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Full Length Research Paper

A comparative study of *Curcuma zedoaria* and *Zingiber zerumbet* plantlet production using different micropropagation systems

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***Curcuma zedoaria* and *Zingiber zerumbet* plantlets could be micropropagated via induction of multiple shoots from *in vitro* shoot explants using different culture systems such as the agar-gelled medium cultures, shake flask system and temporary immersion system (TIS). The immersion period in TIS did not influence shoot multiplication rates of both species. However, proliferation medium (MS plus 0.5 mg/L BA and 0.5 mg/L IBA) supplemented with different sucrose concentration affected the production of multiple shoots but did not affect the fresh and dried shoot biomass for both species when using TIS. The TIS was able to eliminate hyperhydricity problem encountered when using the shake flask system. However, all the *in vitro* plantlets of both species produced by the three culture systems survived and grew normally with no morphological changes after acclimatization. This study indicated that TIS was the best choice of *in vitro* propagation technique for production of normal *C. zedoaria* and *Z. zerumbet* plantlets.**

Key words: Temporary immersion system, medicinal plant, acclimatization, *Curcuma zedoaria, Zingiber zerumbet.*

INTRODUCTION

Zingiberaceae is a family of advanced monocot plants with high medicinal value. Many alkaloids have been detected in Zingiberaceae species (Larsen et al., 1999). *Curcuma zedoaria* and *Zingiber zerumbet* are two important species of the Zingiberaceae family and are cultivated as a traditional herb, vegetable, flavoring agent and perfume in South and Southeast Asian countries (Kapoor, 1990; Ong and Norzalina, 1999; Duke et al., 2003; Lako et al., 2007; Lobo et al., 2009). *C. zedoaria* has been reported to have analgesic (Ali et al., 2004), anti-allergic (Matsuda et al., 2004), anti-inflammtory (Yoshioka et al., 1998), anti metastasic (Seo et al., 2005), antioxidant (Mau et al., 2003) and hepatoprotective properties (Kim et al., 2005). In China, the rhizomes of *C. zedoaria* are used for the treatment of tumors (Matthes et al., 1980). *Z. zerumbet* has been used traditionally for the treatment of stomachache, toothache, fever, sprain and indigestion (Chiu and Chang, 1995; Huang et al., 2005). The species also possesses analgesic, anti-pyretic (Somchit et al., 2005) and anti-inflammatory properties (Somchit and Shukriyah, 2003). Zerumbone is one of the main compounds which was isolated from the *Z. zerumbet* and found to possess chemopreventive activities (Jang et al., 2005).

Germplasm conservation and rapid mass propagation of medicinal plants can be achieved through *in vitro* techniques and cryopreservation (Nalawade et al., 2003) and has been successfully applied to propagate endangered species (Sudhersan et al., 2003). *C. zedoaria* and *Z. zerumbet* are propagated vegetatively by rhizomes but it takes a long time to produce the rhizome. *In vitro* propagation of *C. zedoaria* and *Z. zerumbet* has been reported in gelled medium and liquid shake flask system through shoot proliferation from rhizome bud explants (Stanly and Chan, 2007). However, continuous shoot proliferation in liquid medium using the shake flask system

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Abbreviations: TIS, Temporary immersion system; **MS,** murashige and skoog (1962); **BA,** 6-benzylaminopurine; **IBA,** indole butyric acid.

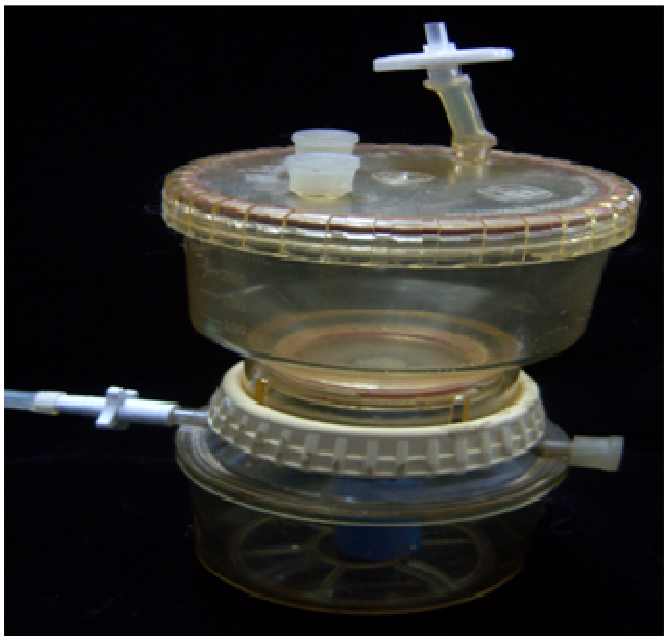

Figure 1. Nalgene® polysulfone filtration system modified as temporary immersion system.

