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In Vitro Propagation of Zingiberaceae Species with Medicinal Properties

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Abstract

***Zingiber officinale* buds from the rhizomes were used to produce *in vitro* shoots. These explants produced the largest number of multiple shoots, 9.8 shoots per explant, when were cultured on MS (Murashige and Skoog 1962) medium supplemented with 2.0 mg/L benzyladenine (BA) and 2.0 mg/L indole butyric acid (IBA). This medium was also found to be suitable for *in vitro* propagation of other Zingiberaceae species: *Alpinia conchigera*, *Alpinia galanga*, *Curcuma domestica*, *C. zedoaria* and *Kaempferia galanga*. Both *C. domestica* and *C. zedoaria* produced more multiple shoots when were cultured in the liquid proliferation medium, MS medium containing 2.0 mg/L BA and 2.0 mg/L IBA. To maintain the *in vitro* plantlets of Zingiberaceae species, they were required to subculture every four weeks. After executing proper acclimatization protocol, *in vitro* plantlets of *Alpinia galanga*, *A. conchigera*, *Curcuma domestica*, *C. zedoaria*, *Kaempferia galanga* and *Zingiber officinale* could be successfully planted in the field with high percentage of survival.**

Key words: Acclimatization, aseptic explants, BA, Zingiberaceae species, IBA, multiple shoots, subculturing

Introduction

Family Zingiberaceae consists of about 50 genera and 1400 species (Hsuan et al. 1998). Zingiberaceae spcies have a wide range of usage. Some of the Zingiberaceae species such as *Zingiber officinale*, *Curcuma domestica*, *Kaempferia galanga*, *Elettaria cardomomum*, *Alpinia galanga* and *Amomum subulatum* are used as food, spices or drink. *C. domestica* is used as dye, while *Hedychium spicatum* is used for making perfume and perfumed powder (Porter 1967; Bateman and Dod 1978; Halevy 1985; Chen 1996). Other Zingiberaceae plants, such as *Hedychium coronarium*, *Costus* spp and *Kaempferia* spp. are famous ornamental plants because of their orchid like and showy flowers (Bateman and Dod 1978). Many of the Zingiberaceae plants have high medicinal value. Some of them have been used as traditional medicine for centuries. *Alpinia conchigera*, *A. galanga*, *C. domestica*, *C. zedoaria*, *K. galanga*, *Z. officinale* have been used for the treatment of cancerous tumor (Mackeen et al. 1977; Itokawa et al. 1987; Liu and Peng 1994; Wang 1994; Yu and Zhui 1995; Pan et al. 1996), reumatism, trauma, swelling, pain, motion sickness, morning sickness, nausea, digestive problem, fever, cough, amenorrhea, hematuria and other minor diseases (Ridley 1967; Anon 1975; Anon 1978; Mowrey and Clayson 1982; Muhamad and Mustafa 1994; Fulder 1996; Teo and Ch'ng 2000). Some of these plants are in short supply, such as *A. galanga*, due to indiscriminate collection from nature and lack of commercial scale cultivation (Anand and Hariharan 1997). Many of the Zingiberaceae plants are rare and are seldom seen and in the danger of extinction (Faujan and Ahmad 1988). Propagation of Zingiberaceae plants using conventional methods is very slow, not productive and always infected with pathogens, for example, the seeds of *Z. officinale* are often infected with *Pseudomonas* spp., *Pythium* spp., *Fusarium* spp. and *Geotrichum* spp. (Mishra and Rath 1988; Bhagyalakshmi and Singh 1988). The objectives of this study was, therefore, to find a common proliferation medium that can induce multiple shoots formation and a micropropagation protocol that can be applied for most of the Zingiberaceae species.

