Optimization of culture condition for callus induction from shoot buds for establishment of rapid growing cell suspension cultures of Mango ginger (*Curcuma mangga*)

Tamil C.M. Sundram[*], M. Suffian M. Annuar and Norzulaani Khalid[*]

Centre for Research in Biotechnology for Agriculture (CEBAR), Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Lembah Pantai, Kuala Lumpur, Malaysia

*Corresponding authors: tgm_tam84@yahoo.co.uk or lani@um.edu.my

Abstract

A systematic approach was taken to establish a rapidly growing suspension culture of *Curcuma mangga (C. mangga)*. Various responses were obtained from shoot bud explants cultured on MS basal medium supplemented with different concentrations of 2,4-D, NAA and IAA either alone or in combinations. Different levels of sucrose were also tested. Rapid growing friable callus obtained from shoot explants cultured on MS basal medium supplemented with 1 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose and 2 g l^{-1} gelrite were selected for the initiation of suspension cultures based on histological morphology. From various media screened, rapid growing suspension cultures were established by using MS liquid medium supplemented with 0.3 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 30 g l^{-1} sucrose, 0.1 g l^{-1} malt extract, 0.5 mg l^{-1} d-Biotin, 100 mg l^{-1} glutamine, 5 mg l^{-1} ascorbic acid and citric acid respectively. Phenolic compounds production was most effectively controlled by the incorporation of ascorbic and citric acid as antioxidants.

Keywords: Embryo, tissue culture, *in vitro*, mango, phenolic compounds.

Introduction

Curcuma mangga Valeton & C. van Zijp belongs to Zingiberaceae family, and it is among 80 species of rhizomatous herbs of *Curcuma* genus. This species of *Curcuma*, manifests most distinct identity of raw mango flavour combined with that of ginger in the rhizome due to the presence of car-3-ene, cis- and trans- dihydroocimene, ocimene and mycrene (Achut and Bandyopadhyaya, 1984; Rao et al., 1989, Singh et al., 2003; Sasikumar, 2005). Traditionally, *C. mangga* was used as medicine to treat stomach pain, fever, debility, bronchitis and asthma, to act as an aphrodisiac and to provide postpartum care (Syu et al., 1998; Hong et al., 2001; Park and Kim, 2002; Abas et al., 2005; Hussain et al., 2008). The natural pigment from this species has also been reported to show antioxidant, anti-inflammatory, antitumour, antitubercular, antifungal, antibacterial and antiprotozoal activities (Richomme et al., 1991; Habibi et al., 2000, Kirana et al., 2003, Chan et al., 2008; Tewtrakul and Subhadhirasakul et al., 2007, Liu and Nair, 2011; Malek et al., 2011). Various secondary metabolites have been isolated from *C. mangga* (Liu and Nair, 2011; Malek et al., 2011), however there is little information on the *in vitro* production of secondary metabolites. Although these compounds can be obtained from field grown rhizomes, enhanced production of diterpenoids from *in vitro* sources is possible to ensure continuous supply of consistently high quality compounds. A stable suspension culture established with a systematic protocol is critical to enable the manipulation of cultures for enhanced production of secondary metabolites. The aim of this study was to establish a cell suspension culture of locally grown *C. mangga* with rapid cell growth to be used as an alternative source for the production of secondary metabolites in future studies. In this process of establishing cell suspension cultures of *C. mangga*, initially a suitable culture medium was selected for the induction of callus from the shoot

explants. The appropriate callus for the initiation of suspension cultures was determined according to histological morphology of the calli and an optimum medium was determined for the establishment of cell suspensions with rapid cell multiplication. Antioxidants were used to overcome browning in the cultures. These cultures will be used as a source for the production of bioactive compounds such as (*E*)-labda-8(17),12-dien-15,16 dial in future studies. (*E*)-labda-8(17),12-dien-15,16 dial (labdene diterpenoids group), is one of the major compounds isolated from rhizomes of *C. mangga* (Abas et al., 2005) with inhibition activity against lung, colon, gastric and breast tumour cell lines (Yunbao and Muraleedharan, 2010).

Results and Discussion

Explant preparation

Since the explants were taken from underground rhizomes, establishment of contamination free culture was a major task (Hosoki and Sagawa, 1997). In this study, rhizomes were sprouted in soil free conditions until shoot buds appeared. As in table 1, rhizomes left to sprout at room temperature under dark condition produced the highest amount of shoot buds. The surfaces of explants usually carry a wide range of microbial contaminants. Thus, the explants must be thoroughly surface sterilized before inoculating on culture medium to avoid infection. Usage of commercial bleach (5.25% sodium hypochlorite as active ingredient), ethanol and mercury chloride (HgCl$_2$) have been reported as very effective sterilizing agents in establishing aseptic buds of many *Zingiberaceae* species (Mrudul et al., 2001; Neeta et al., 2002; Chan and Tong, 2004; Yusuf et al., 2007). Three cm shoot buds (Figure 2(a)) were excised from rhizomes, washed with running tap water and surface sterilized by using

disinfectants with different treatments, as described in Figure 1 and Table 2. Shoot bud explants were first treated with Tween 20, a wetting agent that improves the disinfestation by acting as a surfactant thereby removing surface contaminants such as soil and dust. Hypochlorite is known to be very effective against bacteria. When diluted in water, the concentration of hypochlorite salt used (NaOCl)) leads to the formation of hypochlorous acid (HOCl), whose concentration is correlated with bactericidal activity (Nakagarwara et al., 1998). Bacteria killing by HOCl maybe due at least in part to lethal DNA damage (Wlodkowski et al., 1975; Dukan et al., 1999). Ethanol is also a powerful sterilizing agent but explants were usually exposed to it for only a few minutes due to its extreme phytotoxicity (Afolabi et al., 2009). 70% ethanol washing, prior to disinfection with $HgCl_2$ enhances the contact between $HgCl_2$ and the surface of explants efficiently. Mercury ions in $HgCl_2$ solution function in surface sterilization by interfering with enzymes and protein in the cell membrane and cytoplasm of contaminating pathogenic microorganisms (Smith, 2005). Singh et al., (2009) reported that contamination can be reduced to 5.5% by using 0.1% (w/v) $HgCl_2$. In contrast, our initial study showed that the contamination was high at 80% when 0.1% (w/v) $HgCl_2$ was used. However when the concentration of $HgCl_2$ was increased to 0.5% (w/v), 83% of the cultured explants were found to be dead. This is probably due to the fact that a high concentration of $HgCl_2$ is phytotoxic to plant cells. Out of three surface sterilization treatments carried out, treatment three with 0.3% (w/v) $HgCl_2$ was the most effective with a 77% survival rate (Figure 3) after four weeks in MS medium. This treatment was therefore adopted for subsequent experiments.

