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Preliminary Studies on Optimization of Surface Sterilization Technique and Effects of Selected Carbon Source on *In Vitro* Callus Induction of Mango Ginger (*Curcuma Mangga* Val.)

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ABSTRACT

Curcuma mangga Val., is a traditional ginger with unique mango-like aroma and has medicinal values. Cultivation of this species through conventional methods is prone to diseases and has a low propagation rate. *In vitro* micropropagation is an alternative to produce disease-free *C. mangga* plantlets. Thus, we conducted preliminary study to establish *in vitro* culture, by optimizing surface sterilization technique and identifying optimal carbon source for callus culture initiation. The combination of 70% NaOCl + Tween 20 (5 minutes), 70% ethanol (5 minutes) and 0.3% HgCl2 as disinfectants was found to be the most effective surface sterilization technique out of three methods tested, with an 89.25% survival rate. Next, the effects of carbon sources on callus induction were studied by culturing the explants in 1 mg/L 2,4-D added MS medium supplemented with sucrose or glucose at three different concentrations (15, 30 and 60 g/L). The callus induction percentage, morphology and size obtained varied depending on the treatments. Friable callus was obtained from all the treatments except in treatment T4 with 60g/L glucose, which produced compact callus. The calluses obtained can be indirectly regenerated into *C. mangga* plantlets that can be further used as planting materials of *C. mangga.* Suspension culture could also be initiated using the callus culture. These cultures can be engineered as source for production of essential bioactive compounds

Keywords: Callus, tissue culture, sucrose, glucose, mango ginger, suspension culture.

ABSTRAK

Curcuma manga Val., atau lebih dikenali sebagai temu manga disebabkan aromanya yang menyerupai manga, adalah tumbuhan bernilai perubatan. Namun, penanamannya secara konvensional berhadapan dengan cabaran seperti mudah terdedah kepada penyakit dan kadar pembiakan yang rendah. Mikropembiakan secara *in vitro* menawarkan alternatif yang berpotensi untuk menghasilkan anak benih *C. mangga* yang bebas penyakit. Kajian awal ini bertujuan untuk menetapkan protokol kultur *in vitro* yang optimal melalui penambahbaikan teknik pensterilan dan mengenal pasti sumber karbon yang sesuai untuk penghasilan kalus. Kaedah pensterilan yang didapati terbaik melibatkan rawatan berturutan dengan 70% natrium hipoklorit (NaOCl) + Tween 20 selama 5 minit, 70% etanol selama 5 minit, dan 0.3% merkuri klorida ($HgCl_2$). Kaedah ini menghasilkan kadar kelangsungan hidup sebanyak 89.25%. Seterusnya, kesan pelbagai sumber karbon terhadap induksi kalus dikaji dengan mengkultur eksplan dalam medium MS yang ditambah 1 mg/L 2,4-D, bersama sukrosa atau glukosa dengan tiga kepekatan berbeza (15, 30, dan 60 g/L). Peratusan induksi kalus, morfologi, dan saiz kalus yang diperoleh adalah berbeza bergantung kepada rawatan sumber karbon. Kalus rapuh diperoleh daripada semua rawatan kecuali rawatan T4 dengan glukosa 60 g/L, yang menghasilkan kalus padat. Kalus yang dihasilkan boleh diregenerasi secara tidak langsung menjadi anak benih *C. mangga*, yang boleh digunakan sebagai bahan tanaman *C. mangga*. Kultur suspensi juga dapat dimulakan daripada kultur kalus ini. Kultur ini boleh dimanfaatkan sebagai sumber untuk pengeluaran sebatian bioaktif penting.

Kata kunci: Kalus, tisu kultur, sukrosa, glukosa, temu mangga, kultur gantung.

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INTRODUCTION

Curcuma mangga Valeton & C. van Zijp (*C. mangga*) is an herbaceous perennial plant belonging to Zingiberaceae family. The yellowish-brown rhizome of this plant has a unique mango-like aroma when sliced or crushed (Liu & Nair, 2012). Thus, it is commonly known as mango ginger or 'temu mangga' in Malay and Indonesian languages. The rhizomes are used as spice in many South and Southeast Asian countries. The leaves, on the other hand are added to various dishes for flavouring (Liu & Nair, 2012).