has resulted in hyperhydricity (formerly known as vitrification) (Debergh et al., 1992) in some of the regenerated plants. When hyperhydricity occurred, the *in vitro* plantlets showed glassy appearance and the leaves were thick, elongated, wrinkled and fragile. Hyperhydric shoots did not root properly and eventually did not survive during the acclimatization process (Kevers et al., 2004).

The overall objective of the present study was therefore to develop an optimized protocol for *in vitro* propagation of *C. zedoaria* and *Z. zerumbet* in order to provide consistent normal plantlets. The specific objectives of the current research were to compare an agar-gelled medium, a shake flask system and a temporary immersion system (TIS) on shoot proliferation of *C. zedoaria* and *Z. zerumbet,* and to evaluate the efficiency of TIS in eliminating hyperhydricity problem associated with the shake flask system.

MATERIALS AND METHODS

Plant material and culture conditions

In vitro shoot cultures of *C. zedoaria* and *Z. zerumbet* established from rhizome buds by Stanly and Chan (2007) at School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia were used as study materials. The shoot cultures of both species were cultured in 350 mL glass jars and multiplied using MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/L 6-benzylaminopurine (BA) and 0.5 mg/L indole butyric acid (IBA), the shoot proliferation medium formulated by Stanly and Chan (2007).

Before the experiments, the shoot cultures of both species were cultured on MS basal medium for four weeks to avoid the carry over effect of plant growth regulators. The aerial shoots of about 1.5 cm were selected from the *in vitro* cultures and they were used as explants for all subsequent experiments. These aerial shoots are pseudostems consisting mainly of leave sheaths with one shoot per aerial shoot. The culture medium was autoclaved at 121°C for 11 min and the pH of the medium was adjusted to 5.75 before autoclaving. All the cultures were maintained at 25 ± 2°C in a culture room with continuous lighting provided by cool white fluorescent tubes at 40-42 mmol m^2 s^1.

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 (Figure 1). The modified TIS consisted of two compartments, of which the upper compartment was occupied by the explants and the lower one with 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 (22 µm) so that when the pressure is applied to the lower compartment the medium is pushed to the upper compartment. Thus, the plant materials in the upper compartment were immersed in the liquid medium as long as the pressure is applied. The pressure escapes 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 by sterile 25mm nylon 0.2 µm non-pyrogenic hydrophilic syringe filters (Sartorius) to prevent contamination.

The *in vitro* aerial shoots of *C. zedoaria* were longitudinally cut into two halves and each half shoot (1.5 cm) was used as an explant. Eight half shoot explants were inoculated into the upper compartment of the TIS vessel which contained 50 mL of the liquid MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L IBA in the lower compartment. To determine the suitable aeration duration, the cultures were aerated by an air pump via 0.2 µm non-pyrogenic hydrophilic syringe filters (Sartorius) for 15, 30 and 60 min once a day. The experiment was conducted in a complete randomized design. Three experimental units were used for each trial and eight explants for each experimental unit. The number of shoots produced from each explant was recorded at the end of two weeks of culture period. The data collected were statistically analyzed by one-way ANOVA and the means were compared using Tukey's test (HSD) at p = 0.05 using the software statistical package for the social sciences (SPSS) version 13.