Initiation, growth and characterisation of callus

Callus initiation is the primary stage in many tissue culture processes for the establishment of cell suspension cultures (Kumar and Kanwar, 2007; Ngara et al., 2008), indirect somatic embryogenesis (Kulkarni et al., 2002; Rahman et al., 2006) and other applications. Various plant growth regulator (PGRs) treatments have been used for these purposes in different plant cultures (Afshari et al., 2011). In our present study, several different types and concentrations of auxins were tested for initiation of friable and vigorous growing callus from shoot bud explants to be used as inoculums for suspension cultures. Various auxin concentrations used in this study produced different types of callus morphology and callus induction percentages (Table 3 and Table 4). When cultured on a hormone-free medium, the explants produced roots (Fig 4).In contrast in MS (Murashige and Skoog, 1962) medium supplemented with auxin, cultured explants were swollen after five days of inoculation and callus emerged at the cut edges within 10 days of culture. MS with 1 mg l^{-1} 2,4-D was the most effective medium for friable callus induction at 76% (Fig 5(a)). 1-2 mg l^{-1} 2,4-D was reported to be an effective PGR that produced rapid proliferating friable callus in *C. amada* (Prakash et al., 2004), *Gymnema sylvestris* (Roy et al., 2008) and *Brassica napus* (Jonoubi et al., 2005) *in vitro* cultures. Substitutions with other auxins or increasing or decreasing the concentration of 2,4-D resulted in a reduced percentage of explants producing callus (Table 3 and Table 4). The callus were white, nodular with roots at 0.1 mg l^{-1} 2,4-D (Fig 5(c)) and relatively brownish with a dry friable appearance at 8 mg l^{-1} 2,4-D (Fig 5(e)). However, when MS

in combination with higher or lower concentrations of IAA and NAA were used, the callus morphology and callus induction percentage differed but without any particular pattern (Table 4). In comparison to the natural auxin, IAA, synthetic auxins NAA and 2,4-D induced a better response in terms of the percentage of friable callus induction even at low concentrations (Table 3 and Table 4). Pierik (1990) reported that this result could be attributed to the fact that synthetic PGRs are more stable in their ability to withstand physical and enzymatic degradation than naturally occurring auxins. The callus observed were whitish and globular at lower concentrations of IAA and NAA (1 mg l^{-1} IAA and 1 mg l^{-1} NAA) (Fig 5(g)) and were whitish with a mixed friable and globular appearance at higher concentrations of IAA and NAA (5 mg l^{-1} IAA and 5 mg l^{-1} NAA) (Fig 5(i)). Sucrose concentrations also affected callus morphology and the induction percentage (Table 5). Mucilaginous and white callus were formed when the concentration of sucrose was increased to 8% (Fig 5(k)). This morphology may be caused by the influence of sucrose on the humidity of *in vitro* culture conditions (Lee et al., 2002). In this treatment, the concentration of 2,4-D was maintained at 1 mg l^{-1} due to its response in producing favourable rapid growing friable callus. In plant tissue culture, sucrose is the most commonly used carbohydrate source because of the wide spread of this disaccharide as a transporter molecule, and its high solubility in water. Many *in vitro* studies have proven that sucrose supports near optimum rates of growth and also plays multiple roles in the provision of carbon and energy that promotes cell growth and division (Swedlund & Locy, 1993). The callus doubled in size in less than two weeks when subcultured in a fresh MS medium supplemented with 1 mg l^{-1} 2,4-D. Callus on MS medium supplemented with lower or higher 2,4-D or other PGRs, proliferated slowly (data not shown) and did not produce friable callus. Friable callus is ideal for the initiation of cell suspension cultures in many plants (Lee and Chan, 2004). Slow growth rates were not appropriate for the establishment of suspension cultures. For this reason, the growth kinetics of callus cultured on MS media supplemented with 1 mg l^{-1} 2,4-D was further studied (Fig 6). The growth curve of *C. mangga* callus showed a sigmoidal-type pattern where three growth phases can be distinguished (Figure 6). A sigmoidal growth curve was also reported in the *in vitro* callus culture of other plant species such as *Orthosiphon stamineus* (Lee and Chan, 2004), coffee plant (Santos et al., 2003) and Brazil nut (Serra, 2000). In this report the lag phase where cells of the explants prepare for division and energy accumulation occurred up to day 42 from inoculation, similar to Brazil nut callus culture (Serra, 2000). This was followed by an exponential growth phase, where maximum cell division and increase in cellular area (Abbade et al., 2010) were observed occurring between day 42 and 110. Growth deceleration was observed between day 110 and 120. Lima et al., (2008) reported that growth deceleration occurs as a result of the usage of nutrients and the accumulation of toxic substances in the culture medium. It is appropriate to subculture the callus at the beginning of this phase (Lima et al., 2008). The maximum increase of callus biomass was observed at the late stationary phase, with the initial inoculums of 0.3 g reaching 11 g. Histological studies were also carried out to provide further evidence on fast growing callus of different morphology. Periodic acid Schiff (red stain), specifically stains polysaccharide (starch reserves and walls) meanwhile blue-black naphtol stains

Table 1. Effect of different treatments on rhizome for sprouting.

Treatments	Photoperiod	Temperature (^{o}C)	Duration of sprouting (weeks)	Number of shoot buds (in 4 weeks)
1	16 hours light and 8 hours dark	20 ^{o}C at air-condition	4	5 to 20
2	16 hours light and 8 hours dark	30 ^{o}C at room temperature	4	5 to 20
3	24 hours dark	20 ^{o}C at air-condition	3	40 to 60
4	24 hours dark	30 ^{o}C at room temperature	2	40 to 60

Table 2. Surface sterilization method.