C. mangga is also used as traditional medicine. The extract of the rhizomes is used to treat chest pain, fever, general debility, and womb healing. The starch of the rhizomes is useful in treating abdominal illness. The rhizome is usually chewed or eaten raw with rice as a relief for flatulence, stomach-ache and colic. The leaves are used as heated aromatic herbal bath for ladies in confinement. Besides, the leaves are also used in treating skin diseases as well as for producing essential oil and fragrance (Muchtaromah et al., 2020; Silalahi et al., 2021; Herisman et al., 2022).

Bioactive compounds in *C. mangga* prove to be beneficial as antibacterial, antioxidant, anti-allergic, analgesic, lipid peroxidation, antiviral, cytotoxic and anti-inflammatory (Ruangsang et al., 2010; Rajkumari & Sanatombi, 2017; Dosoky and Setzer et al., 2018; Sundram et al., 2019). Recent reports suggest that *C. mangga* possesses cytotoxic activity against cancer cells (Malek et al., 2011; Karsono et al., 2014; Hong et al.,2016; Sundram et al., 2019). These biological and medicinal activities of *C. mangga* is due to the presence of phenols, terpenoids and alkaloids bioactive compounds such as curcuminoids, (*E*)-labda-8(17),12-dien-15,16-dial, (*E*)-15,16-bisnor-labda-8(17),11-dien-13- on, zerumin A (Malek et al., 2011; Rajkumari & Sanatombi, 2017; Yuandani et al., 2020;).

Pikulthong et al., (2016) reported that in India, *C. mangga* is propagated during rainy season, making their availability seasonal. Traditionally, *C. mangga* is propagated vegetatively by division of rhizomes. However, due to low efficiency of vegetative propagation and requirement of closer planting space, large quantities of rhizomes are required as planting materials (Waman et al., 2021; Rustikawati et al., 2023). Moreover, the production of zingiberaceae species is also affected by other factors such as low propagation rate, soil-borne diseases and deterioration of rhizomes caused by bacteria, fungal and insect attacks. These problems cause heavy losses on the production of *C. mangga* rhizomes (Pikulthong et al., 2016).

In vitro micropropagation technique could serve as an alternative propagation method for mass production of *C. mangga* plantlets. According to Tewelde et al. (2020), *in vitro* micropropagation becomes a viable strategy for mitigating the problem faced in propagating ginger varieties. This technique is useful for mass propagation and conservation of germplasm of vegetatively propagated crops; protection against pests and environmental vagaries; production of bioactive compounds; and genetic improvement of many plant species (Kusumastuti et al., 2014; Anis & Ahmad, 2016).

Plant regeneration from callus facilitates amplification of plant species that have limited plant materials and permits the isolation of rare somaclonal variants. Under certain conditions, explants develop callus with very diverse forms and can be classified into subgroups based on their macroscopic characteristics. Callus formation or somatic embryogenesis can re-differentiate into entire plants under suitable growth media through organogenesis (Ikeuchi et al., 2013). According to Ali et al. (2016), organogenesis from callus is an effective technique for rapid clonal and mass propagation of specific plant species. Callus culture serves as mother culture in initiating cell suspension cultures (Ali et al., 2016). Both these cultures are also currently being explored as an alternative for production of pharmaceutically important

bioactive compounds from various plants species.

Hence, in this preliminary study, surface sterilization methods have been optimised to disinfect the *C. mangga* shoot buds explants from any contaminant impurities. Then, the explants were cultured on Murashige and Skoog (MS) medium supplemented with 1 mg/L of 2,4-Dichlorophenoxyacetic acid (2,4-D) with two different carbon sources. The effects of these carbon sources, sucrose and glucose at varying concentrations on callus induction were tested.

MATERIALS AND METHODS

Plant Material

Fresh rhizomes of *C. mangga* obtained from local farm in Pahang were thoroughly cleaned and sprouted in a soilless condition. *C. mangga* rhizomes were placed in a dark condition at room temperature to induce shooting (Sundram et al., 2012). The rhizomes were daily sprinkled with water for two weeks to induce sprouting of shoot buds. The shoot buds of around 3 ± 0.5 cm in length were collected and used as explants for *in vitro* callus induction.

