

Full Length Research Paper

# Efficient *in vitro* regeneration of *Zingiber zerumbet* Smith (a valuable medicinal plant) plantlets from rhizome bud explants

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This study was conducted to develop an efficient protocol for mass propagation of *Zingiber zerumbet* Smith. Explants from rhizome buds were cultured on Murashige and Skoog (MS) medium supplemented with 6-Benzylaminopurine (BAP) alone (0 to 5 mg/l) or a combination of BAP (0 to 5 mg/l) and indole 3-acetic acid (IAA) (0 to 2 mg/l). MS medium supplemented with a combination of 5.0 mg/l BAP and 2.0 mg/l IAA or 3.0 mg/l BAP and 0.5 mg/l IAA produced the highest mean number of shoots (5.6) per explant as compared to other concentrations. The best shoots length (9.44 cm) was obtained on the medium containing 1.0 mg/l of BAP and 2.0 mg/l IAA. Thus, combined effects of BAP and IAA improved significantly the shoot growth and proliferation. MS medium supplemented with a combination of 5.0 mg/l BAP and 2 mg/l IAA gave the highest number of roots (17). However, longest roots per explant were obtained with 1.0 mg/l BAP alone. The proliferated shoots were green and healthy in appearance. Finally, healthy and complete plants with well developed roots were hardened, acclimatized and planted in the field successfully with a survival rate of 80%.

**Key words:** *Zingiber zerumbet*, shoot multiplication, micropropagation, *in vitro*, acclimatization, medicinal plants, Zingiberaceae.

## INTRODUCTION

The family Zingiberaceae consists of about 50 genera and 1400 species (Hsuan et al., 1998). Some plants of the family are rhizomatous herbs found throughout tropical and subtropical regions with its main distribution in Asia (Khatun et al., 2003). Malaysia is one of the 17 megadiversity centre of the world with 12,500 plant species. Among these indigenous plants, some species produce fragrant flowers and leaves. Ginger rhizomes are rich in secondary metabolites such as oleoresin (Bhagyalakshmi and Singh, 1988). Besides their uses as

spices, *Zingiber* species can also be used in cosmetics and medicines (Balachandran et al., 1990). Furthermore, it has been reported that the ornamental plant market is showing a considerable interest in some species of Zingiberaceae either in landscaping designs or as cut flowers (Faria and Illg, 1995).

*Zingiber zerumbet* Smith is a perennial medicinal herb; it is a part of the Zingiberaceae, closely related to *Zingiber ottensii*, and is believed to be native to India. It is cultivated in India, Sri Lanka, China and throughout South East Asia (Abd Malek et al., 2005). *Z. zerumbet* is known in Malaysia as "lempoyang". It can be found around villages as secondary growth (Burkill, 1966). The ginger grows very well under shade, moist environment and flowering occurs in August and September (Nalawade et al., 2003). The Inflorescence bract is green when young, then turns to red at maturity; corolla lobes and lip are yellow in colour with sweet smell (Abd Malek

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**Abbreviations:** BAP, 6-Benzylaminopurine; IAA, indole 3-acetic acid; MS, Murashige and Skoog (1962) basal medium.

et al., 2005). The herb is also known as shampoo ginger, as the cone-shaped bracts contain a clear liquid which is an excellent natural hair conditioner (Nalawade et al., 2003).

In Malaysia, rhizomes of *Z. zerumbet* are consumed to increase appetite, for the treatment of ulcers, stomach-aches, diarrhea, asthma, rheumatism and as anti-inflammatory (Abd Malek et al., 2005). The rhizome macerated in alcohol is regarded as a novel food factor for mitigating experimental ulcerative colitis (Sakinah et al., 2007). However, in South East Asia, *Z. zerumbet* traditionally is used for the treatment of fever, constipation and to relieve pain (Stanly and Keng, 2007). It possesses antipyretic and analgesic properties (Somchit et al., 2005). It also possesses anti-inflammatory (Somchit and Shukriyah, 2003) and chemo-preventive activities (Nakamura et al., 2004). Abd Malek et al. (2005) noted that the volatile oil of the rhizomes of *Z. zerumbet* contains about 73.08% of zerumbone, 5.93% of  $\alpha$ -humulene, 2.81% of camphene, 2.72% of caryophyllene oxide and 1.31% of camphor. Whereas, Sakinah et al. (2007) noted that zerumbone can be used as a new alternative chemotherapeutic agent for human liver cancer. Furthermore, Baby et al. (2009) reported that zerumbone is a sesquiterpene phytochemical with potential anticancer, anti-inflammatory, anti-HIV and other biological activities.