Disinfectant	Treatment 1 (%) -T1	Treatment 2 (%) -T2	Treatment 3 (%) -T3
Chlorox (v/v)	50 (15 minutes)	50 (15 minutes)	50 (15 minutes)
Ethanol (v/v)	70 (5 minutes)	70 (5 minutes)	70 (5 minutes)
$HgCl_2$ (w/v)	0.1 (30 seconds)	0.5 (10 minutes)	0.3 (5 minutes)

soluble or reserve protein (Fisher, 1968). Red stained cells indicate the presence of polysaccharides as the source of energy that supports proliferation of cell division (Jalil et al, 2008). Histological section of friable white callus cultured on MS solidified medium supplemented with 1 mg l^{-1} and 2 mg l^{-1} 2,4-D showed large cells with large nuclei and dense cytoplasm with islets of protein compounds stained blue-black and polysaccharide stained red (Fig 5(b)). When supplemented with a low concentration of 2,4-D (0.1 mg l^{-1} 2,4-D), cells with dense cytoplasm and nuclei were observed (Fig 5(d)). However these cells (Fig 5(d)) grew slowly due to the absence of polysaccharide which supports cell division. Callus produced in media supplemented with high concentrations of IAA and NAA showed cells with less dense cytoplasm and nuclei (Fig 5(j)). Callus on medium supplemented with low concentrations of IAA and NAA are irregular in shape with small nuclei, less cytoplasmic and the presence of vacuolated cells (Fig 5(h)). While, callus initiated in MS medium supplemented with 8 mgl^{-1} 2,4-D revealed that the cells are largely vacuolated (Fig 5(f)). The presence of vacuolated cells will affect growth of callus, thus reducing the rate of suspension culture propagation when transferred to a liquid medium and the propagation of debris will dominate the culture. Thus, the histological studies show that the ideal callus for the initiation of cell suspension were cells with large nuclei and dense cytoplasm that were cultured on MS medium supplemented with 1 mg l^{-1} 2,4-D. This callus shows the presence of polysaccharides that support the proliferation of cell division. Histological sectioning was also performed for callus initiated on MS medium supplemented with 8% (v/v) of sucrose. The cells also show large nuclei with dense cytoplasm and the presence of few polysaccharides granules (Fig 5(l)).

Determination of optimum maintenance medium for cell suspension culture

When cells were suspended in MS medium supplemented with 1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose, the proliferation rate was rapid, doubling the yield in 10 days after initiation. However, suspension cultures showed tissue browning after the third passage of subculture even though the subculture interval was shortened. Frequent subculture to fresh medium has been reported as a factor favouring culture establishment without tissue browning (Tiwari et al., 2002). Different strengths of MS basal medium and different concentrations of 2,4-D (auxin) were tested to overcome browning. Decreasing the concentration of 2,4-D to 0.5 mg l^{-1} in the MS medium, earlier produced proliferating suspension cells without any sign of tissue browning. Even though, the use of 2,4-D and
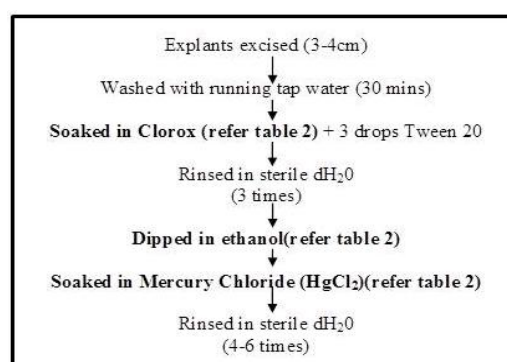
Fig 1. Surface sterilization method.
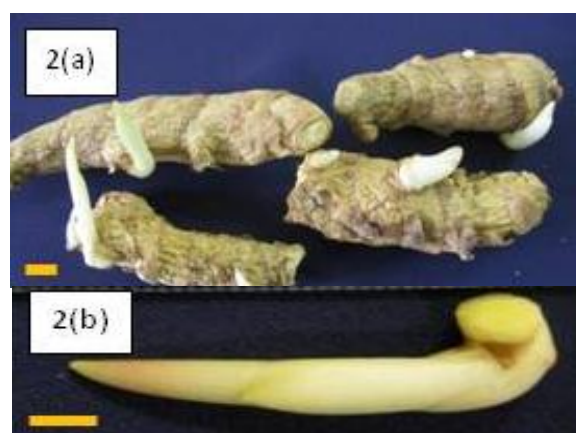
Fig 2. (a) Shoots sprouted from mature rhizomes of *C. mangga* (Bar represent 1 cm). (b) Sprouted buds (3cm to 4cm) collected as explants (bar represent 0.5cm).

NAA in the media as common auxins have encouraged growth and maintained the viability of suspension cultures in this study and also other reports (Endress, 1994), browning was also associated with the use of 2,4-D, especially if high concentrations were added to the medium. This problem was also found in cultures of *Dioscorea* sp. (Viana and Mantell, 1989) and *Aconitum heterophyllum* (Giri et al., 1993). Evans et al., (2003) reported that synthetic auxins like 2,4-D may possess herbicidal property at high concentrations, that inhibits the formation of callus and suspension cells leading to cell browning. However, browning recurred in cells cultured in 0.5 mg l^{-1} 2,4-D supplemented media after the 6th subculture. The addition of ascorbic acid (5 mg l^{-1}) and citric

Table 3. Effect of 2,4-D on callus induction from *C. mangga* shoot bud explants cultured on MS medium with 3% (v/v) sucrose.

2,4-D (mg l^{-1})	Callus induction (%)	Morphology of callus
0.0	0.0	Explant producing roots and shoots without callus formation
0.1	64 ± 2.3	White, globular, compact/nodular with roots
1.0	76 ± 3.2	White, friable, sticky and glassy
2.0	70 ± 3.1	Yellowish white, friable, sticky and glassy
4.0	54 ± 2.9	Yellowish white, dry friable and glassy
8.0	58 ± 3.1	Brownish, dry friable and glassy

Values represent means ± standard deviation (S.D) for 15 cultures per treatment.