Sterilization of Apparatus and Equipment

All glassware were washed with detergent, rinsed several times with tab water, rinsed again using sterile distilled water and finally dried in drying cabinet. In avoiding extraneous contamination due to invading microorganisms, sterile instruments and sterile culture media were used. These media and apparatus were rendered sterile by autoclaving at 121 °C for 20 minutes.

Aseptic transfers were performed in laminar air flow chamber, equipped with hot beads sterilizer. In order to control the airborne bacteria in the culture room, ultraviolet (UV) lamp was switched on for 15 minutes, prior to culture or subculture activities in the chamber. Autoclaved forceps and scalpels were placed in hot beads sterilizer at 250°C before being used. The surface of laminar air flow chamber was sterilized by wiping them with 70% ethanol. Prior to aseptic transfer, all equipment and materials to be used inside the chamber were sprayed and wiped with 70% ethanol.

Explants Surface Sterilization

The surface sterilization methods were manipulated to get an optimized surface sterilization technique, with lowest percentage of contamination. The shoot buds used as explants were excised when they reach the height of 3 ± 0.5 cm in length. The explants were washed several times under running tap water. Then, the explants were soaked in sodium hypochlorite (NaClO) at the different concentrations, of either 50% (v/v) (surface sterilization method 1 (SM1)), 70% (v/v) (SM2), and 100% (v/v) (SM3) for 15 minutes, with an addition of 5 drops of Tween 20. All these NaClO soaked explants were then washed with sterile distilled water for 5 minutes before being soaked in 70% ethanol for 5 minutes. Later, the explants were soaked in 0.3% (w/v) mercuric chloride ($HgCl_2$) for 4 minutes and rinsed with sterile distilled water for 3-4 times. Under aseptic conditions, the outer sheaths of explants were removed and cut into small pieces of about 0.5 cm, prior to inoculation on media. Observation on the contamination and the survival percentage of the cultures under each treatment were recorded. The survival percentage is calculated as follow:

$$\frac{Number\ of\ explants\ survived}{Total\ number\ of\ explants\ inoculated} \times 100\%$$

Culture Medium and Callus Initiation

Murashige and Skoog basal salts (MS) was used as main culture medium in this study. According to Sundram et al. (2012), MS medium supplemented with 1 mg/L 2,4-D induced the highest percentage of *C. mangga* friable callus. Thus, in this study, 2,4-D was used as plant growth regulator at a standardized concentration of 1 mg/L for callus induction. In identifying the effect of carbon source on callus induction of *C.*

mangga, the 0.5 cm sliced explants were then cultured on MS + 1 mg/L 2,4-D media with either sucrose (table 2.0) or glucose (table 3.0) at three different concentrations (15 g/L, 30 g/L or 60 g/L). For every treatment, there were eight replicates in total (8 plates of same medium) used with 5 pieces of sliced explants cultured on each plate. The pH of the media was adjusted between 5.7 to 5.8 and then solidified with 2 g/L agar and sterilized at 120°C for 20 min. All cultures were maintained at the photoperiod of 24 hours dark at 25 ± 1°C for 16 weeks. Observation of the cultures were done biweekly. Contaminated and necrotic culture were discarded. Meanwhile, subculturing was done every four weeks. Observations of the callus morphology, the growth and callus induction percentage were recorded at week 16. The callus induction percentage was calculated as follow:

$$\frac{Number\ of\ explants\ initiating\ callus}{Total\ number\ of\ explants\ inoculated} \times 100\%$$

RESULTS AND DISCUSSION

Explant Surface Sterilization

A suitable explant selection is one of the vital aspects for a successful micropropagation of any plants (Seran, 2013). Stable and healthy *in vitro* regenerated plantlets are largely affected by the source and the regeneration mode of the explants (Mohanty et al., 2013). Shoot buds are chosen as explant due to their high regenerative potential, organogenic ability and genetic stability in maintaining characteristic of parent plant (Shaik & Kanth, 2018).

