

The Effects of Culture Systems and Explant Incision on *In vitro* Propagation of *Curcuma zedoaria* Roscoe

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ABSTRACT

Three types of culture systems, solid medium system, liquid shake flask system, and temporary immersion system (TIS) were used for testing their efficiency in propagating *Curcuma zedoaria* plantlets. The proliferation medium used in shoot multiplication was the Murashige and Skoog medium supplemented with 0.5 mg/L 6-benzylaminopurine (BA) and 0.5 mg/L Indole-3-butyric acid (IBA). Among the three systems used, the liquid shake flask system significantly induced more shoot formation and larger shoots from the shoot explants of *C. zedoaria*. Meanwhile, divided shoot explants produced significantly higher number of shoots than the undivided shoot explants. The *in vitro* plantlets derived from the three different culture systems produced healthy and morphologically similar to the mother plants after acclimatization and being transferred to the field.

Keywords: Acclimatization, *Curcuma zedoaria*, liquid shake flask system, temporary immersion system

INTRODUCTION

Curcuma zedoaria Roscoe is one of the important medicinal plants belonging to the family *Zingiberaceae*. It is known as temu kuning in Malaysia and temu puteh in Java, Indonesia. In particular,

C. zedoaria has anti-tumour (Kim *et al.*, 2000), hepatoprotective (Matsuda *et al.*, 1998), anti-inflammatory (Jang *et al.*, 2001) and analgesic properties (Navarro *et al.*, 2002). It is used as a colouring and flavouring agent (Islam, 2004). The rhizomes oil of *C. zedoaria* is used as a tonic and also in perfume. The rhizomes are traditionally used as appetizer, anti-helmenthic, anti-pyretic, for the treatment of leucoderma, piles, tuberculosis and also against enlargement of spleen (Bharalee *et al.*, 2005).

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The market demand of *C. zedoaria* is continuously increasing due to its multiple uses. The conventional way of propagation cannot fulfill the increasing market demand. Besides this, the conventional propagation of *C. zedoaria* by rhizome cutting is also season dependent and it requires a long time to be built up for the commercial quantities. However, the *in vitro* culture techniques are used to overcome the present demands of most of the medicinal and aromatic plants (Rahman *et al.*, 2004). Meanwhile, the tissue culture technique could be used as an alternative method for the mass propagation of *C. zedoaria* in the production of useful secondary metabolite. The *in vitro* propagation protocols have been successfully developed for many species of the *Zingiberaceae* family, such as *Alpinia galanga* (Borthakur *et al.*, 1999), *Kaempferia galanga* (Shirin *et al.*, 2000), *Curcuma longa* (Rahman *et al.*, 2004), *Zingiber officinale* (Sharma & Singh, 1997; Khatun *et al.*, 2003), as well as *Curcuma zedoaria* and *Zingiber zerumbet* (Stanly *et al.*, 2010).

The most common method of micropropagation involves proliferation of the shoots via solid medium. However, this conventional way of propagation usually involves a high production cost and is time consuming. To overcome these problems, the use of shake cultures using liquid medium has been promoted. It is reported that liquid medium is ideal for micropropagation because it reduces production cost and is suitable for automation (Aitken-Christie & Davis, 1995). The liquid culture system

can provide much more uniform culturing conditions and the culture media can be changed easily. Furthermore, it allows a close contact with the tissue which stimulates and facilitates the uptake of nutrients leading to better shoot and root growth. However, a continuous contact of the plant tissues with the liquid medium will normally cause hyperhydricity (Hussey, 1986) which is responsible for poor growth and substantial losses during and after *in vitro* culture. In contrast, temporary immersion system (TIS) has the advantages over the solid and liquid cultures. TIS generally improves the quality of plant tissues as they can perform better during the acclimatization process compared to the plantlets which are obtained from semi-solid or from liquid medium. Therefore, the main objective of the study was to develop an optimized protocol for *in vitro* propagation of *C. zedoaria*. The specific objectives were to compare shoot proliferation between solid, liquid and temporary immersion system (TIS) and to study the effects of explant type on the shoot proliferation of *C. zedoaria*.