Table 4. Effect of IAA and NAA on callus induction from *C. mangga* shoot bud explants cultured on MS medium with 3% (v/v) sucrose.

IAA(mg l^{-1})	NAA (mg l^{-1})	Callus induction (%)	Morphology of callus
1	1	62 ± 1.8	Whitish and globular
3	1	55 ± 2.0	Whitish and globular
5	1	58 ± 6.1	Whitish, globular and friable
1	3	42 ± 3.4	Whitish, globular and friable
1	5	57 ± 5.2	Whitish, globular and friable
5	5	70 ± 1.7	Whitish, globular and friable
3	3	68 ± 2.0	Whitish, globular and friable
5	3	67 ± 2.3	Whitish, globular and friable
3	5	66 ± 2.4	Whitish, globular and friable

Values represent means ± standard deviation (S.D) for 15 cultures per treatment.

acid (5 mg l^{-1}) as antioxidants and further decreasing 2,4-D concentration to 0.3 mg l^{-1} produced proliferating yellowish white and vigorous suspension cell cultures (Fig 8) without further tissue browning. Bushra et al., (2009), reported that the browning and subsequent death of explants and cultures could be attributed to the oxidation of polyphenols. Treatment with antioxidants like ascorbic acid and citric acid seemed to inhibit the polyphenoloxidase enzyme that converts the phenols to typical brown polymersis. Therefore this is one of the effective strategies to control the phenolic browning of *in vitro* cultures (Pizzocaro et al., 1993). The combination of 2% ascorbic acid and 2% citric acid reduced the percentage of browning in *F. religiosa* L. culture (Siwach et al., 2011). MS medium augmented with 0.3 mg l^{-1}, 2,4-D, 0.1 mg l^{-1} NAA, 100 mg l^{-1} glutamine, 0.1 mg l^{-1} d-Biotin, 30 g l^{-1} sucrose , 0.1 g l^{-1} malt extract and antioxidant agents, 5 mg l^{-1} ascorbic acid and citric acid respectively was further used as standardized medium(M2) for suspension cultures. Meanwhile other media used in this study did not overcome cell browning. 100 mg l^{-1} glutamine and 0.1 mg l^{-1} d-biotin were also added to the media. Glutamine is an amino acid added as a nitrogen source and also to increase embryogenic callus formation (Peterson and Smith, 1991). Ahmed et al. (2009) reported that glutamine at different concentrations has been used in order to reduce phenolic exudation from leaf callus. Meanwhile biotin acts as a cofactor for a small number of enzymes involved in carboxylation, decarboxylation, and transcarboxylation reactions that are concerned with fatty acid and carbohydrate metabolism (Knowles, 1989; Alban et al., 2000). Since prior results had indicated that suspension cultures of *C. mangga* could grow well in an MS medium supplemented with vitamins and augmented in 0.3 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 100 mg l^{-1} glutamine, 0.1 mg l^{-1} d-Biotin, 30 g l^{-1} sucrose , 0.1 g l^{-1} malt extract and added with antioxidant agent of 5 mg l^{-1} ascorbic acid and 5 mg l^{-1} citric acid, growth kinetics experiments were undertaken using this medium. The cell cultures were maintained by subculturing at 10 day intervals. The growth of cells in the suspension culture was found to proliferate more vigorously compared to cells on the solidified culture
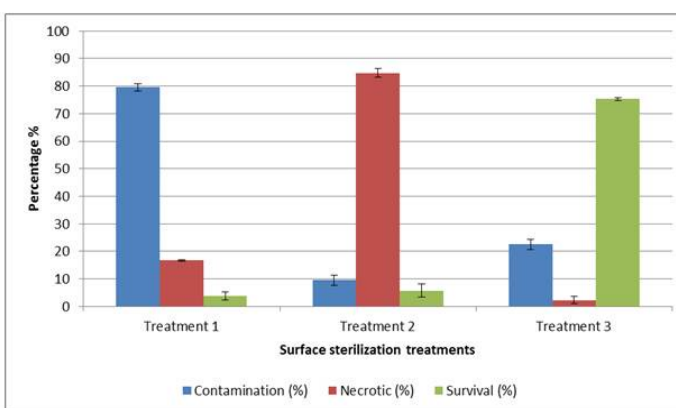

Fig 3. Percentage of surviving explants under different methods of surface sterilization. Values are mean ± standard deviation for 4 replicates each treatment.

Fig 4. Root from shoot bud explants of *C. mangga* on MS media without any phytohormones (Scale: Bar = 1 mm).

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Table 5. Effect of sucrose on callus induction from *C. mangga* shoot bud explants cultured on MS medium with 1mg/l 2,4-D.

Sucrose (%) (v/v)	Callus induction (%)	Morphology of callus
3	76 ± 3.2	White, friable, sticky and glassy
8	65 ± 2.7	White, mucilaginous, wet and in lumps

Values represent means ± standard deviation (S.D) for 15 cultures per treatment.

medium (Fig 7). This was probably due to the agitation and loosening of cells which would encourage further cell division. In this study, suspension cultures showed typical growth kinetics when 5 ml (SCV) inoculums were cultured in 50 ml of culture medium. The growth of cell suspension cultures reached a maximum growth (45ml SCV) 21 days after initiation and subsequently became stationery and started to decline after day 28. The same growth kinetics pattern was reported by Lee and Chan et al (2004) in *in vitro* *Orthosiphon stamineus* cell suspension growth kinetics.