Fungus, bacteria, and other microbial contaminants from the explant source and environment could cause contamination of *in vitro* culture. According to Efzueni et al., (2014) many rhizomatic species were sprouted in soilless conditions prior to culture as to reduce the potential of soil microorganisms infecting the explants. Therefore, in our study the shoot buds were grown and maintained in soilless condition to avoid soil-borne microorganisms' contaminants. However, as the rhizomes were originally harvested from underground soil, they are still likely to be exposed to soil-borne contamination (Shivakumar, 2019). Hence, to avoid any further contamination of *in vitro* cultures, proper surface sterilisation of shoot bud explants was done using three disinfectants as mentioned in table 1.0. The survival rate and types of contaminating microorganisms causing the death of explants in the *in vitro* cultures are also summarized in table 1.0.

In all the three surface sterilization treatments undertaken, only concentration of NaOCl was varied while concentration of ethanol and $HgCl_2$ were kept constant. Based on results obtained as shown in Table 1.0, SM2 recorded the highest survival rate of explants at 89.25%. Hence, this method was applied for all subsequent experiments. The contamination was found to be caused by bacteria. Meanwhile, explants treated using SM3 method has 73.0% survival rate, and the culture contamination were found due to either bacteria or fungal. In comparison to the other two methods, SM1 had the lowest survival rate, with only 57.5 percent of explants surviving. The contaminations could be due to ineffective concentrations of sterilants used, handling error or endogenous contaminant from explant sources. Explant browning was also detected when SM3 was used, which leads to explant cell death.

Sterile water, detergent solution, antibacterial agents, antifungal agents, sodium hypochlorite (NaOCl), ethanol, mercuric chloride ($HgCl_2$), and antibiotics are commonly used surface sterilisation agents. They play important role in limiting the growth of contaminants *in vitro* (Smith, 2012). Pikulthong et al., (2016) established contamination-free cultures of *C. longa* and *C. mangga* by initially soaking the rhizome in liquid soap for 5 minutes and then rinsing them several times under running tap water. The explants were then treated in 70% ethanol for 30 seconds, soaked in 20% sodium hypochlorite with Tween 20 for 20 minutes, and washed three to four times with sterilised

distilled water prior to culturing onto medium (Pikulthong et al., 2016). These steps resulted in contaminant-free *C. longa* and *C. manga* plantlets.

In this study, the optimum regime for surface sterilization was obtained through treatment SM2, using 70% NaOCl for 15 minutes. NaOCl -containing commercial bleach (Clorox) is a commonly used non-hazardous disinfectant agent for the surface sterilisation of plant materials. that has been proven to kill bacteria. According to Oyebanji et al. (2009) and Yildiz et al., (2012) perhaps micromolar concentrations of NaOCl are sufficient to significantly reduce bacterial populations. Seran (2013) reported that the effectiveness of NaOCl on surface sterilisation improves proportionally with its concentration and exposure period, up to a certain optimal point. In addition, Tween 20 acts as a surfactant which helps to improve the disinfection process by removing any surface contaminants (Sundram et al., 2012).

Next, the explants were treated with 70% ethanol. Ethanol is a powerful sterilizing agent and is phototoxic to plants (Abubakar & Pudake, 2019). According to Tewelde et al. (2020), the effectiveness of 70% ethanol decreases as exposure time increases. Thus, the rhizome buds were only soaked for 5 minutes in 70% ethanol, as prolonged exposure can cause tissue damage due to the alcohol's phytotoxicity. Besides, longer exposure to ethanol can lead to reverse osmosis in explants. Consequently, the cell will shrink, resulting in plasmolysis (Sundram et al., 2012).

Primary sterilizing agents include NaOCl and ethanol, followed by $HgCl_2$ as a secondary agent. $HgCl_2$ showed a clear effectiveness when combined with another sterilization agents. Tewelde et al. (2020) reported on the efficiency of 70% ethanol along with $HgCl_2$ in sterilizing explants of ginger species. $HgCl_2$ is a strong sterilizing agent and important in controlling both bacterial and fungal contamination. However, long exposure to this agent resulted in death and desiccated cultures. Besides, treatment in $HgCl_2$ for more than 10 minutes can cause explant browning and death (Devi et al., 2018).

Browning was also observed in this study, as shown in Figure 1 (d). This browning effect is due to the phenolic oxidation response (Zuraida et al., 2016). During explants excision, leaching of the phenolic compounds readily oxidizes and become phytotoxic in causing cell death and necrosis. Subsequently, ethylene influences in the oxidation of explants and higher levels of ethylene can lead to explant death. Larger explants tend to produce more ethylene than the smaller explants. Thus, the browning of larger explants is significantly correlated with the lower percentage of survival (Silva et al., 2015). The production of phenolic compounds leading to cell browning can most effectively be mitigated through the incorporation of ascorbic and citric acid as antioxidants (Sundram et al., 2020).