MATERIAL AND METHODS

Plant Material and culture media

The *in vitro* shoot cultures of *C. zedoaria* obtained from the Plant Tissue and Cell Culture Laboratory, School of Biological Science, Universiti Sains Malaysia were used as the study material. The apical shoot explants were inoculated into a 200ml conical flask containing Murashige and Skoog (1962) solid medium supplemented with 0.5 mg/L 6-benzylaminopurine (BA)

and 0.5 mg/L Indole-3-butyric acid (IBA), the optimum shoot proliferation medium formulated by Stanly and Chan (2007). All the cultures were kept at $25 \pm 2^\circ\text{C}$ in a culture room under continuous lighting provided by cool white fluorescent tubes, with intensity of $40\text{-}42 \text{ mmol m}^{-2} \text{ s}^{-1}$. More shoots were proliferated by subculturing onto the same proliferation medium at every four-week interval.

For the solid medium, 7.5g/L agar (Algas, Chile) was added into the MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L IBA. The same constituent was used for the liquid medium (without gelling agent). The pH of the medium was adjusted to 5.7 – 5.8 for both the liquid and gelled medium prior to autoclaving at 121°C for 11 minutes. A volume of 30 ml liquid medium was dispensed in a conical flask for shake flask system and 100ml of the liquid medium was used for the temporary immersion system.

Description of Temporary Immersion System (TIS)

Reusable Nalgene® polysulfone filtration system (Nalge Nunc International, USA, catalogue number-KH06730-52) was modified and used as the TIS vessel. The modified TIS consisted of two compartments, of which the upper compartment was occupied by the shoot explants and the lower one by the liquid medium. The two compartments of the Nalgene® polysulfone filtration system was modified by connecting each of the compartments with a tube fitted with a filter (0.2 μm) so that when

the pressure was applied to the lower compartment, the medium would be pushed to the upper compartment. Thus, the plant materials in the upper compartment were immersed in the liquid medium as long as the pressure was applied. The pressure can escape through the outlet on the top of the vessel. This process helps to aerate the medium and agitate the plants. When the pressure is removed, the medium returns to the lower compartment. The air that enters the vessel is filtered through sterile 25mm nylon non-pyrogenic hydrophilic syringe filters (0.2 μm) (Sartorius) so as to prevent contamination.

The Effects of the Solid and Liquid Culture Systems on the Shoot Proliferation of Curcuma zedoaria

For this purpose, three aseptic shoots (1.5 cm) were cultured into each 200 ml conical flask containing 30ml of MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L IBA, the shoot proliferation medium, and solidified with 7.5 g/L agar (Algas, Chile). For the liquid medium, three aseptic shoots were inoculated in a 200 mL conical flask containing 30 mL of the liquid shoot proliferation medium. The shake flasks were agitated on a rotary shaker (Panasonic G Series, Speed Controller, DUX940W) at 120 rpm. Five experimental units were used for the solid and liquid cultures, respectively. All the cultures were kept at $25 \pm 2^\circ\text{C}$ under a continuous cool white fluorescent light with an intensity of $40\text{-}42 \text{ mmol m}^{-2} \text{ s}^{-1}$. The biomass and the number of shoots produced from each explant were

recorded after three weeks of culturing. The means were compared using Independent T-test at $p \leq 0.05$.

Temporary Immersion System (TIS) and Shake Flask System

The shoot biomass and the multiple shoots formation were compared between the temporary immersion system and the shake flask system. Five shoots were inoculated into each temporary immersion vessel containing 100ml liquid shoot proliferation medium with an immersion period of 15 minutes once a day. For the shake flask system, five aseptic shoots were inoculated into 250 ml conical flask containing 100ml liquid medium of the same constituents. Five units of temporary immersion vessels and shake flasks were used respectively. All the shake flask cultures were agitated on an orbital shaker at 120 rpm. The temporary immersion and shake flask cultures were maintained at $25 \pm 2^\circ\text{C}$ under continuous cool white fluorescent light, with an intensity of $40\text{-}42 \text{ mmol m}^{-2} \text{ s}^{-1}$. After three weeks of culturing, the increases in the biomass and number of shoots produced from each explant were determined and the data were analyzed using the Independent T-test at $p \leq 0.05$.