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Materials and Methods

Establishment of aseptic explants

The rhizomes of *A. conchigera*, *A. galanga*, *C. domestica*, *C. zedoaria*, *K. galang* and *Z. officinale* were collected from various parts of Penang, Malaysia. Buds from the rhizomes were used as explants. The explants were thoroughly washed with detergent several times and rinsed under running tap water. They were then surface sterilized in $HgCl_2$ solution (100 mg/L) under continuous agitation for 5 min. They were then rinsed three times with sterile distilled water. The buds were subsequently surface-sterilized in 15% Clorox® with a few drops of Tween 20 under constant agitation for 15 min, followed by washing three times with sterile distilled water. The buds were further surface-sterilized again in 10% Clorox® for 10 min, and rinsed three times with sterile distilled water. They were then cultured in each 150 ml jam jar containing MS (Murashige and Skoog 1962) medium supplemented with 30 g/L sucrose and 7.5 g/L agar (Algas, Chile) for 10 days. The pH of the medium was adjusted to 5.7-5.8 prior to autoclave at 121℃ and 1.06 kg/cm^2 for 13 min. All the cultures were maintain at 25±2℃ in a culture room with continuous lighting provided with cool white fluorescent lamps at 35 μmol m^{-2} s^{-1}.

Induction of multiple shoots of *Zingiber officinale*

Aseptic buds of *Z. officinale* were transferred aseptically from the basic MS medium onto MS medium supplemented with different concentration of BA and IBA ranging from 0, 2, 4, 6, 8 and 10 mg/L in 6x 6 factorial block design. Three buds were cultured in each 150 mL jam jar and 10 units of jam jars were used for each medium combination. The number of shoots produced was recorded after 4 weeks. Data obtained were analyzed using two-way ANOVA and the means were compared using Duncan's Multiple Range Test at p=0.05.

Effect of reduced concentration of BA and IBA on shoot proliferation of *Zingiber officinale*

The aseptic buds of *Z. officinale* were cultured on MS medium supplemented with lower concentration of BA and IBA (1.0, 1.5, 2.0, 2.5, 3.0 mg/L) in 5 x 5 factorial block design. Three buds were cultured in each 150 mL jam jar and 10 units of jam jars were used for each combination medium. The number of shoots produced was recorded after 4 weeks. Data obtained were analyzed using two-way ANOVA and the means were compared using Duncan's Multiple Range Test at p=0.05.

Application of shoot proliferation medium of *Zingiber officinale* for multiple shoots formation of other Zingiberaceae species

The aseptic buds of *Z. officinale*, *A. conchigera*, *A. galanga*, *C. domestica*, *C. zedoaria* and *K. galanga* were cultured on MS medium supplemented with 2 mg/L BA and 2 mg/L IBA, the most optimum medium for multiple shoots formation of *Z. officinale*. Three bud explants were cultured into 150 mL jam jar containing the shoot proliferation medium and 10 units of jam jars were used for each species. The number of shoots formed from the bud explant of each species was recorded after 4 weeks of culture. The data analyzed using one-way ANOVA and the effect of the proliferation medium on the various Zingiberaceae species was determined using Duncan's Multiple Range Test at p=0.05.

Effect of liquid culture medium on shoot proliferation

Aseptic buds of *Z. officinale*, *A. conchigera*, *A. galanga*, *C. domestica*, *C. zedoaria* and *K. galanga* were cultured in liquid MS medium supplemented with 2 mg/L BA and 2 mg/L IBA. The bud explants of each species were also cultured on MS solid medium supplemented with 2 mg/L BA and 2 mg/L IBA. The number of shoots produced from each bud explant from the liquid and solid medium was recorded after 4 weeks of culture and compared using student-t test at p=0.05.

Effect of subculture cycle period on formation of multiple shoots

Four weeks old *in vitro* shoots of *Z. officinale*, *A. conchigera*, *A. galanga*, *C. domestica*, *C. zedoaria* and *K. galanga* were cultured on MS solid medium supplemented with 2 mg/L BA and 2 mg/L IBA, the proliferation medium. Three shoots were cultured in each 150 mL jam jar containing the proliferation medium and 20 units of jam jars were used for each species. Half of the cultures of each species were subcultured after 4 weeks while the remainders were subcultured after 8 weeks. The number of shoots formed from each explant was determined after the 4-week and 8-week cycle period. The data collected was analyzed using student-t test at p=0.05.