Callus Induction of *Curcuma Mangga*

Effect of Sucrose on Callus Induction

Callus was successfully induced in all the treatments (Figure 2). However, the callus induction percentage, morphology and size of callus varied depending on the different concentration of sucrose used as summarized in Table 2.0. Treatment T1 using 15 g/L sucrose, induced lowest percentage of callus (25%) with smaller sized callus when compared to other treatments. The colour of the callus appeared to be brownish as shown in figure 2 (a). Meanwhile, highest rate of callus initiation was obtained under treatment T2 (30 g/L sucrose), with 50% explants successfully formed callus. The callus was pale white and friable as in figure 2 (b). As for treatment T3 with 60 g/L sucrose), yellowish callus with a green bud-like structure was observed as shown in figure 3 (c). According to Ali et al., (2016), a friable callus can also be used for shoot induction with supplementation of 6-Benzylaminopurine BAP or Kinetin.

Sucrose has a significant influence on the growth of callus as it is the most common carbon sources used in *in vitro* cultures (Salvi et al., 2002). It acts as an energy source in

maintaining osmotic potential in plants. Besides, sucrose is also added to ensure optimal development during *in vitro* growth of plants (David et al., 2016). Higher concentration of sucrose gives a favourable response to the explants, as it causes an increase in the biomass and influence organogenesis. However, concentration of sucrose higher than the optimal level causes osmotic shock to the explants, resulting lower percentage of callus formation (Verma et al. 2016). Treatment with 30.0 g/L of sucrose substantially has maximum rate of survival and highest callus induction percentage as compared to other sucrose concentration tested in this study. In a study on the effect of sucrose concentration on *C. aeruginosa* conducted by Loo (2006), the optimum callus growth was achieved with the 30g/l sucrose as compared to other sucrose concentration used.

Effect of Glucose on Callus Induction
Callus was successfully induced in all the treatments (Figure 3). However, the callus induction percentage, morphology and size of callus varied depending on the different concentration of glucose used as summarized in Table 3.0.

In treatment T4, all cultured explants turned into friable and creamy white callus as shown in Figure 3(a). Meanwhile, treatment T5 and T6 shared the same rate of callus initiation success. Callus obtained through treatment T5 had the same morphology as treatment T4, friable and creamy callus. However, they are larger in size (Figure 3(b)). Friable callus is highly desirable for initiating a fine cell suspension culture in liquid medium (Bhojwani & Dantu, 2013). Nevertheless, with higher concentration of glucose (60 g/L) under treatment T6, the callus appeared to be compact (Figure 3(c)) or wet (Figure 3(d)).

Glucose as carbon source has effects on cell growth and secondary metabolite production. According to Guo et al. (2007), the plating efficiency for protoplast of ginger (*Z. officinale*) was found to be much higher after being cultures on a medium supplemented with glucose than sucrose. As a result, glucose is generally preferable to sucrose for cell division and colony formation from protoplast. Besides, a study on the effect of carbon sources on callus culture of spiral ginger conducted by Wani et al. (2014) found that glucose with 30 g/L and 60 g/L yielded the highest callus growth followed by sucrose and fructose respectively. Generally, glucose can be alternatively used as *in vitro* carbon sources for plant tissue culture.

CONCLUSION

In conclusion, this study successfully developed an optimal protocol for surface sterilization treatment using 70% NaOCl + 5 drops of Tween 20 (5 minutes), 70% ethanol (5 minutes) and 0.3% $HgCl_2$, with a survival rate of 89.25%. Culturing shoot buds of *C. mangga* in MS media supplemented with different concentrations of sucrose and glucose as carbon sources, results in different morphology and percentages of callus induction. In this study, MS medium + 1 2,4-D supplemented with 30.0 g/L of sucrose recorded 50% callus induction, with friable callus being produced. Whereas, when the concentration of sucrose was increased to 60.0 g/L, it caused an increase in the size of callus and influenced callogenesis, where a green bud-like structure was formed. A friable callus was also observed in MS media supplemented with 15.0 g/L and 30.0 g/L of glucose. Nevertheless, MS media with 15.0 g/L glucose recorded the highest rate of callus induction (100%). Higher concentration of glucose at 60.0 g/L led to production of compact and wet calli. Different carbon sources have different effects on morphology and percentage of callus induction, as they have inevitable role in plant cell and tissue culture media due to heterotrophy of the culture cells. Besides, they partially affect the rate of cell division and the degree of cell morphogenesis. Further study can be done to test the effect of other carbon sources such as mannitol and xylose on callus induction of *C. mangga*.