*Zingiber zerumbet* is traditionally propagated by rhizome. The rhizome cannot be stored for a long time, as it is susceptible to fungal diseases, which affect the quality of the tubers (Nalawade et al., 2003). Thus, micro-propagation can be used as an alternative method for producing pathogen-free plants in a large number with homogeneity and good quality tubers (Poonsapaya and Kraisintu, 1993). Nalawade et al. (2003) reported that the use of *in vitro* propagation technique led to the production of large number of pathogen-free uniform clones of elite, rare and important native medicinal plants so as to be reintroduced in their natural habitats and thus promoting safe exchange of germplasm across inter-national borders. With this in view, the aim of this study was to develop tissue culture system for the mass production and conservation of this important medicinal species.

## MATERIALS AND METHODS

The rhizomes of *Z. zerumbet* were collected from the Agricultural Conservatory Park, Universiti Putra Malaysia. Buds from the rhizomes were excised and used as explants. The explants were washed thoroughly under running tap water to remove adhering soil particles. To control the microbial contamination, a composite approach of washing the explants first with 60% Clorox and addition of 6 to 7 drops of Tween 20 for 30 min was employed. Subsequently, the explants were thoroughly washed once with sterile distilled water. Sterilized explants were then dissected to remove one layer of leaf sheaths under aseptic conditions. Then, the excised explants were immersed again in 20% Clorox with the addition of 6 to 7 drops of Tween 20 for another 15 min and thoroughly washed 7 times with sterilized distilled water. Outer

leaves were removed aseptically and explants of 0.5 to 0.8 mm were inoculated onto the culture medium.

## Culture medium and incubation conditions

All the media used in this study were based on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose. The medium was adjusted to pH 5.8 before solidifying with 4.5 g/L gelrite and autoclaved at 121°C, 15 psi for 20 min. All cultures were kept under 16 h photoperiod at a photosynthetic flux of 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , provided by cool fluorescent lamps and maintained at  $25 \pm 2^\circ\text{C}$ . Different concentrations of BAP (0, 1, 3 and 5 mg/l) were added either alone or in combination with IAA (0, 0.5, 1.0, 2.0 mg/l) to the MS medium. Plantlets with well developed roots were removed from the medium, then washed thoroughly under running tap water to remove adhering solid MS medium, and transplanted to plastic pots containing sterilized peat moss soil and kept in a 50% shaded net house. The plants were covered by a transparent perforated polyethylene bags in order to maintain a high humidity and to avoid plant dehydration by water loss. The plants were frequently watered to keep high level of humidity. The polyethylene bags were then removed after 7 days and the plantlets were acclimatized for another three weeks. Then, the percentage of survival rate was recorded.

## Data collection

After eight weeks of culture, the following parameters were recorded: number of shoots, number of leaves, number of roots, shoots and root length, and survival rate was calculated.

## Statistical analysis

Collected data was analyzed using the SPSS statistical package software version 10.0 (Chicago, USA). Analysis of variance (ANOVA) with mean separation by Duncan's multiple range test ( $P < 0.05$ ) was used to compare means of different treatments. Each experiment was repeated thrice.

## RESULTS AND DISCUSSION

The explants of *Z. zerumbet* cultured on MS medium supplemented with different concentrations of BAP alone (0.0, 1.0, 3.0 and 5.0 mg/l) or in combination with IAA (0.0, 0.5, 1.0 and 2.0 mg/l) produced both shoots and roots, simultaneously whereas, roots induction was lowest (4.00) for explants cultured on MS medium containing higher concentrations of BAP alone (5 mg/l) and highest (17) in medium containing combination of 5 mg/l of BAP and 2 mg/l of IAA (Table 1). Such type of simultaneous production of shoot and roots were reported earlier for *Z. zerumbet* by Stanly and Keng (2007) and on other Zingiberaceae species (Balachandran et al., 1990; Chan and Thong, 2004; Bharalee et al., 2005; Yusuf et al., 2011). This study suggests that the use of combination medium will shorten the time for plant regeneration. When BAP was used alone, the maximum number of shoots (3.6) was obtained from explants on MS medium with 5 mg/l BAP. With an increase in the concentration of BAP, the number and

**Table 1.** Effect of growth regulators on *in vitro* multiplication of the number of shoots, shoot length and number of roots of *Z. zerumbet* grown on MS medium