Acclimatization

In vitro plantlets of *Z. officinale*, *C. domestica*, *C. zedoaria*,

A. galanga, *A. conchigera* and *K. galanga* were removed from the solid culture medium and the roots were washed under running tap water to remove any remaining agar. Each plantlet was then planted into the Jiffy® pellet (Jiffy Products Limited, Norway) and placed in the growth incubator (Hotech Instruments Corp., Model 624) regulated at 25±2℃ with continuous lighting with an intensity of 9 μ mol m^{-2} s^{-1} for two weeks. The survived plantlets were transferred to pots containing top soil: sand: organic soil (2:2:1). The percentage of survival was recorded after four weeks.

Results and Discussion

Establishment of aseptic explants

The buds from the rhizomes of *A. conchigera*, *A. galanga*, *C. domestica*, *C. zedoaria*, *K. galang* and *Z. officinale* obtained from the field that were surface-sterilized with 100 mg/L mercuric chloride and double sterilization with Clorox® solution did give a satisfactory result. The best double stage Clorox sterilization by using 15% Clorox® solution for 15 mins at the first stage followed by 10% Clorox® solution for 10 mins at the second stage could establish 62-81% aseptic bud explants with 63-89% survival rate (Table 1). This result showed that explants from different plant species respond differently to the surface-sterilizing protocol. For the establishment of aseptic tissues of Zingiberaceae plants, a few sterilizing agents had been tried but Clorox® solution, a commercial bleach which contained 5.25% sodium hypoclorite together with mercury chloride solution (100 mg/L) were found to be the best for reducing explants contamination and maintaining reasonably high survival rate. Chan and Chan (2002) also found that the use of mercury chloride with two-stage surface sterilization using Clorox® solution was very effective for establishing aseptic shoots of *Cyperus aromaticus*.

Multiple shoot formation of *Zingiber officinale*

Aseptic bud explants of *Z. officinale* cultured on MS medium supplemented with 0, 2, 4, 6, 8, 10 mg/l BA and IBA showed varying degree of multiple shoot formation. Multiple shoots were formed from all the bud explants that were cultured on MS medium supplemented with different combination of BA and IBA (0-10 mg/L). The buds formed multiple shoots even on MS medium without the addition of any growth regulators, BA and IBA, with an average of 4.8 shoots per bud explant within 4 weeks. Ikeda and Tanabe (1989) also found that decapitated crown section of ginger formed multiple shoots on basic MS medium. Sharma and Singh (1997) found that *Z. officinale* formed multiple shoots on MS medium supplemented with only BA or kinetin. While in our study, MS medium containing 2.0 mg/L BA and 2.0 mg/L IBA induced the highest number of multiple shoots (9.8 shoots per bud), formed from *Z. officinale* buds within 4 weeks. MS medium containing 2 mg/L BA and 4 mg/L IBA also induced the formation of reasonably high number of shoots with an average of 8 shoots per bud explant within the same period of time. When the amount of BA and IBA added into MS medium increased, the number of shoots formed from each explant was reduced. MS medium supplemented with only IBA (2-10 mg/L) without BA induced few multiple shoots i.e. between 1.5 and 3.7 shoots from each bud. Results indicated that MS medium supplemented with 2.0 mg/L BA and 2.0 mg/L IBA was the most optimum for multiple shoots formation of *Z. officinale* using bud explants (Table 2). The multiple shoots formed were healthy with normal leaves and roots (Figure 1). However, Noguchi and Yamakawa (1988) reported that numerous buds were induced in apical meristem culture of *Z. officinale* after treatment with high concentration of NAA. While Bhagyalakshmi and Singh (1988) found that IBA was more effective for bud meristem culture of *Z. officinale*.