Conflict of interest: Authors declare no conflict of interest

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Table 1.0: Survival rate of explants under different surface sterilization treatments

Surface Sterilization methods	**Disinfectants**	**Percentage of survival (%)**	**Forms of contamination**
SM1	50% NaOCl (v/v) 70% ethanol (v/v) 0.3% $HgCl_2$ (w/v)	57.5	Bacteria (Figure 1 (c)) Fungus (Figure 1(b)) Browning (Figure 1 (d))
SM2	70% NaOCl (v/v) 70% ethanol (v/v) 0.3% $HgCl_2$ (w/v)	89.25	Bacteria (Figure 1 (c))
SM3	100% NaOCl (v/v) 70% ethanol (v/v) 0.3% $HgCl_2$ (w/v)	73.00	Bacteria (Figure 1 (c)) Fungus (Figure 1 (b))

Table 2.0: Effects of different sucrose concentration on callus induction of *C. mangga* shoot bud explants cultured on MS medium with 1 mg/l 2,4-D at week 16.

Treatments	**Sucrose concentration (g/L)**	**Callus induction (% ± S.E)**	**Morphology of the callus**	**Size of the callus (cm)**
T1	15.0	25 ± 0.0	Friable and brownish callus (Figure 2 (a))	1.7 x 2.2
T2	30.0	50 ± 0.0	Friable and pale white callus (Figure 2 (b))	2.1 x 3.4
T3	60.0	37.5 ± 0.0	Friable, dry and yellowish callus. A green bud-like structure was observed (Figure 2 (c))	3.2 x 4.7

Table 3.0: Effects of different glucose concentration on callus induction of *C. mangga* shoot bud explants cultured on MS medium with 1 mg/l 2,4-D at week 16.

Treatment	**Glucose concentration (g/L)**	**Callus induction (% ± S.E)**	**Morphology of the callus**	**Size of the callus (cm)**
T4	15.0	100 ± 0.0	Friable and creamy white callus (Figure 3 (a))	1.5 x 2.6
T5	30.0	50 ± 0.0	Friable and creamy white callus (Figure 3 (b))	3.5 x 3.7
T6	60.0	50 ± 0.0	Compact, wet and pale white callus (Figure 3 (c&d))	1.5 x 2.4