Materials and methods

Establishment of aseptic explants

The fresh yellow rhizomes of *C. mangga* Val. were purchased from a local farm. The rhizomes were washed, air dried and placed in paper boxes to allow shoots to sprout to about 3 to 4 cm in length and were used as explants. The rhizomes were placed in different conditions as in Table 1 to obtain optimal sprouting. Suitable explants were chosen and rinsed under running tap water for 10 minutes. The summary of the general sterilization method is shown in the Fig 1 and different treatments used are shown in Table 2. Tween-20 was added to reduce surface tension. The surface sterilized shoots had their external leaves removed and were finally dried on filter papers under laminar air flow prior to inoculation on media. The contamination, necrotic and survival percentage of cultures were calculated using the following formula:

$$\frac{\text{No. of cultures contaminated or necrotic or survived}}{\text{Total number of cultures inoculated}} \times 100\%$$

Determination of culture media for callus induction and proliferation

The shoot buds explants were trimmed and sliced into 1 mm pieces cross sections. Explants were cultured on a MS basal medium with 2 g l^{-1} gelrite were augmented with different concentrations of sucrose and phytohormones combinations (Table 3, 4 and 5). Media were adjusted to pH 5.7 and were autoclaved at 120°C for 20 minutes. The cultures were maintained in the dark at 25 ± 1 °C. For every treatment 15 replicates (15 plates of same medium) were used with 10 explants cultured on each plate. Observations on the morphology of the calli induced were recorded after six weeks. The calli induction percentage was calculated using the following formula:

$$\frac{\text{No. of explants initiating calli}}{\text{Total number of explants inoculated}} \times 100\%$$

Rapid growth of friable callus was assessed using fresh weight fortnightly. Each treatment had four replicates and was repeated twice.

Histology of calli

Histology techniques using resin developed by the ORSTOM – CIRAD team LRGAPT France (Maril et al., 1995) were used. The samples were sliced and then fixed for 24 to 48 hours at room temperature in Glutaraldehyde-Paraformaldehyde-caffeine (GPC) fixative (mixture of 50 ml 0.2M phosphate buffer, 20 ml paraformaldehyde, 4 ml 25% glutaraldehyde, 1 g caffeine and topped up to 100 ml with distilled water at pH 7.2). The samples were then dehydrated in ethanol: 30% (30 min); 50% (45 min); 70% (45 min); 80% (60 min); 90% (60 min); 95% (60 min) and twice in absolute ethanol for 60 minutes each. The tissues were then prepared for filtration with basic resin (Leica Historesin Embedding Kit) for 24 to 48 hours at 4°C under slight vacuum. The specimens were embedded and the resin was allowed to be fully polymerized and were sliced using a microtome. Specimens were stained with 1% periodic acid for five minutes, rinsed with distilled water at pH 4.5 and the submerged in Schiff's reagent for 20 minutes in the dark. Finally, for counter staining, Napthol Blue Black was used at 60°C for five minutes and viewed under a light microscope.

Determination of optimum maintenance medium for liquid suspension culture

Callus was suspended in a liquid medium to enhance proliferation. For initiation, only friable callus was used. Prior to any new treatment, 1 g of the friable callus was proliferated in a liquid medium with same concentration as callus induction medium (MS medium supplemented with 1mg l^{-1} 2,4-D and 30g l^{-1} sucrose). However, due to cell browning, after the 3rd subculture, cells were suspended in a liquid medium with MS augmented with various concentrations of 2,4-D (0.05 – 0.5 mg l^{-1}) and sucrose(20-50 g l^{-1}) with 100 mg l^{-1} glutamine, and 0.1 mg l^{-1} malt extract. The pH of the medium was adjusted to 5.8 before autoclaving at 120 °C for 20 minutes. The cultures were incubated at 25 ± 1 °C at 90 rpm on a shaker under indirect light at 18 hours photoperiod. The cultures were subcultured into fresh liquid medium every 10 days and were observed periodically under the microscope. After the 6th subculture, the medium was again changed because of tissue browning. The suspension cultures were suspended in liquid MS salts augmented with various concentrations of 2,4-D (0.1 – 0.5 mg l^{-1}), 0.1 mg l^{-1} NAA, 100 mg l^{-1} glutamine, 0.1 mg l^{-1} d-Biotin, 30 g l^{-1} sucrose , 0.1 g l^{-1} malt extract and antioxidant agents of ascorbic acid (0 or 5 mg l^{-1}) and citric acid (0 or 5 mg l^{-1}). Stable suspension cultures were used to determine the growth curve. The growth of the suspension cultures was measured by determining the settled cell volumes (SCV) every three days over a period of one month.

