The Production of Zerumbone in Adventitious Roots Culture of *Zingiber zerumbet* Smith

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Abstract

Root cultures were established through adventitious roots obtained either from direct or indirect organogenesis. The frequency of root response, number of roots per explant, root length, and zerumbone production were influenced by the concentrations and the types of auxins, initial root inoculum and the strength of the basic Murashige and Skoog (MS) salt in the culture media. It was crucial to decide the type of root explant and optimum media that supported both growth and bioactive compound production in the root cultures. In our study, we found that there was a noncorrelation in the optimised media for growth and zerumbone production in the root cultures of medicinal ginger Zingiber zerumbet Smith. Full strength (MS) medium was the optimum media for specific growth rates whereas zerumbone accumulation was higher in half strength MS medium for adventitious roots from direct (AdRD) and indirect (AdRId) organogenesis. AdRD was chosen over AdRId although the specific growth rate achieved was higher in the latter ($7.2 \times 10^{-2}\mu$) than the former ($5.5 \times 10^{-2}\mu$) based on the zerumbone accumulation performance. Subsequently, these AdRD root cultures were elicitated with methyl jasmonate which showed ten-folds increase in zerumbone production than the controls. This study could provide a scalable protocol for the production of zerumbone from adventitious root culture in the future.

Keywords: herbs, lempoyang, , bioactive compounds, root culture

Mahanom J., N. H. Alwakil, B. Chin Tan , N. Khalid, M. S. Mohamad Annuar.2024. The Production of Zerumbone in Adventitious Roots Culture of *Zingiber zerumbet* Smith . *Iranian Journal of Plant Physiology ,14* (1),4879- 4890.

Introduction

Zerumbone (ZER) is a well-studied bioactive compound and has been applied in medical and cosmetic industry. Reports on antimicrobial, anti-inflammatory anti-cancer properties and skin whitening have been published (Oh et al., 2018; Girisa et al., 2019). A patented property of ZER as a chemo preventive agent in hepatocarcinogenesis and suppressor of tumor promoter cells has also been filed (Akhtar et al., 2019; Jalili et al., 2020). Zerumbone can be found abundantly in *Zingiber zerumbet* Smith, which belongs to the Zingiberaceae family.

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Received: June, 2023
Accepted: December, 2023

Zerumbone could be obtained conventionally from rhizome but subjected to natural calamities and diseases such as root rot. Unpredictable climatic changes will affect the production of secondary compounds *in vivo* including the ZER (Holopainen et al., 2018). Alternatively, establishment of *in vitro* plant cell cultures including callus, cell suspension and root cultures in controlled environment is critical (Sarin, 2005). In comparison to callus and cell suspension cultures, adventitious root cultures (AdR) are more stable under culture environment, require less initial inoculum, and the bioactive compounds are more amenable for extraction (Sivakumar, 2006). The formation of AdR is controlled by multiple endogenous factors and culture conditions such as plant growth regulators, media components, photoperiod and inoculum size (Cui et al., 2020).

Besides regulating the AdR formation, these factors also influence the production of plant secondary metabolites. Therefore, developing an optimal growth condition for sustainable AdR culture and bioactive compound production is essential. Bioactive compound production in *in vitro* culture could be enhanced through the use of elicitors or precursors (Kannan et al., 2020). In this work, we aimed to develop an efficient protocol for propagating *Z. zerumbet* adventitious root cultures and concurrently evaluate zerumbone production. Subsequently, enhancement of ZER through methyl jasmonate elicitation will be investigated.

Materials and Methods

Initiation of adventitious root cultures

Adventitious roots (AdR) were initiated through direct (AdRD) and indirect (via an intermediary callus phase; AdRId) organogenesis. Shoot buds (5 cm) of *Z. zerumbet* were surface sterilized and sliced horizontally and used as explant for AdRD initiation in Murashige and Skoog (MS) (Murashige & Skoog, 1962) basal medium supplemented with 3 % (w/v) sucrose, 2 g/l phytagel, indole-3-butyric acid (IBA), and 1-naphthaleneaceatic acid (NAA) at 0.5, 1, 2, 3, 5, 7, 9 mg/l] or callus induction medium for AdRId [MS basal medium supplemented with 1.0 mg/l d-biotin, 3 % sucrose, 2 g/l phytagel, 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/l indoleacetic acid (IAA), and 1 mg/l NAA] (Jalil et al., 2015). The media was adjusted to pH 5.7 prior to autoclaving. All cultures were maintained at 25 ± 1 °C under a dark photoperiod. The frequency of root induction, number and length of AdR formed were recorded after 2 months of culture. All experiments were carried out in triplicate cultures (5 explants per petri dish) and repeated twice.