Table 1. Percentage of aseptic bud explants and their survival rate for various species of Zingiberaceae plants after surface-sterilized with 100 mg/L mercury chloride followed by two-stage Clorox® surface- sterilization.

Zingiberaceae species	Aseptic explants (%)	Survival explants (%)
Alpinia conchigera	62.3 ± 7.7	73.7 ± 6.9
Alpinia galanga	62.0 ± 7.9	62.5 ± 7.1
Curcuma domestica	67.6 ± 8.3	70.5 ± 5.8
Curcuma zedoaria	77.6 ± 5.6	65.9 ± 8.8
Kaempferia galang	76.3 ± 5.3	76.0 ± 5.2
Zingiber officinale	81.2 ± 4.8	88.7 ± 6.7

Table 2. Effect of MS medium supplemented with BA and IBA (0-10 mg/L) on multiple shoots formation of *Zingiber officinale* over a period of four weeks.

Concentration of growth regulators (mg/L)		Number of shoots/bud explant
BA	IBA	
0.0	0.0	4.75 def
	2.0	1.50 k
	4.0	3.67 defghij
	6.0	3.50 defghij
	8.0	2.92 hijk
	10.0	2.42 jk
2.0	0.0	4.75 def
	2.0	9.75 a
	4.0	8.00 b
	6.0	4.47 defgh
	8.0	4.25 defghi
	10.0	4.50 defgh
4.0	0.0	6.50 c
	2.0	4.67 defg
	4.0	4.92 de
	6.0	4.42 defgh
	8.0	2.92 hijk
	10.0	2.42 jk
6.0	0.0	5.17 cd
	2.0	3.75 defghij
	4.0	3.25 efghijk
	6.0	3.75 defghij
	8.0	3.00 ghijk
	10.0	3.17 fghijk
8.0	0.0	3.25 efghijk
	2.0	3.75 defghij
	4.0	2.50 ijk
	6.0	3.25 efghijk
	8.0	2.75 hijk
	10.0	2.75 hijk
10.0	0.0	3.00 ghijk
	2.0	2.75 hijk
	4.0	2.75 hijk
	6.0	2.17 jk
	8.0	2.50 ijk
	10.0	2.33 jk

Mean number of shoots followed by the same alphabets are not significantly different at p=0.05 (DMRT)

Effect of reduced concentration of BA and IBA on shoot proliferation of *Zingiber officinale*

With the addition of lower concentration of BA and IBA, the number of shoots produced from each of the bud explant of *Z. officinale* was reduced. The number of shoots formed from each bud explant of *Z. officinale* was between 3 and 5 shoots when the bud explants were cultured on MS medium supplemented with (0-1.5 mg/L) BA plus (0-3.0 mg/L) IBA. The highest number of shoots (8 per explant

Figure 1. Normal and healthy multiple shoots induced from the bud explant of *Z. officinale* on MS + 2.0 mg/L BA + 2.0 mg/L IBA.

Table 3. Effect of Combination of BA and IBA (1-3 mg/L) in MS medium on shoot proliferation of *Zingiber officinale* over a period of four weeks.

Concentration of growth regulators (mg/L)		Number of shoots/bud explant
BA	IBA	
0.0	0.0	3.2 d
1.0	1.0	3.2 d
	1.5	3.8 d
	2.0	3.5 d
	2.5	3.8 d
	3.0	4.3 d
1.5	1.0	3.9 d
	1.5	3.8 d
	2.0	4.7 cd
	2.5	4.3 d
	3.0	4.3 d
2.0	1.0	6.4 bc
	1.5	6.4 bc
	2.0	8.3 a
	2.5	6.4 bc
	3.0	6.8 b
2.5	1.0	5.3 cd
	1.5	5.0 cd
	2.0	5.3 cd
	2.5	4.3 d
	3.0	4.0 d
3.0	1.0	4.8 cd
	1.5	4.0 d
	2.0	4.4 d
	2.5	4.1 d
	3.0	4.0 d