PGRs (mg/l)	Number of shoot	Length of shoot	Number of root
MSO	2.00 <sup>e*</sup>	2.70 <sup>g</sup>	4.30 <sup>i</sup>
1.0 BAP	2.00 <sup>e</sup>	3.30 <sup>g</sup>	6.00 <sup>h</sup>
3.0 BAP	3.30 <sup>d</sup>	5.00 <sup>f</sup>	11.30 <sup>g</sup>
5.0 BAP	3.60 <sup>cd</sup>	3.20 <sup>g</sup>	4.00 <sup>i</sup>
1.0 BAP + 0.5 IAA	4.00 <sup>cd</sup>	7.44 <sup>e</sup>	13.80 <sup>d</sup>
1.0 BAP + 1.0 IAA	4.00 <sup>cd</sup>	7.44 <sup>e</sup>	13.40 <sup>de</sup>
1.0 BAP + 2.0 IAA	4.10 <sup>bc</sup>	9.44 <sup>a</sup>	13.40 <sup>de</sup>
3.0 BAP + 0.5 IAA	5.60 <sup>a</sup>	9.00 <sup>ab</sup>	12.60 <sup>ef</sup>
3.0 BAP + 1.0 IAA	4.80 <sup>ab</sup>	8.19 <sup>cd</sup>	12.20 <sup>fg</sup>
3.0 BAP + 2.0 IAA	5.00 <sup>a</sup>	7.65 <sup>de</sup>	15.80 <sup>b</sup>
5.0 BAP + 0.5 IAA	5.20 <sup>a</sup>	8.15 <sup>cd</sup>	14.80 <sup>c</sup>
5.0 BAP + 1.0 IAA	5.40 <sup>a</sup>	8.72 <sup>bc</sup>	16.00 <sup>b</sup>
5.0 BAP + 2.0 IAA	5.60 <sup>a</sup>	9.17 <sup>ab</sup>	17.00 <sup>a</sup>

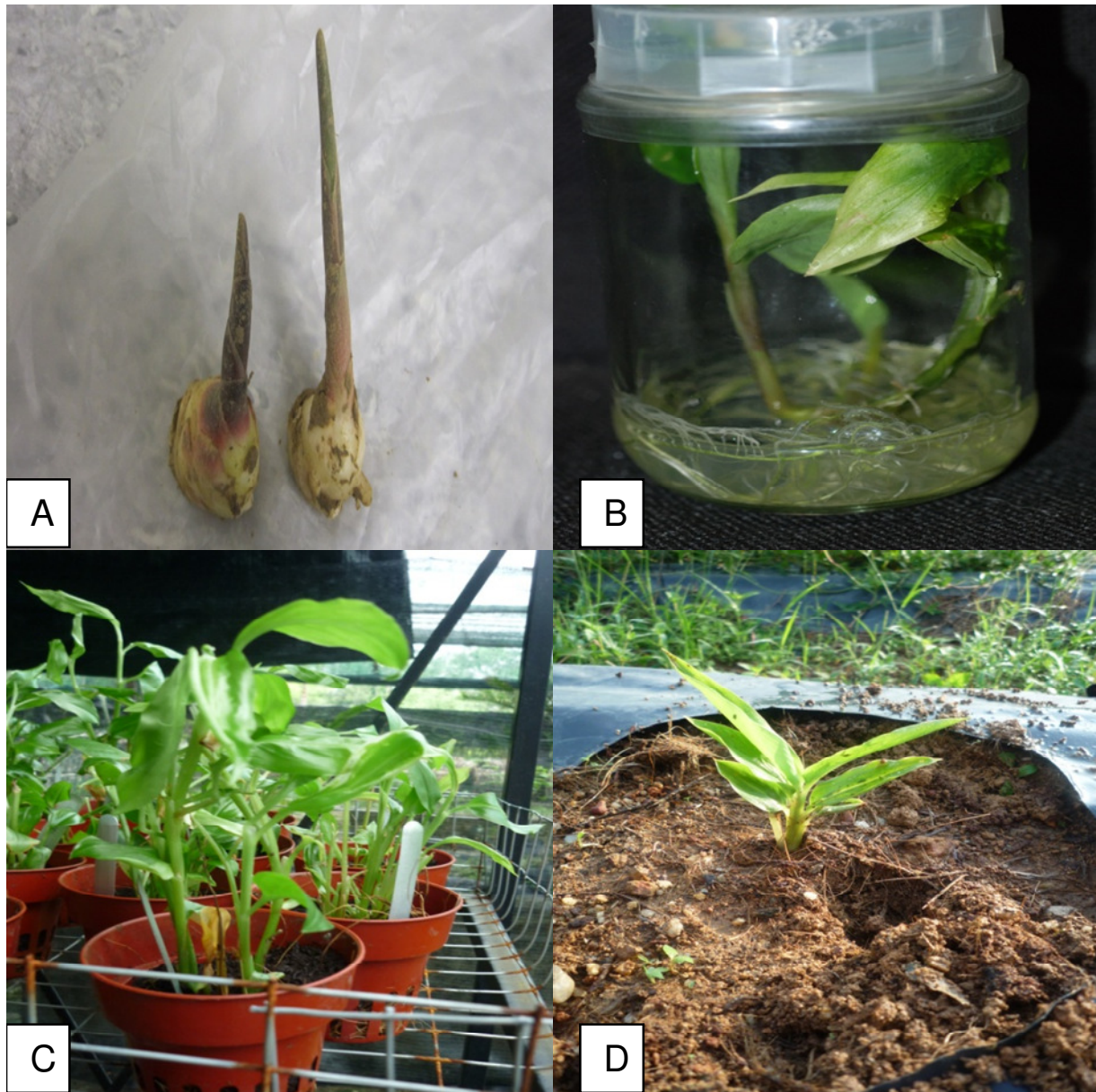
\*Means followed by the same letter(s) within each column are not significantly different at  $P \leq 0.05$ , according to Duncan's multiple range test for each parameter. PGRs, plant growth regulator.