Conclusion

Suspension cultures of *C. mangga* could be established from friable callus derived from shoot bud explants. This friable callus were produced by culturing on MS medium supplemented with 1.0 mg l^{-1} 2,4-D. Histological sectioning showed that this callus has dense cytoplasmic cells and a
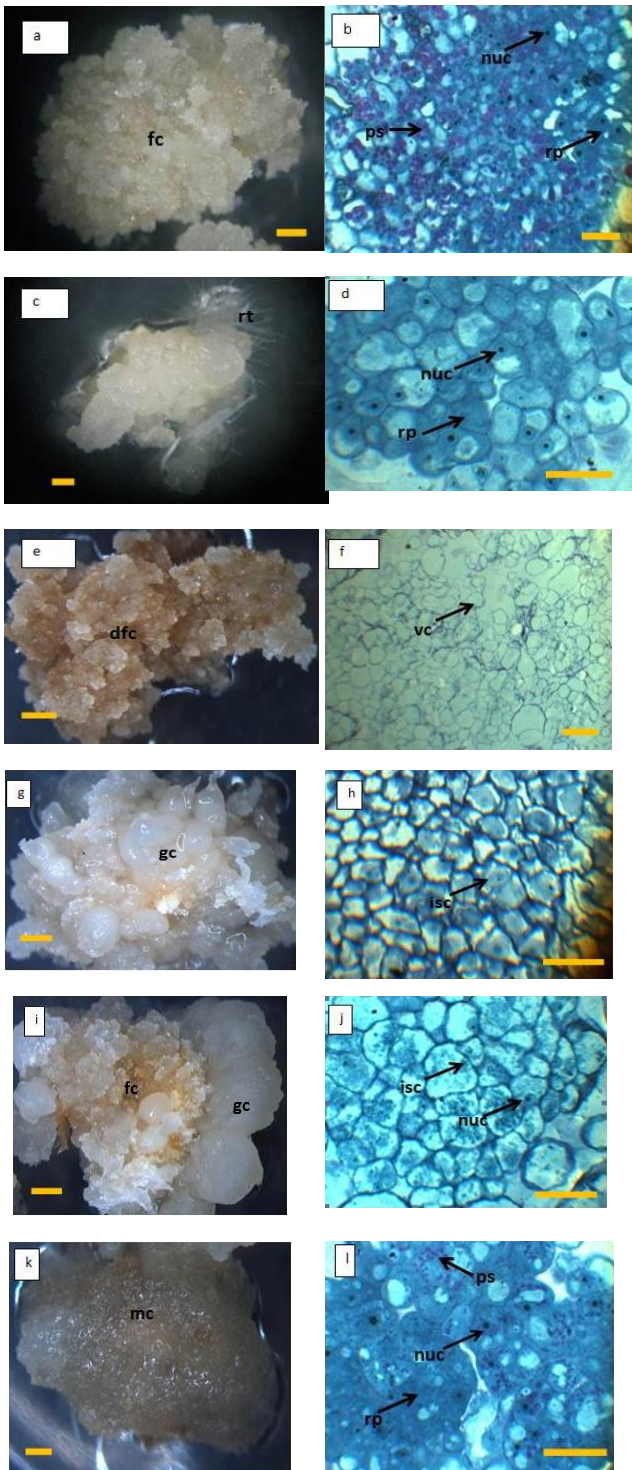
Fig 5: Morphology and histology of *C. mangga* calli in MS medium. (5a) White and friable callus induced on MS with 1mgl⁻¹ 2,4- D media (Scale: Bar = 1mm). (5b) Histology of dense cells of callus with large nucleus on MS with 1mgl⁻¹ 2,4- D media (Scale: Bar = 50µm). (5c) White, nodular and rooting callus on MS with 0.1mgl⁻¹ 2,4-D media (Scale: Bar = 1mm). (5d) Histology of dense cells of callus with large nucleus initiated on MS with 0.1mgl⁻¹ 2,4-D media(Scale: Bar = 50µm). (5e) Brownish, dry friable callus on MS with 8mgl⁻¹ 2,4-D media (Scale: Bar = 1mm). (5f) Histology of callus with large vacuolated cells on MS with 8mgl⁻¹2,4-D media (Scale: Bar = 50µm). (5g) Whitish and globular callus on MS with 1mgl⁻¹ IAA and 1mgl⁻¹ NAA media (Scale: Bar = 1mm). (5h) Histology of callus with irregular shaped cells with small nucleus and cytoplasm on MS with 1mgl⁻¹ IAA and 1mgl⁻¹ NAA media (Scale: Bar = 50µm). (5i) Whitish, globular and friable callus on MS with 5mgl⁻¹ NAA and 5mgl⁻¹ IAA media (Scale: Bar = 1mm). (5j) Histology of callus with small nucleus and cytoplasm on MS with 5mgl⁻¹ NAA and 5mgl⁻¹ IAA media (Scale: Bar = 50µm). (5k) Mucilaginous, wet and large lump callus on MS with 8% sucrose in 1mgl⁻¹ 2,4-D media (Scale: Bar = 1mm). (5l) Histology of dense cells of callus with large nuclei on MS with 5mgl⁻¹ NAA and 5mgl⁻¹ IAA media (Scale: Bar = 50µm). **dfc**: dried friable callus; **fc**: friable callus; **gc**: globular callus; **isc**: irregular shaped cells; **mc**: mucilaginous callus; **nuc**: nucleus; **ps**: polysaccharide; **rp**: reserved protein; **rt**: root; **vc**: vacuolated cells
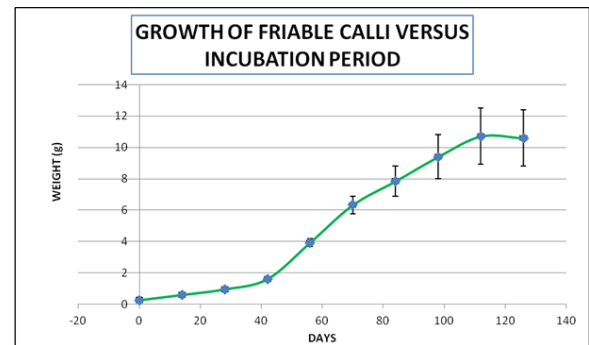

Fig 6. Fresh weight of *C. mangga* callus culture in MS medium supplemented with 1mg/L 2,4-D. Values represent means ± standard deviation (SD) for 4 replicates per treatment.
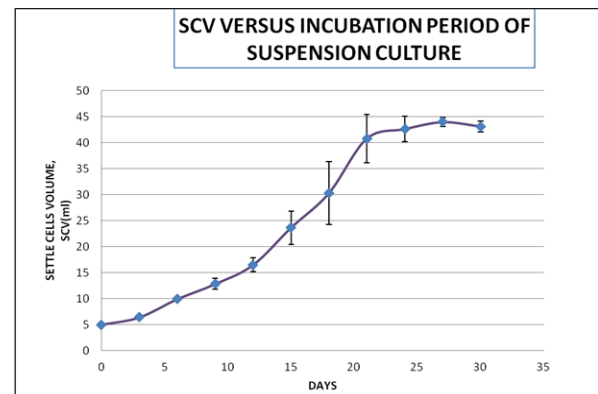

Fig 7. Fresh weight of *C. mangga* suspension culture in M2 medium. Values represent means ± standard deviation (SD) for 8 replicates per treatment.

distinct nucleus with the presence of polysaccharide and reserved protein that supports rapid cell growth. The suspension cultures of *C. mangga* can be established and maintained by inoculating friable callus into MS liquid medium supplemented with 0.3 mg l⁻¹ 2,4-D, 0.1 mg l⁻¹ NAA, 5 mg l⁻¹ ascorbic acid, 5 mg l⁻¹ citric acid, 0.1 mg l⁻¹ d-Biotin, 100 mg l⁻¹ glutamine, 0.1 g l⁻¹ malt extract and 30 g l⁻¹sucrose. Ascorbic acid and citric acid were added as antioxidant agents to overcome cell browning. For future studies, suspension cultures established in this medium can be used as a source for the production of secondary metabolites.
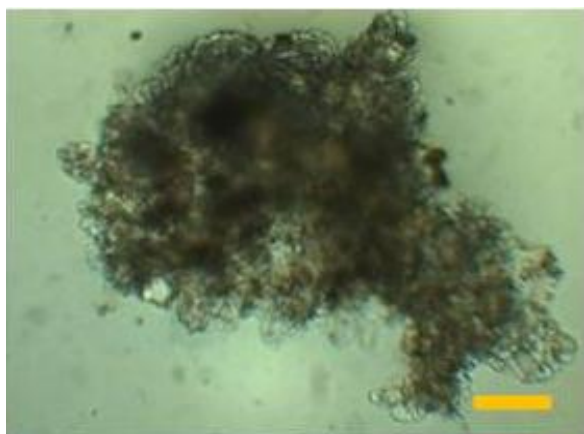
Fig 8. Rapid growing suspension cells in M2 medium (Scale: Bar = 100μm)

Acknowledgements

References

Abas F, Lajis NH, Shaari K, Israf DA, Stanslas J, Yusuf UK (2005) A labdane diterpene glucoside from the rhizomes of *Curcuma mangga*. J Nat Prod 68: 1090-1093.