The Effects of Shoot Incision on Shoot Proliferation

In order to investigate the effect of shoot incision on the shoot multiplication of *C. zedoaria*, the shoot explant used either undivided whole aseptic shoots or the shoot were divided into two halves, with each

half shoot used as an explant. The divided and undivided shoots were cultured into a 250 mL conical flask containing liquid proliferation medium. Three shoot explants were used for each flask and six experimental units were used for each explant type. The increments in the biomass and the number of shoots per explant were recorded after three weeks of culturing and the data were analyzed using the Independent T-test at $p \leq 0.05$.

Rooting and Acclimatization

Seventy-five *in vitro* shoots were separated into individual micro-shoot and inoculated into basic MS medium for rooting. The rooted plantlets of *C. zedoaria* were thoroughly washed with tap water to remove traces of the nutrient medium. Old yellowish leaves were removed and the explants were transferred into plastic containers (30 x 40 cm) containing a mixture of organic soil and sand (1:1) with a relative humidity of 80-90% under green house conditions ($28 \pm 2^\circ\text{C}$ during day time and $24 \pm 2^\circ\text{C}$ during night time).

RESULTS AND DISCUSSION

Three types of the culture systems, solid medium system, liquid shake flask system, and temporary immersion system (TIS) were used to evaluate their effects on the *in vitro* propagation of *C. zedoaria*. After three weeks of culturing on the MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L IBA, the number of shoots produced per explant and the biomass of the formed shoots were found to be varied depending

on the culture system used. An average of 2.2 ± 0.3 shoots per explant was obtained from the shake flask liquid medium system (Fig.1A) which was higher than 1.7 ± 0.3 shoots per explant obtained on the solid medium (Fig.1B). The result of this study also showed that the explants cultured on the liquid medium produced bigger shoots with an average of 1.4 ± 0.1 g per shoot biomass as compared to 0.58 ± 0.1 g per shoot biomass cultured on the solid medium (Fig.2). This result indicated that the shoot explants of *C. zedoaria* which was cultured in the liquid medium produced a significantly higher number of shoots and a heavier

biomass (bigger shoot) as compared to that cultured on the solid medium (Fig.1c). As a solidifying agent, agar was found to have effects on the growth and development of *in vitro* cultures (Scholten & Pierik, 1998). The main reason was that the gelling agent of the solid medium provided less oxygen to the cultured explants as dissolved oxygen was hindered by the agar (Kohlenbach & Wemicke, 1978). In contrast, the use of agitated shake flask stimulated and facilitated the nutritional and hormonal uptake, leading to better shoot and root growth (Ziv, 1989; Smith & Spomer, 1994; Sandal *et al.*, 2001). Moreover, a continuous