length of shoots per explant increased (Table 1). Therefore, it appears that a higher concentration of BAP would have a positive effect on *in vitro* shoot multiplication of *Z. zerumbet*. The proliferated shoots were green and healthy in appearance (Figure 1B and C). The role of BAP in shoots proliferation has been reported in other Zingiberaceae species (Ikeda and Tambe, 1989; Balachandran et al., 1990; Smith and Hamil, 1996; Rout et al., 2001; Panda et al., 2007; Mohanty et al., 2011; Abdelmageed et al., 2011). The presence of BAP and IAA in the medium markedly improved the number of proliferating shoots. Besides the number of shoots induced, BAP and IAA accelerated mean shoot length considerably (Table 1). The highest shoot multiplication was found in the medium containing 5.0 mg/l BAP + 2.0 mg/l IAA and also 3.0 mg/l BAP + 0.5 mg/l IAA, which produced nearly 5.6 shoots per explant, whereas, the longest shoots length (9.44) were obtained on the media containing 1.0 mg/l of BAP + 2.0 mg/l of IAA and 5.0 mg/l of BAP + 2.0 mg/l of IAA, respectively (Table 1). It was observed that cytokinin was required in optimal quantity for shoot proliferation in some species of Zingiberaceae, but inclusion of low concentration of auxins along with cytokinin triggered the rate of shoot proliferation (Rout and Das, 1997; Sharma and Singh, 1997). Further, Stanly and Keng (2007) reported that MS medium supplemented with 6 mg/l BAP induced the formation of multiple shoots of *Z. zerumbet*. Earlier, it was reported that the highest numbers of shoots were obtained on MS medium supplemented with 5 to 10 mg/l BAP from *Zingiber officinale* Roscoe (Noguchi and Yamakawa, 1988). Faria and Illg (1995) noted that the addition of 2.25 mg/l of BAP with 1.0 mg/l of IAA induced a high rate of shoot proliferation of *Zingiber spectabile*.

For *Curcuma haritha*, Bejoy et al. (2006) found that the

best shoot multiplication and root system were achieved on MS medium supplemented with 1.0 mg/l of BAP and 0.5 mg/l of IAA. This result confirmed this study, which indicated the importance of IAA hormone in the induction of roots. Moreover, Anish et al. (2008) reported that MS medium supplemented with IAA at 0.5 mg/l in a combination with BAP seems to be optimum for rooting of *Boesenbergia pulcherrima*. In the same trend, Vincent et al. (1992) reported that the highest number of shoots per explant was obtained with cultured axillary buds of *Kaempferia galangal* on MS medium supplemented with 0.50 mg/l of BAP and 3.0 mg/l kinetin after 120 days of incubation.

MS medium supplemented with 5 mg/l BAP and 2.0 mg/l IAA produced significantly the highest mean number (17.0) of roots per explant (Table 1) whereas, MS medium supplemented with 1.0 mg/l BAP produced the longest roots (5.20) per explant followed by MS medium without growth regulators (4.90) (Table 2). This result indicate that IAA promoted the growth of roots. Higher concentration of auxin, in the range that normally stimulates elongation of shoots, causes a significant inhibition of root growth (Hopkins and Hüner, 2004). Bejoy et al. (2006) found that the shoot multiplication and root systems were obtained on MS medium supplemented with 1.0 mg/l of BAP and 0.50 mg/l of IAA. This result is comparable to this study, which indicates the importance of IAA hormone in the induction of roots. According to Anish et al. (2008), MS medium supplemented with IAA at 0.50 mg/l in combination with BAP seems to be optimum for rooting of *B. pulcherrima*. In this study, the IAA hormone was found to have a positive effect on root induction, especially when used in combination with BAP. According to Sharma (2006), IAA and IBA are usually used for easier-to-root herbaceous