Comparison of agar-gelled medium culture, shake flask system and TIS on multiple shoot formation of *C. zedoaria* and *Z. zerumbet*

For agar-gelled medium culture, eight half shoots (1.5 cm) of *C. zedoaria* were inoculated into 250 mL conical flask containing 50 mL MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L IBA, the shoot proliferation medium, and solidified with 0.8% agar (Algas, Chile). For shake flask system, eight half shoots (1.5 cm) were inoculated into 250 mL conical flask containing 50 mL of the liquid shoot proliferation medium. The shake flask cultures were agitated on an orbital shaker at 120 rpm. Eight half shoots (1.5 cm) of *C. zedoaria* were inoculated into each temporary immersion vessel containing 50 mL of the liquid shoot proliferation medium with an immersion period of 15 min once a day. Three experimental units were used for each of the culture system and eight explants were used for each experimental unit. The experiment was carried out using complete randomized design. The number of shoots produced by each explant was recorded after two weeks of culture and data was analyzed by one-way ANOVA and the means were compared using Tukey's test (HSD) at p = 0.05. Similar experiment was carried out for *Z. zerumbet* with whole shoots as explants as it

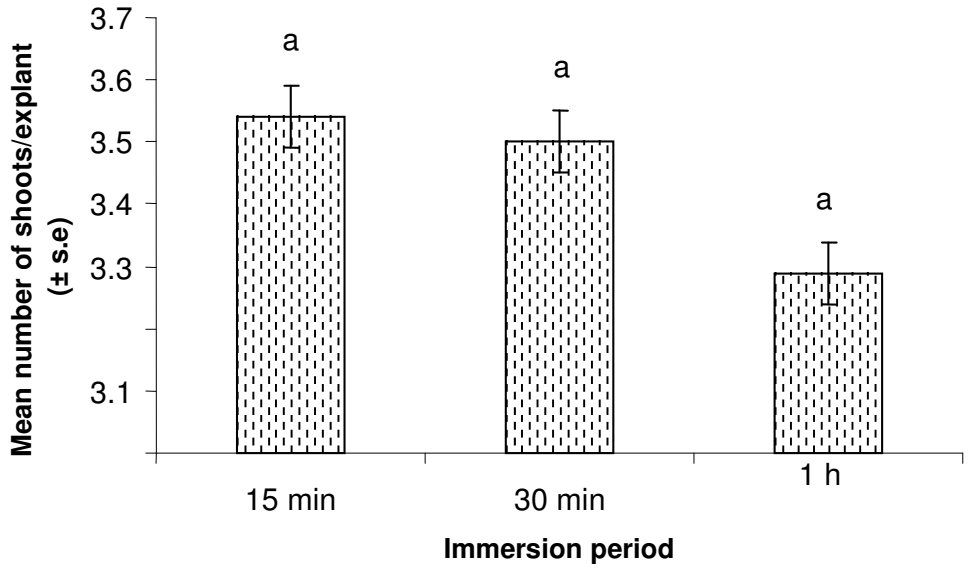

Figure 2. Effect of immersion period using TIS on multiple shoot formation of *C. zedoaria*. Mean values with same letter are not significantly different, HSD (p = 0.05).

was reported by Stanly and Chan (2007) that whole shoots performed better than half shoots for *Z. zerumbet*.

Effect of sucrose on multiple shoot formation and shoot biomass of *C. zedoaria* and *Z. zerumbet*

Two different sucrose concentrations (30 and 15 g/L) were tested for this experiment. Eight half shoots of *C. zedoaria* were inoculated into each TIS vessel and three units of TIS vessels were used for each sugar concentration supplemented into the shoot proliferation medium. The TIS cultures were aerated for 15 min once a day. For the shake flask system, eight half shoots of *C. zedoaria* were inoculated into 250 mL conical flasks containing 50 mL of the liquid proliferation medium supplemented with 30 and 15 g/L of sucrose, respectively. The shake flask cultures were agitated on an orbital shaker at 120 rpm. Three units of conical flasks containing eight explants each were used for each concentration of sucrose. Similar experiment was carried out for *Z. zerumbet* using whole shoot as explants. The number of shoots produced by each explant, shoot height and the shoot biomass were recorded after two weeks of culture and the data was analyzed using independent T-test at p = 0.05 for each species and each of the culture system with the aid of SPSS 13 software.