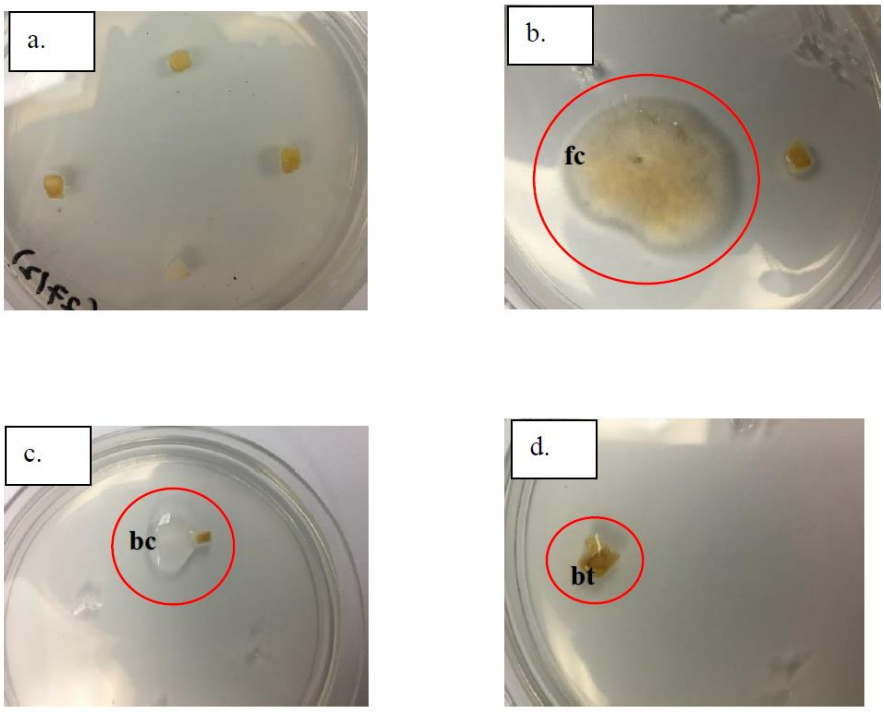

Figure 1: Observation on *in vitro* cultured *C. mangga* shoot bud explants treated with three different surface sterilization methods. (a) Healthy explants with no contamination observed.

(b) fc: Fungal contaminated explants (fc). (c) Bacterial contaminated explants (bc). (d) Browning on explants tissues (bt).

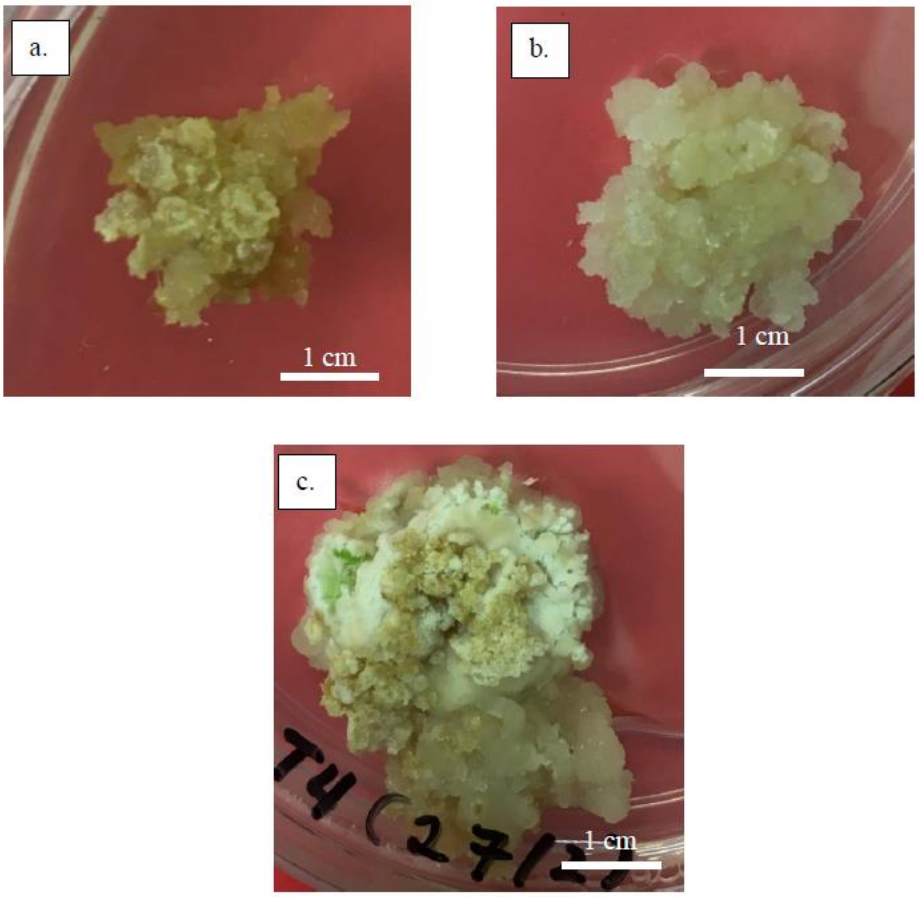

Figure 2: Morphology of *C. mangga* callus in MS medium supplemented with 1 mg/L of 2,4-D and different concentrations of sucrose after 16 weeks. (a) Friable and brownish callus produced on T1 (MS + 1 mg/L of 2,4-D with 15.0 g/L sucrose); (b) Friable and pale white callus produced on T2 (MS 1 mg/L of 2,4-D with 30.0 g/L sucrose); (c) Friable and dry callus with green bud-like structure produce on T3 (MS 1 mg/L of 2,4-D with 60.0 g/L sucrose).