Mean number of shoots followed by the same alphabets are not significantly different at p=0.05 (DMRT)

was produced on MS medium containing 2.0 mg/L BA and 2.0 mg/L IBA. When more than 2.0 mg/L BA with various concentration of IBA (1.0-3.0 mg/L) was added to the MS medium, the number of shoots formed from each explant was also reduced (Table 3). This again indicated that MS medium containing 2.0 mg/L BA and 2.0 mg/L IBA was the optimum shoot proliferation medium for *Z. officinale*.

Application of shoot proliferation medium of *zingiber officinale* for multiple shoots formation of other zingiberaceae species

When the optimum proliferation medium of *Z. officinale* was used as the culture medium for the five other Zingiberaceae species (*A. conchigera*, *A. galanga*, *C. domestica*, *C. zedoaria*, *K. galanga*), all the bud explants of these plants formed multiple shoots. This proliferation medium induced the formation of an average of 7.2 shoots per bud explant of *K. galanga* within 4 weeks. The multiple shoots formed were healthy with normal leaves and roots (Figure 2). The number of shoots produced from the other Zingiberaceae species (*A. conchigera*, *A. galanga*, *C. domestica* and *C. zedoaria*) were lesser, with 3 to 5 shoots formed from each bud explant within the same period of time but significantly not different from each other (Figure 3). However, Sharma and Singh (1997) reported that only 1-4 shoots could be induced from microrhizom of *Z. officinale* using MS medium containing 8.0 mg/L BA and 75 g/L sucrose after 50-60 days of culture. Chang and Criley (1993) reported that BA was effective in propagation of *A. purpurata*. But Lim (2000) found that combination of BA and IBA was not effective for multiple shoots formation of *A. galanga* and high concentration of BA caused the tissue of *A. galanga* to become necrotic. Anand and Hariharan (1997), on the other hand, found that MS medium containing 1.0 mg/L BA induced multiple shoots formation in *A. galanga*, but the number was reduced as the concentration of BA was increased. Hence, this clearly indicated that the same cytokinin growth regulator not necessary induced multiple shoot formation even in the same plant species.

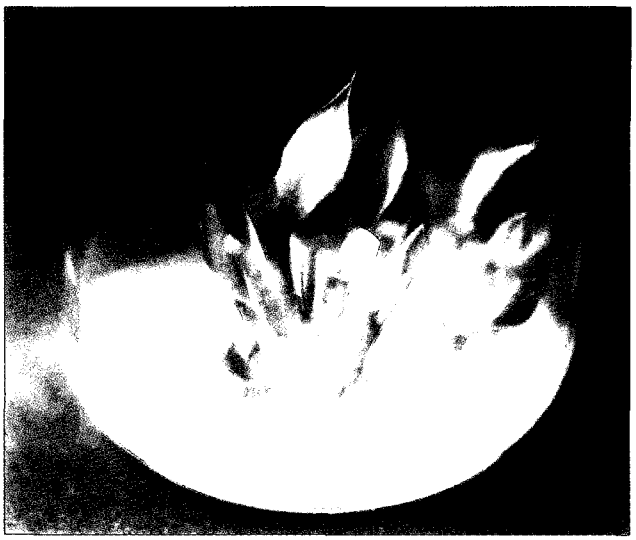

Figure 2. Multiple shoots of *K. galanga* on proliferation medium MS + 2.0 mg/L BA + 2.0 mg/L IBA.