Acclimatization

In vitro plantlets of *C. zedoaria* and *Z. zerumbet* (shoot height 3-5 cm, roots 4-6 and leaves 2-3) were selected from TIS, shake flask system and agar-gelled medium for the acclimatization process. The *in vitro* plantlets (n = 24 from each culture system) were removed from the culture vessels and washed thoroughly with tap water and transplanted into thermocole trays (30 x 40 cm) containing a mixture of organic soil and sand (1:1) with relative humidity of 80-90% under green house conditions (28 ± 2°C during day time and 24 ± 2°C during night time). The experiment was carried out using complete randomized design at p = 0.05. The plantlets were watered twice a day (morning and evening) with tap water. Shoot height was measured after four weeks from the base of the plant to the tip of the leaves. The percentage of surviving plants was recorded after four weeks in the green house.

RESULTS

Effect of immersion period in TIS on multiple shoot formation of *C. zedoaria*

Result revealed that the different immersion period did not showed any significant difference on the number of multiple shoots formation. Shoot explants of *C. zedoaria* produced an average of 3.5 shoots per half shoot after being immersed in the liquid proliferation medium for 15 or 30 min once a day for two weeks. An average of 3.3 shoots per half shoot was produced at 60 min immersion period and the number of shoots formed was not significantly different from those produced in 15 and 30 min of immersion period (Figure 2). The multiple shoots formed in the TIS were normal with well developed roots. Since different immersion period did not showed significant difference on multiple shoot formation therefore, 15 min of immersion period once a day was used for further experiments with *C. zedoaria* and *Z. zerumbet*.

Comparison of agar-gelled medium, shake flask system and TIS on the multiple shoot formation of C. zedoaria and Z. zerumbet

More *C. zedoaria* shoots (5.8shoots/explant) could be produced in the shake flask system and was found to be significantly different from those produced using the agar-gelled medium. An average of 4.7shoots/explant was

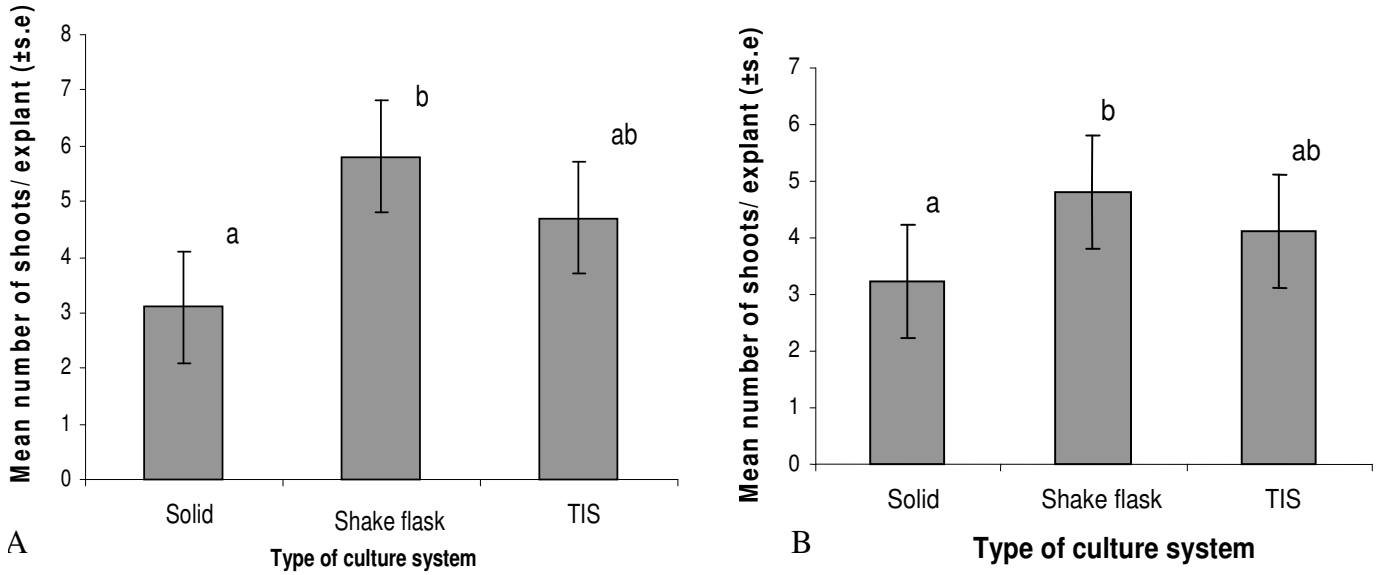

Figure 3. Comparison between solid, shake flask system and TIS on multiple shoot formation (A) *C. zedoaria* and (B) *Z. zerumbet*. Mean values with different letters are significantly different, HSD (p = 0.05).