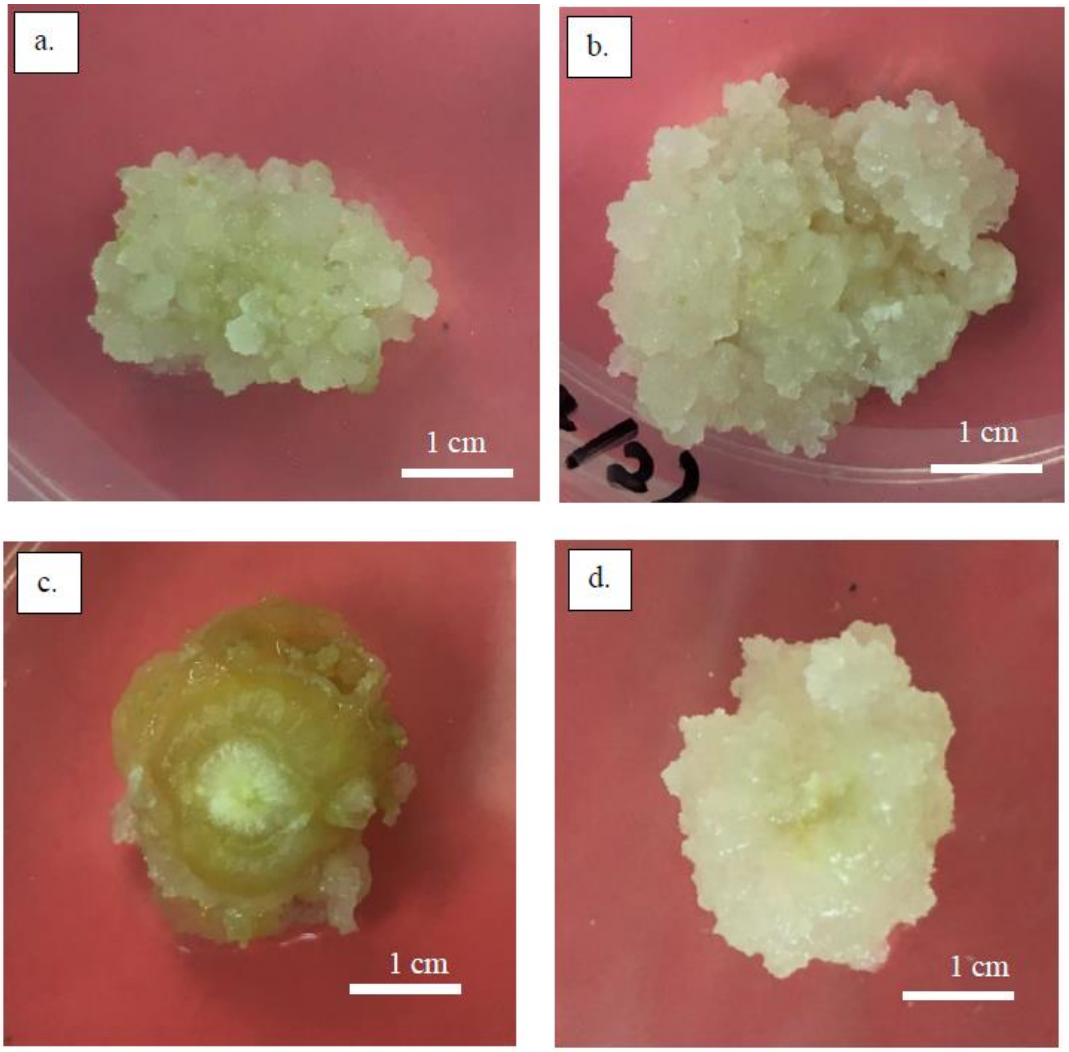

Figure 3: Morphology of *C. mangga* callus in MS medium supplemented with 1 mg/L of 2,4-D and different concentrations of sucrose after 16 weeks. (a) Friable and creamy white callus produced onT4 (MS+ 1 mg/L of 2,4-D with 15.0 g/L glucose); (b) Friable and creamy white callus produced onT5 (MS+ 1 mg/L of 2,4-D with 30.0 g/L glucose); (c & d) Compact and white wet callus produce on T6 (MS + 1 mg/L of 2,4-D with 60.0 g/L glucose).