Abbade LC, Paiva PD, Paiva R, Graciano M (2010) Growth curve and biochemical analyses of callus of Ipe-branco (*Tabebuia roseo* alba (Ridl.) Sand.). Naturalia Rio Claro 33: 45-56.

Achut SG, Bandyopadhyaya C (1984) Characterization of mango like aroma in *Curcuma amada* Roxb. J Agr Food Chem 32: 57-59.

Afolabi AS, Oyebanji OB, Nweke O, Odebunmi O, Galadima NB, Idris MS, Nnodi UN, Ogbadu GH (2009) Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds. Afr J Biotechnol 8 (20): 5395-5399.

Afshari RT, Angoshtari R, Kalantari S (2011) Effects of light and different plant growth regulators on induction of callus growth in rapeseed (*Brassica napus L.*) genotypes. Plant Omics J 4(2): 60-67.

Ahmed ABI , Rao AS, Rao MV (2009) Somatic Embryogenesis and Plant Regeneration from Cell Suspension Culture of *Gymnema sylvestre* (Retz) R. Br. Ex Roemer & Schultes. KMITL Science and Technology Journal 9 (1): 18-26.

Alban C, Job D, Douce R (2000) Biotin metabolism in plants. Annu Rev Plant Phys 51: 17-47.

Bushra S, Anwar F, Ashraf M (2009) Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules 14(6): 2167-2180.

Chan EWC, Lim YY, Wong LF, Lianto FS, Wong SK, Lim KK, Joe CE, Li TY (2008) Antioxidant and tyrosinase inhibition properties of leaves and rhizomes of ginger species. Food Chem 109: 477-483.

Chan LK, Thong WH (2004) *In vitro* propagation of Zingiberaceae species with medicinal properties. J Plant Biotechnol 6: 181-188.

Dukan S, Belkin S, Touati D (1999) Reactive oxygen species are partially involved in the bactericidal action of hypochlorous acid. Arch Biochem Biophys 367: 311–316.

Endress R (1994) Plant Cell Biotechnology. Springer publisher, Verlag, Berlin, Heidelberg, New York, pp: 35.

Evans DE, Coleman JOD and Kearns A (2003) Plant Cell Culture. Bios scientific publishers, London and New York.

Fisher DB (1968) Protein staining of ribboned epon section for light microscopy. Histochemie 16: 92-96.

Giri A, Ahuja PS, Ajay Kumar PV (1993) Somatic embryogenesis and plant regenaration from callus culture of *Aconitum heterophyllum* Wall. Plant Cell Tiss Org 32: 213-218.

Habibi Z, Eftekhar F, Samiee K, Rustaiyan A (2000) Structure and antibacterial activity of a new labdane diterpenoids from *Salvia leriaefolia*. J Nat Prod 63: 270-271.

Hong CH, Kim Y, Lee SK (2001) Sesquiterpenoids from the rhizome of *Curcuma zedoaria*. Arch Pharm Res 24: 424-426.

Hosoki T, Sagawa Y (1997) Clonal propagation of ginger (*Zingiber officinale* Rosc.) through tissue culture. HortScience 12: 451-452.

Hussain Z, Tyagi RK, Sharma R, Agarwal A (2008) Genetic diversity in *in vitro*-conserved germplasm of *Curcuma Longa* as revealed by RAPD markers. Biol Plantarum 52: 627-633.

Jalil M, Wong WC, Othman RY, Khalid N (2008) Morphohistological examination on somatic embrogenesis of *Musa acuminata* cv. Mas (AA). Sci Hortic-Amsterdam 117: 335-340.

Jonoubi P, Mousavi A, Majd A, Salmanian AH, Jalali JM (2005) Efficient regeneration of *Brassica napus* L. hypocotyls and genetic transformation by *Agrobacterium tumefaciens*. Biol Plantarum 49: 175-180.

Kirana C, Record IR, McIntosh GH, Jones GP (2003) Screening for antitumor activity of 11 species of Indonesian Zingiberaceae using human MCF-7 and HT-29 cancer cells. Pharm Biol 41: 271-276.

Knowles JR (1989) The mechanism of biotin-dependent enzymes. Ann Rev Biochem 58: 195-221.

Kulkarni VM, Varshny LR, Bapat VA, Rao PS (2002) Somatic embryogenesis and plant regeneration in a seeded banana [*Ensete superbum* (Roxb.) Cheesman]. Curr Sci India 83: 939-941.

Kumar S, Kanwar JK (2007) Plant regeneration from cell suspension in *Gerbera gamesonii* Bolus**.** J Fruit Ornam Plant Res 15: 157-166.

Lee K, Jeon H and Kim M (2002) Optimization of mature embryo based *in vitro* culture system for high-frequency somatic embryogenic callus induction and plant regeneration from Japonica Rice Cultivars. Plant Cell Tiss Org 71: 237-244.

Lee WL, Chan LK (2004) Establishment of *Orthosiphon stamineus* cell suspension for cell growth. Plant Cell Tiss Org 78: 101-106.

Lima EC, Paiva R, Nogueira RC, Soares FP, Emrich EB, Silva AAN (2008) Callus induction in leaf segments of *Croton urucurana* Baill. Cienc Agrotec 32(1): 17-22.

Liu Y, Nair MG (2011) Labdane diterpenes in *Curcuma mangga* rhizomes inhibit lipid peroxidation, cyclooxygenase enzymes and human tumour cell proliferation. Food Chem 124: 527-532.