produced in the TIS and the number of shoots produced was found to be not significantly different with that of shake flask system and the agar-gelled system (Figure 3A). Similar trend was observed in *Z. zerumbet* (Figure 3B). The multiple shoots with well developed roots were formed in each culture system. The plantlets from the three culture systems showed similar height range of 3-5 cm (*C. zedoaria*) and 2.5-4 cm (*Z. zerumbet*) (Data not presented). However, through observation it was found that the plantlets cultured in TIS and shake flask system produced bigger leaves as compared to agar-gelled medium. TIS and shake flask system were found to produce significantly higher number of shoots as compared to the agar-gelled cultures for both the species. Therefore, TIS and shake flask system were used for further experiments.

Effect of sucrose on the multiple shoot formation and shoot biomass of *C. zedoaria* and *Z. zerumbet*

Proliferation medium supplemented with 30 and 15 g/L sucrose using the TIS showed significant effect on the number of multiple shoots produced in *C. zedoaria*. The proliferation medium supplemented with 30 g/L of sucrose induced higher number of multiple shoots (3.4 shoots/explant) as compared to the medium supplemented with 15 g/L (2.6 shoots/explant). However, the proliferation medium supplemented with 30 and 15 g/L did not affect multiple shoots production in *C. zedoaria* using the shake flask system (Figure 4A). The proliferation medium supplemented with 30 g/L sucrose did not affect significantly the shoot biomass (fresh weight- 4.91 g and dry weight- 1.23 g) when compared to 15 g/L sucrose (fresh weight 4.76 and dry weight 0.98 g) using TIS. Similarly, the shake flask system also did not show any significant difference in the fresh weight and dry weight of the multiple shoots with different concentration of sucrose supplemented into the shoot proliferation medium (Table 1).

The proliferation medium supplemented with 30 g/L sucrose produced significantly higher number of multiple shoot in *Z. zerumbet* (3.2 shoots/explant) as compared to the 15g/L sucrose (1.7shoots/explant) using the TIS. However there was no significant difference in the number of multiple shoots produced under the proliferation medium supplemented with 30 g/L sucrose (5.1 shoots/ explant) and 15 g/L of sucrose (4.0 shoots/explant) using shake flask system (Figure 4B). Similarly, there was no significant difference in the fresh weight and dry weight of the multiple shoots in the proliferation medium supplemented with different sucrose concentration, 30 g/L sucrose (fresh weight- 2.31 g and dry weight- 0.67 g) and 15 g/L sucrose (fresh weight- 1.78 g and dry weight- 0.55 g) using the TIS. Similar trend was observed in the shake flask system (Table 1).

Acclimatization

In vitro plantlets of *Z. zerumbet* and *C. zedoaria* from each culture system were successfully acclimatized and showed 100% surviving rate when transferred to the soil. The plantlets of *C. zedoaria* and *Z. zerumbet* derived from the three culture systems were morphologically similar to their respective mother plants. The plantlets from the three culture system reached a similar height range of 13-15 cm (*C. zedoaria*) and 8-10 cm (*Z. zerumbet*) after 4 weeks of acclimatization (data not