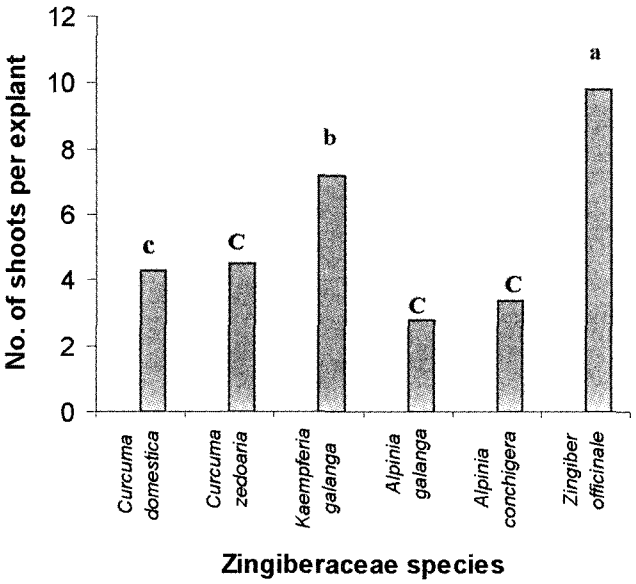

Figure 3. Effect of proliferation medium MS + 2.0 mg/L BA + 2.0 mg/L IBA on formation of multiple shoot of different Zingiberaceae species.

Effect of liquid culture medium on shoot proliferation

For *Z. officinale*, *K. galanga*, *A. galanga* and *A. conchigera*, there was no signifancant differences in multiple shoots formation in the liquid MS medium containing 2.0 mg/L BA and 2.0 mg/L IBA as compared to the solid medium with the same combination of growth regulators. But, for both *C. domestica* and *C.zedoaria*, the number of multiple shoots formed was 1.5 times more using the liquid MS medium containing 2.0 mg/L BA and 2.0 mg/L IBA than the solid medium (Table 4). The fast proliferation of *C. domestica* and *C. zedoaria* shoots in liquid medium (Figure 4) could be due to continuous agitation of liquid medium on the orbital shaker enabled higher aeration and more nutrient up-take by the plant tissues. Hu and Wang (1983) explained that continuous agitation of liquid medium could change the physiological condition of the plant that encouraged shoot growth. In our study, the shoots formed on solid medium

Table 4. Effect of solid and liquid medium of MS + 2 mg/L BA + 2 mg/L IBA on multiple shoots formation of Zingiberaceae species.

Zingiberaceae species	No. of shoots formed per bud explant on MS + 2 mg/L BA + 2 mg/L IBA	
	Solid medium	Liquid medium
Alpinia conchigera	2.7 a	2.0 a
Alpinia galanga	3.4 b	4.0 b
Curcuma zedoaria	3.9 c	6.3 d
Curcuma domestica	3.9 e	6.0 f
Kaempferia galanga	7.0 g	8.0 g
Zingiber officinale	7.5 h	9.2 h

Mean values within the same row followed by the same alphabets was not significantly different at p=0.05, DMRT.

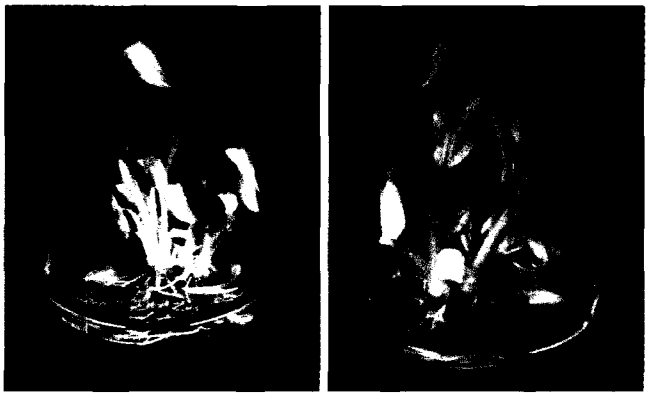

Figure 4. Multiple shoots formation of *C. domestica* (left) and *C. zedoaria* (right) on liquid MS containing 2.0 mg/L BA and 2.0 mg/L IBA.