**Figure 1.** Stages of *in vitro* multiplication of *Z. zerumbet*. (a) Rhizome buds as a source of explants; (b) induction of multiple shoots from axillary buds on MS medium supplemented with 5.0 mg/l BAP and 2.0 mg/l IAA after eight weeks of subculture; (c) established plant following transplantation of regenerated plantlets into plastic pots containing sterilized peat moss under nethouse conditions; (d) established plant under field conditions.

plants and NAA for more recalcitrant woody plants, and they mentioned that the efficacy of different auxins also depends on the explants type and exposure to light.

The number of rhizomes per explant showed significant differences among the different treatments (Table 2). The highest number of rhizomes formed was obtained on MS media supplemented with the following combinations: 5.0 mg/l BAP + 0.5 IAA mg/l, 5.0 mg/l BAP + 1.0 mg/l IAA and 5.0 mg/l BAP + 2.0 mg/l IAA, respectively. This result is comparable to those noted on other Zingiberaceae species (Rout et al., 2000; Anisuzzaman et al., 2008).

This study revealed that rhizomes induction in *Z. zerumbet* needs further works.

With regards to number of leaves per explant, MS medium containing 5.0 mg/l BAP + 2.0 mg/l produced significantly the highest number of leaves per explants (6.60) (Table 2), which indicate that cytokinin alone or in combination with auxin (BAP and IAA) had a significant response on the number of leaves per explant of this species. This result confirmed earlier findings of Borthakur et al. (1999) for *Alpinia galanga*, Yusuf et al. (2011) for *Boesenbergia rotunda* and Waseem et al.

**Table 2.** Effect of growth regulators on *in vitro* multiplication of length of roots, number of rhizomes and number of leaves of *Z. zerumbet* grown on MS medium

PGRs (mg/l)	Length of root	Number of rhizome	Number of leaf
MSO	4.90 <sup>ab*</sup>	1.00 <sup>d</sup>	2.00 <sup>g</sup>
1.0 BAP	5.20 <sup>a</sup>	1.00 <sup>d</sup>	2.70 <sup>f</sup>
3.0 BAP	4.60 <sup>bc</sup>	1.30 <sup>c</sup>	3.00 <sup>ef</sup>
5.0 BAP	2.20 <sup>i</sup>	1.30 <sup>c</sup>	3.30 <sup>e</sup>
1.0 BAP + 0.5 IAA	3.20 <sup>gh</sup>	1.40 <sup>c</sup>	4.40 <sup>cd</sup>
1.0 BAP + 1.0 IAA	3.70 <sup>ef</sup>	2.00 <sup>b</sup>	4.35 <sup>d</sup>
1.0 BAP + 2.0 IAA	4.05 <sup>de</sup>	2.00 <sup>b</sup>	4.20 <sup>d</sup>
3.0 BAP + 0.5 IAA	3.00 <sup>h</sup>	2.00 <sup>b</sup>	4.40 <sup>cd</sup>
3.0 BAP + 1.0 IAA	3.52 <sup>fg</sup>	2.00 <sup>b</sup>	4.80 <sup>bcd</sup>
3.0 BAP + 2.0 IAA	3.80 <sup>def</sup>	2.00 <sup>b</sup>	5.00 <sup>bc</sup>
5.0 BAP + 0.5 IAA	3.80 <sup>def</sup>	3.00 <sup>a</sup>	5.20 <sup>b</sup>
5.0 BAP + 1.0 IAA	4.00 <sup>de</sup>	3.00 <sup>a</sup>	5.40 <sup>b</sup>
5.0 BAP + 2.0 IAA	4.20 <sup>cd</sup>	3.00 <sup>a</sup>	6.60 <sup>a</sup>

\*Means followed by the same letter(s) within each column are not significantly different at  $P \leq 0.05$ , according to Duncan's multiple range test for each parameter.

(2011) for *Chrysanthemum morifolium* L.

Acclimatization of plantlets can be considered as one of the most important phase in tissue culture techniques. In this investigation, the mortality rate of the plantlets with well developed roots that were acclimatized and hardened was low. On average, 80% of *in vitro* transferred plantlets survived in potted soil and did not show any morphological abnormalities (Figure 1D). In conclusion, this protocol is a step forward towards improvement of the propagation of *Z. zerumbet*, species with a very important medicinal value.

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