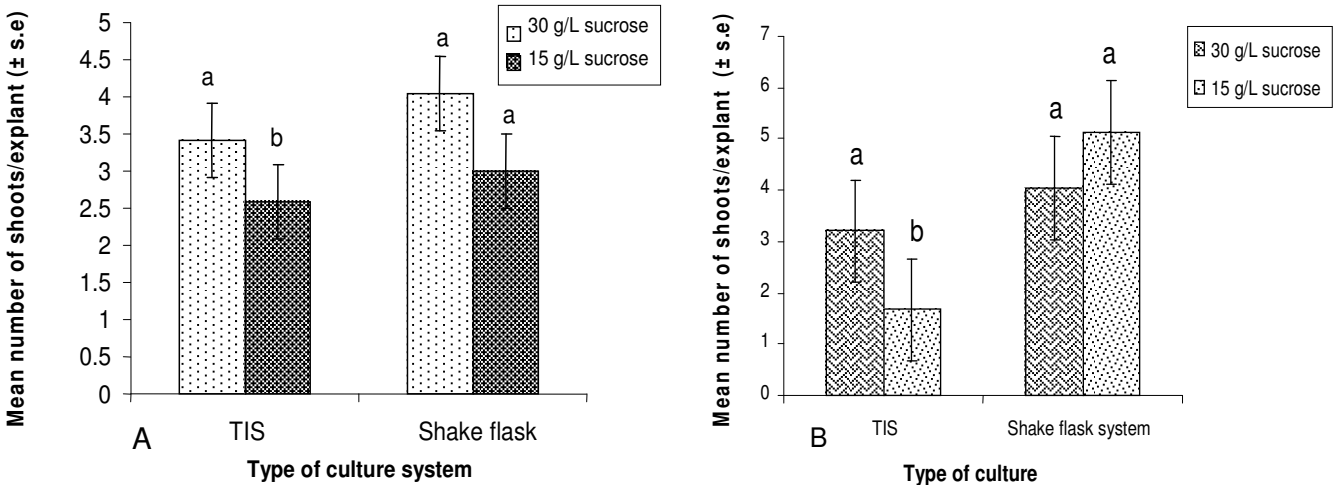

Figure 4. Effect of sucrose supplemented into the shoot proliferation medium on multiple shoots formation of (A) *C. zedoaria* and (B) *Z. zerumbet* using TIS and shake flask system. Mean values for each culture system with same letters are not significantly different using independent T-test (p=0.05).

Table 1. Effect of sucrose supplemented into shoot proliferation medium on shoot biomass of *C. zedoaria* and *Z. zerumbet* using TIS and shake flask system.

Culture system	Concentration of sucrose (g/L) in culture medium	Fresh weight of shoots (g) ± s.d.		Dry weight of shoots (g) ± s.d.	
		C. zedoria	*Z. zerumbet*	*C. zedoria*	*Z. zerumbet*
TIS	30	4.91 ± 1.75 a	2.31 ± 0.42 a	1.23 ± 0.49 a	0.67 ± 0.30a
	15	4.76 ± 1.07 a	1.78 ± 0.34 a	0.98 ± 0.12 a	0.55 ± 0.26a
Shake flask	30	9.94 ± 3.26 a	2.88 ± 1.09 a	1.34 ± 0.61 a	0.48 ± 0.14a
	15	6.01 ± 0.90 a	3.63 ± 0.25 a	0.78 ± 0.11 a	0.56 ± 0.18a

Mean weight (fresh or dried) values for each species in each culture system followed by the same letter are not significantly different, independent t-test (p = 0.05).

presented). All the plantlets produced via TIS grew normally after acclimatization (Figure 5).

DISCUSSION

TIS has been successfully used for micro-propagation of several plant species (Saare-Surminski et al., 2008; Lorenzo et al., 1998; Alvard et al., 1993). This system offers certain advantages such as reduction of hyper-hydricity in tissues, minimizing labor cost and reduce culture contamination (Yoeup and Chakrabarty, 2003). It provides better aeration and the removal of undesired gases like carbon dioxide and ethylene during the immersion cycles (Ilczuk et al., 2005). Zobayed and Saxena (2003) reported that the TIS allowed better scaling up for mass production of plantlets and many plant species showed better results in this system. In our study, *C. zedoaria* and *Z. zerumbet* showed significant difference between the numbers of multiple shoots produced under the three different culture systems (agar-gelled medium, shake flask system and TIS).

The TIS and shake flask system produced significantly higher number of multiple shoots as compared to the agar-gelled cultures. These findings are consistent with Rodrigues et al. (2006) who reported that the TIS showed higher shoot multiplication in *Heliconia achampneiana* than the agar-gelled cultures. The number of shoots produced from the shoot explants were not significantly different either using the shake flask system or TIS. The assumed reason for the higher number of shoots of *C. zedoaria* and *Z. zerumbet* in shake flask system and TIS as compared to the gelled medium was due to better availability of nutrients as well as faster and efficient uptake of nutrients from the liquid medium in both the shake flask and TIS. However, the shoot cultures of *Prunus* and *Malus* species using TIS and semi-solid cultures did not show any difference in multiplication rate (Damiano et al., 2005). This report suggested that TIS system was not appropriate for all plant species but could be used for the rapid multiplication of Zingiberaceae species.