Malek SNA , Guan SL, Sok LH, Hashim Y,Norhanom AW, Jean-FFW, Syed AAS (2011) Phytochemical and Cytotoxic Investigations of *Curcuma mangga* rhizomes. Molecules 16: 4539-4548.

Maril S, Engelmann F, Chabrillangel N, Huet C and Michaux-Ferrière N (1995) Histocytological study of apices of coffee (*Coffe Racemosa* and *C. Sessiliflora*) in

vitro plantlets during their cryopreservation using the encapsulation-dehydration technique. Cryoletters 16: 289-298.

Mrudul VS, John CK, Rajani SN (2001) Factors affecting *in vitro* microrhizome production in turmeric. Plant Cell Tiss Org 64: 5-11.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol 15: 473-497.

Nakagarwara S, Goto T, Nara M, Ozawa Y, Hotta K, Arata Y(1998) Spectroscopic characterisation and the pH dependence of bacterial activity of the aqueous chlorine solution. Anal Sci 14: 691-698.

Neeta DS, Leela G, Susan E (2002) Micropropagation and field evaluation of micropropagated plants of turmeric. Plant Cell Tiss Org 68: 143-151.

Ngara R, Rees J, Ndimba BK (2008). Establishment of sorghum cell suspension culture system for proteomics studies. Afr J Biotechnol. 7: 744-749.

Park SY, Kim DSHL (2002) Discovery of natural products from *Curcuma longa* that protect cells from beta-amyloid insult: A drug discovery effort against Alzheimer's disease. J Nat Prod 65: 1227-1231.

Peterson G, Smith R (1991). Effect of abscicic acid and callus size on regeneration of American and International rice varieties Plant Cell Rep 10: 35-38.

Pierik, RLM (1990) Cultivo *in vitro* de las plantas superiors. Multiprensa publisher, Madrid, España, pp: 326.

Pizzocaro F, Torreggiani D, Gilardi G (1993) Inhibition of Apple polyphenoloxidase (ppo) by ascorbic acid, citric acid and sodium chloride. J Food Process Pres 17: 21-30.

Prakash S, Elangomathavan R, Seshadri S, Kathiravan K, Ignacimuthu S (2004) Efficient regeneration of *Curcuma amada* Roxb plantlets from rhizome and leaf sheath explants. Plant Cell Tiss Org 78: 159-165.

Rahman M, Asaduzzaman M, Nahar N, Bari MA (2006) efficient plant regeneration from cotyledon and midrib derived callus in eggplant (*Solanum melongena* L.). J Bioscience 14: 31-38.

Rao AS, Bandaru R, Ramachandran S (1989) Volatile aroma components of *Curcuma amada* Roxb. J Agr Food Chem 37(3): 740-743.

Richomme P, Godet MC, Foussard F, Toupet L, Sevenet T, Bruneton J (1991) A novel leishmanicidal labdane from *Polyalthia macropoda*. Planta Med 57: 552-554.

Roy A , Ghosh S, Chaudhuri M, Saha PK (2008) Effect of different plant hormones on callus induction in *Gymnema sylvestris* R.Br. (Asclepiadaceae). Afr J Biotechnol 7(13): 2209-2211.

Santos CG, Paiva R, Paiva PDO, Paiva E (2003) Indução e análise bioquímica de calos obtidos de segmentos foliares de *Coffea arabica* L. Cultivar Rubi. Cienc Agrotec 27(3): 571-577.

Sasikumar B (2005) Genetic resources of Curcuma: diversity, characterization and utilization. Plant Genetic Resources 3(2): 230-251.

Serra AGP, Paiva R, Paiva PDO (2000) Análises bioquímicas de calos formados de explantes foliares de castanheira-do-Brasil (*Bertholletia excelsa* H.B.K). Cienc Agrotec Lavras 24(4): 833-840.

Singh A, Kandasamy T and Odhav B (2009) *In vitro* propagation of *Alternanthera sessilis* (sessile joyweed), a famine food plant. Afr J Biotechnol 8 (21): 5691-5695.

Singh N, Singh J, Kaur L, Sodhi NS, Gill BS (2003) Morphological, thermal and rheological properties of starch from different botanical sources – a review. Food Chem 81: 219-231.

Siwach P, Anita RG and Kavitha K (2011) Effect of season, explants, growth regulators and sugar level on induction and long term maintenance of callus cultures of *Ficus religiosa* L. Afr J Biotechnol 10(24): 4879-4886.

Smith RH (2005) Plant Tissue Culture: Techniques and Experiments 2nd edn. Elsevier Publishers, New Delhi.

Swedlund B and Locy RD (1993) Sorbitol as the primary carbon source for the growth of embryogenic callus of maize. Plant Physiol 103: 1339-1346.

Syu WJ, Shen CC, Don MJ, Ou JC, Lee GH Sun CM (1998) Antiplatelet aggregation constituents from *Annona purpurea*. J Nat Prod 61: 1531-1534.

Tewtrakul S, Subhadhirasakul S (2007) Anti-allergic activity of some selected plants in the Zingiberaceae family. J Ethnopharmacol 109: 535-538.

Tiwari SK, Tiwari KP, Siril EA (2002) An improved micropropagation protocol for teak. Plant Cell Tiss Org 71: 1-6.

Viana AM, Mantell SH (1989) Callus induction and plant regeneration from excised zygotic embryo of seed-propagated yams *Dioscorea composite* Hemsl. and *D. cayenenesis* Lam. Plant Cell Tiss Org 16: 113-122.

Wlodkowski TJ, Rosenkranz HS (1975) Mutagenicity of sodium hypochlorite *for Salmonella typhimurium*. Mutat Res 31: 39-42.

Yunbao L, Muraleedharan GN (2010) Labdane diterpenes in *Curcuma mangga* rhizomes inhibit lipid peroxidation, cyclooxygenase enzymes and human tumour cell proliferation. Food Chem 124: 527-532.

Yusuf N, Khalid N, Ibrahim H (2007) Establishments of tissue culture for selected medicinal *Curcuma* spp. Malaysian J Sci 26(1): 85-91.