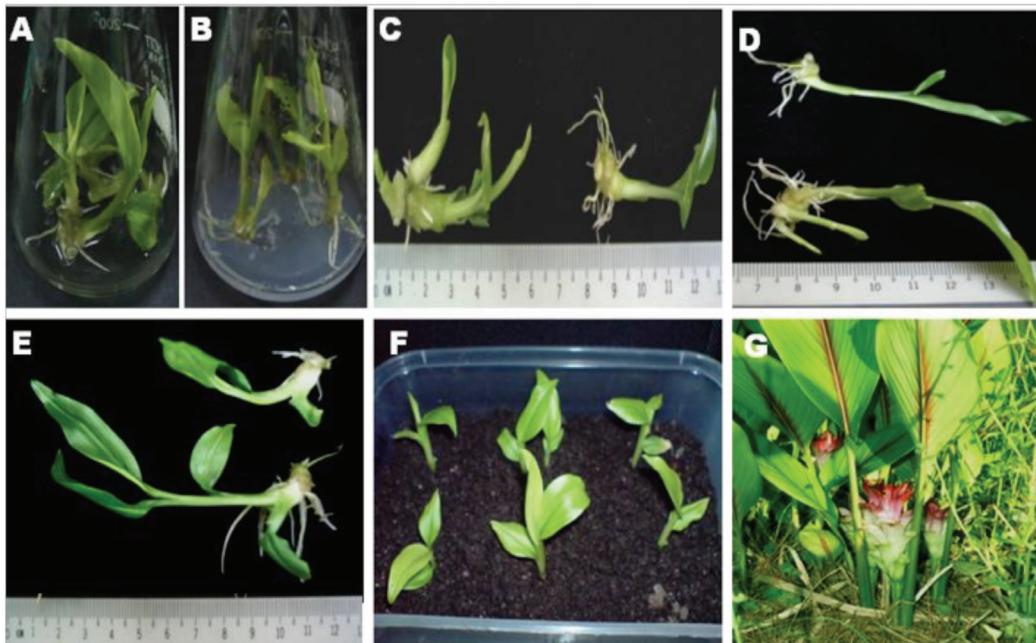
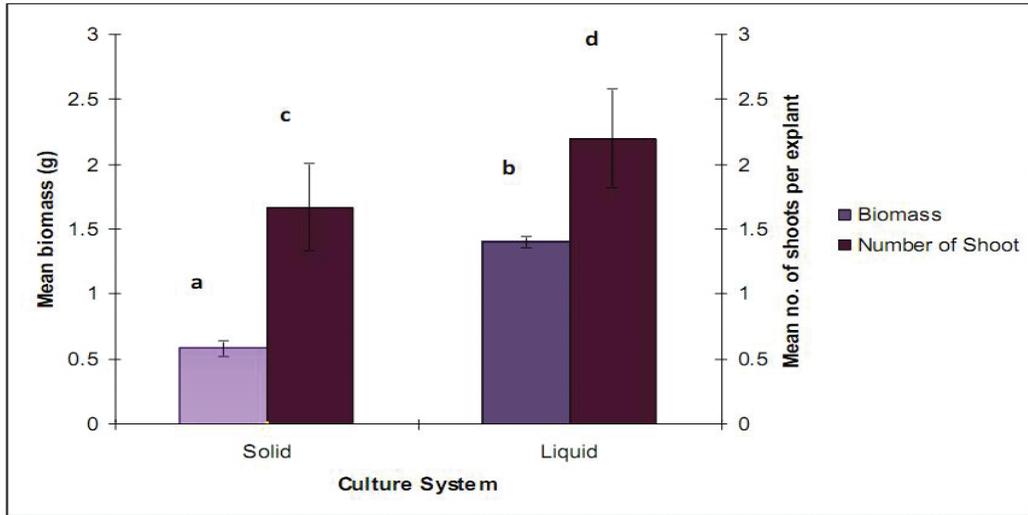


Fig.1: The effects of the culture system and explant types on the *in vitro* micropropagation of *Curcuma zedoaria* after three weeks of culture; A) Multiple shoots formation on liquid medium; B) Multiple shoots formation on solid medium; C) Multiple shoots and shoots size of explants cultured on solid medium (Right) and liquid medium (Left); D) Multiple shoots and shoots size of the explants cultured in TIS (Top) and shake flask (Bottom); E) Shoot size derived from the undivided (Top) and divided (Bottom) shoot explants cultured in shake flask; F) Plantlets acclimatized in the soil mixture containing black soil:sand (1:1) (Day 14); and G) Mature plant established in the soil under field conditions



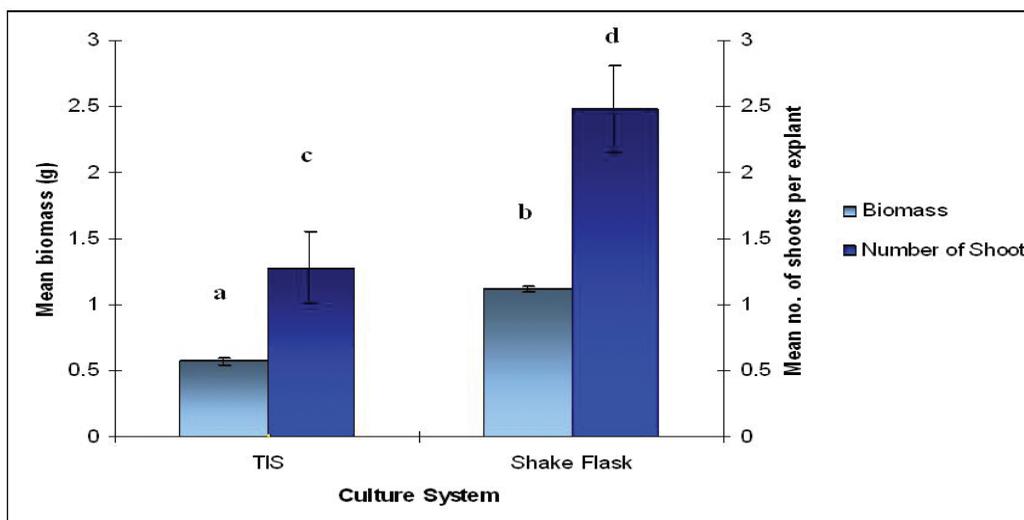
The mean value of each parameter, followed by different alphabet, are significantly different using the Independent T-test, $p \leq 0.05$.