Both *C. zedoaria* and *Z. zerumbet* produced normal multiple shoots with well developed roots using TIS.

Figure 5. Two months old normal *Z. zerumbet* from TIS after being transferred to soil.

Gontier et al. (2005) also found that *Ruta graveolens* showed similar shoot growth pattern in the temporary immersion and permanent immersion cultures. The TIS derived microshoots of *Olea europaea* was found to be equal to those obtained from Erlenmeyer flasks. However the shoot growth and its quality were better in temporary immersion cultures (Grigoriadou et al., 2005). Chakrabarty et al. (2007) on the other hand reported that the number of shoots produced by the apple root stock explants (M9 EMLA) using temporary immersion bioreactor was lower as compared to the continuous immersion bioreactor. However the TIS increased the shoot vigor and produced normal plantlets. Wawrosch et al. (2005) also found that the total number of multiple shoots of *Charybdis numidica* produced by TIS was only half the total number of multiple shoots produced by the shake flask system and was significantly lower than the semi-solid cultures.

Our results indicated that both *C. zedoaria* and *Z. zerumbet* showed significant difference in number of multiple shoots formed but not the shoot biomass when cultured in the proliferation medium supplemented with different sucrose concentration (30 and 15 g/L) using TIS. In shake flask system, the different concentration of sucrose present in the medium did not affect the shoot biomass and the number of multiple shoots produced from both species. The multiple shoots biomass obtained from shake flask system and TIS were not significantly different for both species (*C. zedoaria* and *Z. zerumbet*). In the present study both species showed higher fresh weight and lower dry weight in shake flask system. This findings are consistent with Chakrabarty et al. (2007) who explained that the higher fresh biomass and lower dry biomass of the shoots of apple root stock 'M9 EMLA' obtained from continuous immersion cultures due to the fact that the leaves contained more water and the dry biomass were similar to those obtained from temporary immersion cultures. They also reported that the leaves of the proliferating apple shoots under continuous immersion system were smaller than those produced in temporary immersion cultures which were also observed in both *C. zedoaria* and *Z. zerumbet*. Escalona et al. (1999) also reported that the pineapple shoots did not showed any difference in the dry biomass in the continuous liquid cultures and TIS cultures and explained that this might be due to the reduction in leaf development in TIS cultures.

TIS derived plantlets of *C. zedoaria* and *Z. zerumbet* showed similar morphology to the plantlets derived from agar-gelled medium, shake flask system as well as conventionally propagated plants. Eight to 10% of the *in vitro* plantlets were found to be hyperhydric when subcultured continuously in the shake flask system. This was due to the enhanced water uptake in shake flask system resulting in the high tendency of the *in vitro* shoots towards hyperhydricity. However, the hyperhydric plantlets became normal again when subcultured into agar-gelled MS medium for another two weeks. All the *in vitro* plantlets produced from the three different micro-propagation systems grew normally as that of the mother plants after acclimatization. The well developed root system might be assumed as major factor to the proper acclimatization of plantlets from all the three tested system. Lorenzo et al. (2001) also reported that the sugarcane plantlets obtained from conventional method, solid culture medium and TIS showed similar growth

pattern after acclimatization.

Our study enabled us to conclude that TIS system was the best of the three systems tested for the in *vitro* propagation of both *C. zedoaria* and *Z. zerumbet.* It was reported that TIS was effective in reducing the production costs as compared to shake flask system and agar-gelled medium for sugar cane (Lorenzo et al., 1998). This was achieved by eliminating the use of gelling agent, one of the expensive components of the culture media. Besides, the agar-gelled cultures needed large number of containers and space which could be avoided by using TIS vessels. In addition, agar-gelled cultures are labor intensive as compared to TIS cultures. By using TIS with low immersion frequency, better shoot proliferation could be achieved with less labor input as compared to the gelled medium, and normal plantlets without hyperhydricity problem could be obtained as compared to the shake flask system.

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