 ^{ab}	3.5±0.5 ^a	9.8±1.4 ^a	1.3±0.1 ^b	2.8±0.3 ^a
3.0	1.0	2.2±1.0 ^a	3.4±0.6 ^a	10.7±2.9 ^a	1.2±0.2 ^b	2.5±0.5 ^a
5.0	0.5	1.8±0.3 ^{ab}	3.2±0.6 ^a	11.2±2.3 ^a	1.3±0.3 ^b	2.4±0.7 ^a
5.0	1.0	1.5±0.2 ^{ab}	2.8±0.6 ^a	7.1±3.4 ^a	1.0±0.2 ^b	2.1±0.3 ^a

*Means followed by the same letter(s) within each column are not significantly different at $P \leq 0.05$, according to Duncan's New Multiple Range Test (DNMRT). *PGR: plant growth regulator.

significant as compared to the control with 2.2 ± 1.0 shoots formed. The multiple shoots formed were healthy with normal-looking leaves and roots (Figure 1B). The ratio of auxin and cytokinin determines the type of culture established. In general, a high cytokinin to auxin ratio favours shoot formation whereas a high auxin to cytokinin ratio favours root formation. Bejoy et al. (2006) reported that the combination of BAP and NAA indicated the healthier shoots multiplication for *Curcuma haritha* compared to other hormone combinations.

In general, the explants that were inoculated in the combination media were observed to have better shoot and root formation compared with single media. According to research on micropropagation of ginger

conducted by Kambaska and Santilata (2009), a comparatively lower response was observed when BAP was added alone in the medium. However, the addition of auxin such as NAA, IBA or IAA indicated a positive response to the shoot growth. Besides that, cytokinin and auxin balance is important for the final overall effect on growth and development, thus the results may be expected to differ as compared to addition of cytokinin or auxin alone in media. However, in higher concentration of NAA the shoot formation was inhibited and only roots were formed.

In a previous study by Bharalee et al. (2005), shoot multiplication of *Curcuma caesia* was induced in MS medium supplemented with 4 mg/L BAP + 0.5 mg/L NAA

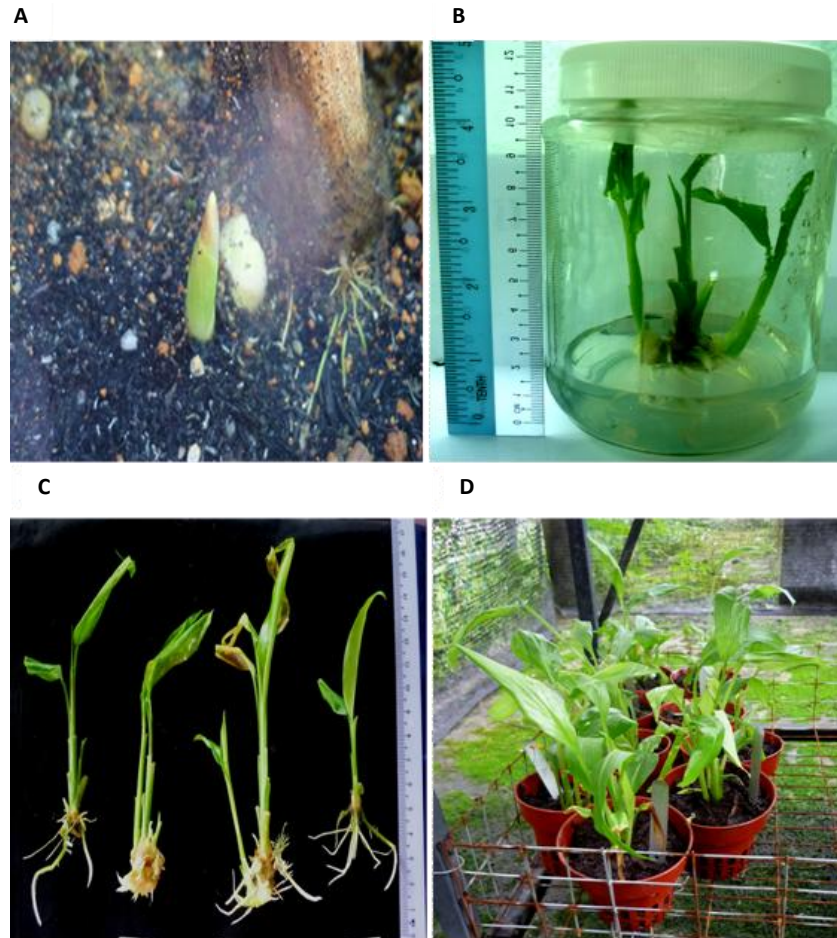


Figure 1. (A) Fresh rhizome bud of *Curcuma mangga* used for surface sterilization (B) Multiple shoots formation of *C. mangga* in MS medium supplemented with 3.0 mg/L BAP and 1.0 mg/L NAA after 4 weeks of subculture (C) Plantlets of *C. mangga* after 4 weeks subculturing in rooting hormone (D) Plantlets of *C. mangga* after 4 weeks of acclimatization in shade net house.

which produced 3.5 shoots per explant while MS medium supplemented with 1 mg/L BAP + 0.5 mg/L NAA was optimum for *C. zedoaria* with 4.5 shoots per explant. Sunitibala et al. (2001) reported that MS medium containing 2 mg/L BAP + 1.0 mg/L NAA shows optimum clonal propagation of turmeric by rhizome bud culture.

Most of the research on micropropagation used combination of cytokinin and auxin to obtain better results for both root and shoot multiplications. However, the research on *C. mangga* by Hutami and Purnamaningsih (2003) used a combination of cytokinins which were BAP and thidiazuron. According to their findings, the MS medium supplemented with 3 mg/L BAP + 0.5 mg/L thidiazuron yielded 1.8 shoots.

The amount is slightly lower than with the combination of BAP and NAA. This indicated that combination of cytokinin and auxin showed better results for shoot and root multiplication of *C. mangga* compared to combination of only cytokinins.

Acclimatization

Acclimatization of plantlets is the crucial phase where plantlets are in transition from *in vitro* phase to *in vivo* phase. A major problem is a many *in vitro* plantlets did not survive if the acclimatization protocol was not carried out properly. According to Chithra et al. (2005), most of the *in vitro* plantlets usually produced leaves lacking epicuticular wax and with ineffective control of stomatal function. These conditions enhance water loss when they were transferred to the soil. For this research, the well-developed explants established from the rooting stage were transferred to *in vivo* stage. After four weeks, the *in vitro* plantlets of *C. mangga* were successfully acclimatized with 75% of plantlet survival (Figure 1D).

In conclusion, plant tissue culture is now a well established technology which has made significant contributions to the improvement of agricultural crops in general. The successful production of multiple shoots,

roots and *in vitro* rhizome formation depended on the nutrient medium and the culture environment. This study had showed a developed protocol on plant tissue culture of *C. mangga* and can be used for a large scale production and germplasm conservation of the species.

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