Fig.2: The effects of the solid and liquid media on the shoot proliferation and shoot biomass of *Curcuma zedoaria*

shaking of the medium provided ample oxygen supply to the shoot explants and a better aeration which ultimately enhanced growth and multiplication (Mehrotra *et al.*, 2007). Furthermore, the liquid medium allowed a greater uptake of nutrient and plant growth regulators due to the large surface of absorption provided by the partially submerged shoots (Arshad *et al.*, 2005). The higher rates of shoot multiplication and improved growth in different plants using the liquid medium have also been reported (Rizvi *et al.*, 2007; Douglas, 1984; Nadgauda *et al.*, 1990; Liu *et al.*, 2004). In a different study by Murch *et al.* (2004) the liquid culture was proven to be more efficient for the production of biomass than the solid medium and there was no hyperhydricity observed in any of the cultures grown in the liquid medium.

Comparative studies between temporary

immersion system (TIS) and shake flask system revealed that shoot multiplication and growth were more efficient in shake flask system (Fig.1D). The shake flask system yielded 2.5 ± 0.3 shoots per explant, whereas the temporary immersion system produced 1.3 ± 0.25 shoots per explant (Fig.3). Similarly, Wawrosch *et al.* (2005) observed that the total number of the multiple shoots of *Charybdis numidica* produced by TIS was only half of the total number of the multiple shoots produced by the shake flask system. A higher multiplication rate in the shake flask system has been explained as attributing to the continuous contact between the shoots and the liquid medium, which enables a constant supply of nutrients as well as a continuous aeration to the explants. Here, the result of this study showed that the liquid shake flask system was better than TIS. However, in



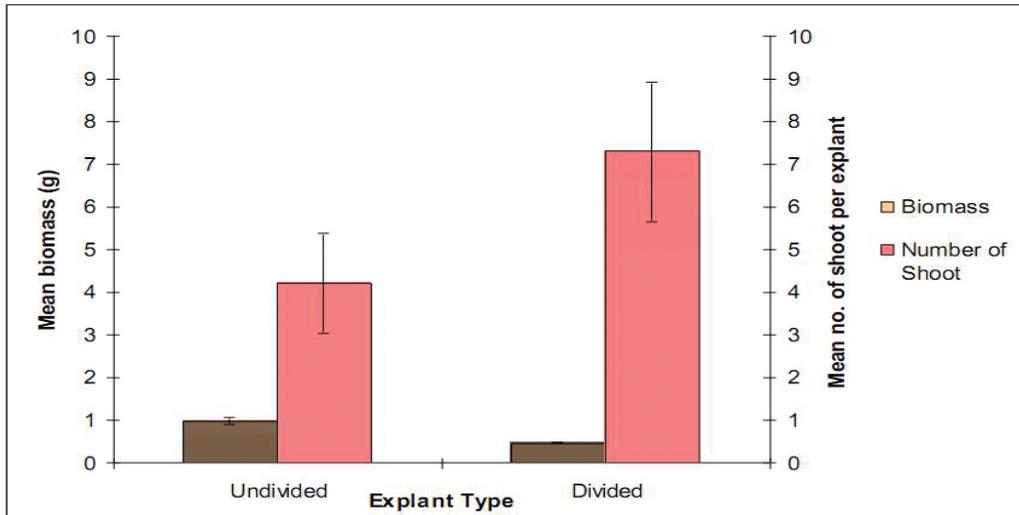
The mean value of each parameter, followed by different alphabet, is significantly different using the Independent T-test, $p \leq 0.05$.