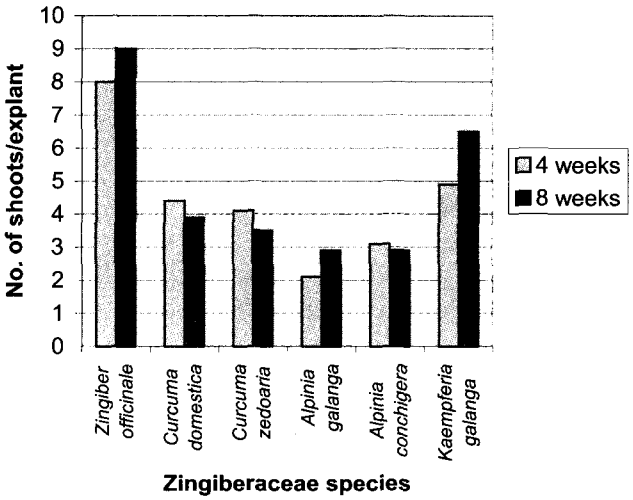

Figure 5. Effect of subculture duration on shoot proliferation of Zingiberaceae species.

were green and normal. After a long period of culturing in the liquid medium, shoots become vitrified, but became normal again when they were transferred to the solid proliferation medium. However, the *Z. officinale* shoots produced in liquid medium did not survive well even after transfer to the solid medium. Rout and Das (1997) found that liquid medium was not effective for the rooting of *Z. officinale* compared with solid medium.

Effect of subculture cycle period on shoot proliferation

For *Z. officinale*, *C. domestica*, *C. zedoaria* and *A. conchigera*, the number of multiple shoots formed after four weeks subculture and eight weeks subculture cycle was not significantly different, but for *K. galanga* and *A. galanga*, the number of multiple shoots formed by four week was less than after eight weeks subculture (Figure 5). This result indicated that each *in vitro* plant species required different period for subculturing. For example, Rahmad's study (1999) showed that every six week subculturing cycle was suitable for obtaining the best proliferation rate for the gerbera cultures. While, Nallammai (1997) found that subculturing *Anubia nana* every five weeks was the best subculturing cycle for maintaining normal and healthy plantlets.

Acclimatization protocol

Many *in vitro* plantlets did not survive if the acclimatization protocol was not carried out properly. This was mainly due to low relative humidity, high light intensity and higher temperature of the outside environment as compared to the *in vitro* conditions. Besides, most of the *in vitro* plantlets usually produced leaves lacking epicuticular wax and with ineffective control of stomatal function, and these conditions enhanced water loss when they were transferred to the soil (George and Sherrington 1984). For our study, each of the *in vitro* plantlet of Zingiberaceae species was planted into the Jiffy® pellet after removing from the culture vessels and placed in the growth incubator regulated at 25

Table 5. The survival rate of the *in vitro* plantlets of Zingiberaceae species after acclimatized in the growth chamber regulated at 25 ±2℃ with continuous lighting with an intensity of 9 μmol m^{-2} s^{-1} for two weeks and transferred to soil.

Zingiberaceae species	Survival rate of the acclimatized plantlets (%) ± s.d
Alpinia conchigera	83.3 ± 11.5
Alpinia galanga	76.7 ± 5.8
Curcuma zedoaria	80.8 ± 10.0
Curcuma domestica	83.0 ± 11.5
Kaempferia galanga	90.0 ± 23.5
Zingiber officinale	86.0 ± 5.8

±2℃ with continuous lighting with an intensity of 9 μmol m^{-2} s^{-1} for two weeks. Thereafter they were transferred to pot containing top soil: sand: organic soil (2:2:1). This acclimatization protocol enabled high percentage (77-90%) of the *in vitro* plantlets of *Z. officinale*, *C. domestica*, *C. zedoaria*, *K. galanga*, *A. galanga* and *A. conchigera* to survive and were successfully planted in the outside environment (Table 5). Morphology of the *in vitro* plantlets was identical to their mother plants.

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