Fig.3: A comparison between TIS and shake flask system on the multiple shoot formation and biomass of *Curcuma zedoaria*

the previous study Stanly *et al.* (2010), TIS was reported as the best choice of the *in vitro* propagation technique for the production of normal *C. zedoaria* and *Z. zerumbet* plantlets. In addition, Murch *et al.* (2004) reported that both the fresh and dry weights of *Crescentia cujete* plantlets grown in TIS were significantly higher than those cultured in the semi-solid medium and the shake flask culture. Moreover, in a different study Grigoriadou *et al.* (2005) reported an equal number of microshoots per explant of *Olea europaea* derived from the TIS and liquid flask cultures. This indicates that the efficiency of a culture system is dependent on many cultural factors and plant species.

The results obtained indicated that dividing the shoot explants longitudinally into halves could enhance the formation of multiple shoots. After 3 weeks of culturing,

the divided shoot explants produced an average of 3.6 ± 1.0 shoot per half explant and hence approximately 7.0 ± 2.0 shoots could be obtained from a single shoot. On the other hand, the undivided shoot yielded 4.2 ± 1.1 shoots per explant. Biomass produced by the divided shoot was 0.98 ± 0.1 g while undivided shoot was only 0.46 ± 0.1 g (Fig.4). The divided shoot explants produced bigger shoots as compared to the ones derived from the whole shoot (Fig.1E). The superiority of the divided shoot over the non-divided shoots was already reported for *Alocasia longiloba* 'Watsoniana' by Chan and Chong (2010) who observed that when the shoot explants were divided longitudinally into halves and cultured into the liquid proliferation medium using the shake flask system, a total of 10 to 12 buds were produced from each whole shoot



The mean value of each parameter, followed by different alphabet, is significantly different using the Independent T-test, $p = 0.05$.

Fig.4: The effects of shoot incision on the multiple shoots formation and biomass of *Curcuma zedoaria*

within four weeks as compared to the non-divided shoot explants which produced only 3-4 buds within the same duration.

After three week of culture in basal MS medium, all the micro-shoots *C. zedoaria* produced roots. The *in vitro* plantlets of *C. zedoaria* with well-developed roots were removed from the rooting medium and washed under running tap water in order to remove adhered medium. This is important because the agar contains sucrose and other nutrients that can serve as a medium for growth of disease-causing organisms which may eventually cause the rotting of roots. At this stage, the plantlets are normally delicate and extra care has to be taken when they are being transferred from the culture vessels to the external environment. Plantlets grown *in vitro* have been continuously exposed to a unique microenvironment that has been selected to provide minimal stress and

optimum conditions for plant multiplication. Plantlets developed within the culture vessels are normally grown under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with high level of humidity. They may readily lose water when exposed to ambient conditions. Hence, these delicate plantlets need to undergo an acclimatization process before they can be transferred to the field. The washed *C. zedoaria* plantlets were successfully acclimatized and all (100%) the plantlets survived when they were transferred to the soil. The well-developed root system may be assumed as major factor to a proper acclimatization of the plantlets from all the three tested systems. Most of the plants showed normal and healthy growths after 14 days of acclimatization (Fig.1F). Lorenzo *et al.*

(1998) also reported that the sugarcane plantlets obtained using the conventional method, solid culture medium and TIS showed a similar growth. The plantlets of *C. zedoaria* derived from the three culture systems were morphologically similar to their respective mother plants (Fig. 1G).

CONCLUSION

It can be concluded that the shake flask system using liquid medium proved to be more efficient than the solid medium and the temporary immersion system in promoting the shoot proliferation and shoot biomass of *C. zedoaria*. However, normal and healthy plantlets could be produced from all the three different culture systems. More research is needed to improve the regeneration efficiency that will provide a better regeneration system for the micropropagation and transformation of this elite medicinal plant.

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