Initiation of AdR suspension cultures

Initiation of adventitious root in liquid culture were done by transferring AdRD and AdRId (0.5 g) into liquid MS medium containing 3 % sucrose and supplemented with different concentrations of IBA or NAA (1, 2, 3, 5, 7, 9 mg/l). The cultures were incubated at 25 ± 1 °C under continuous shaking condition of 80 rpm in a 16 h light: 8 h dark or dark condition. Fresh (FW) and dry weight (DW) of roots was measured after two months of culture. All experiments were carried out in triplicate cultures.

Multiplication and growth of adventitious root culture

Different inoculum sizes (0.5, 1.0, 2.0, 3.0, and 5.0 g) and medium salt strengths (0.5, 1.0, and 2.0 strength of MS) were tested for AdRId and AdRD. The initial inoculum for medium salt strength treatment was at 0.5 g FW. The FW of roots was recorded at a five-day interval for a period of 30 days, whereas specific growth rate (the changes of settled cell volume in natural log) was recorded for 25 days of culture. AdR suspension cultures were then multiplied on the optimized media and maintained under the same condition as described previously. All experiments were carried out in triplicate cultures.

Extraction of zerumbone from adventitious root culture

Roots for all treatments were collected at day 25 for AdR suspension culture initiation and multiplication. The harvested AdR cultures were oven-dried at 38°C for 48h before being ground into a fine powder. The powder (0.1 g) was then extracted using Soxhlett method and the crude extract was evaporated using a rotary evaporator

(BÜCHI Rotavapor R-114, USA). The extract was dissolved in 10 ml dichloromethane (Merck, USA) and kept at 4 ºC in a chiller for usage. Each analysis was repeated three times.

Histological examination of roots

The tissue specimens of two months old roots (AdRD and AdRId) were fixed in formaldehyde-absolute ethanol-acetic acid (FAA) solution consisting of 5% (v/v) formaldehyde, 45% (v/v) absolute ethanol and 5% (v/v) glacial acetic acid for 24 h. The samples were then dehydrated in an ethanol series; 30% for 30 min, 50% for 45 min, 70% for 45 min, 80% for 60 min and 100% for 120 min. The specimens were then embedded in basic resin Technovit 7100 (Kulzer, Germany) and cut into 3.5 µm thick sections. Fine sections were double stained with Naphthol Blue Black (Sigma, USA) [1 g Naphthol Blue Black in 100 ml 7% (v/v) acetic acid] and periodic acid [1% (w/v) (Fisher, 1968).

Identification of zerumbone

The mixture was filtered through a 0.45µm PTFE filter (Sartorius 13CR, USA), whereas liquid medium from AdR suspension cultures was filtered using Whatman filter paper No.1. An injection volume of 20 µl was applied for each sample and the eluent was monitored at 254 nm in a high-performance liquid chromatography (HPLC) system (Waters, USA) equipped with a W600E multisolvent delivery system, W2489UV/Visible detector, in-line de-gasser, guard and reverse columns (Chromolith RP-18 encapped, 100-4.6mm, Merck USA), and W2707 auto sampler controlled by Empower2 software. The solvent for elution was 0.1% (v/v) phosphoric acid (A) and acetonitrile (B). The guard column and column were flushed with pure acetonitrile before and after use. The ZER compound was identified by matching their retention times (10.6-10.8) to commercially available standard, ZER (Sigma, USA).

Elicitation of AdR culture by using methyl jasmonate (MeJA)

Based on optimised parameters, adventitious roots through direct regeneration (AdRd) were selected for elicitation using 800 µM MeJA after 15 days incubation. For control, an equivalent volume of sterilized distilled water was added to the cultures. The crude extracts of AdRd culture of *Z. zerumbet* roots without elicitation (control) and treated (elicited) were subjected to HPLC for compound identification followed by quantification using standard calibration curve using zerumbone (Sigma) as standard.

Statistical analysis

All data were analyzed by one way ANOVA and Duncan test at a significance level of $p < 0.05$.

Results

Initiation of AdRD and AdRId organogenesis

For the indirect organogenesis, we first induced callus from shoot buds before transferring to AdR initiation medium. Callus was formed after two weeks of initiation (Fig. I. A) and multiplied into friable callus within 1-2 months of culture (Fig. I. B). Short and tiny roots were generated from the friable callus after two weeks (Fig. I. C), whereas shoot bud explant for direct organogenesis started to produce roots after one week of culture (Figs. II. A and B). Subsequently, it multiplied to form masses of roots after two months (Fig. II. C).

Histological examination was performed on roots generated from both methods to support the different growth rate between AdRId and AdRD. Results showed that AdRId had less meristematic cells and lack of vascular bundle (Fig. I. D) in comparison with AdRD. The apical and elongation regions in AdRD contained dense cells and defined vascular bundle (Fig. II. D) showing resemblance to *in vivo* roots. Nonetheless, both types of roots possessed root cap with similar root anatomy.

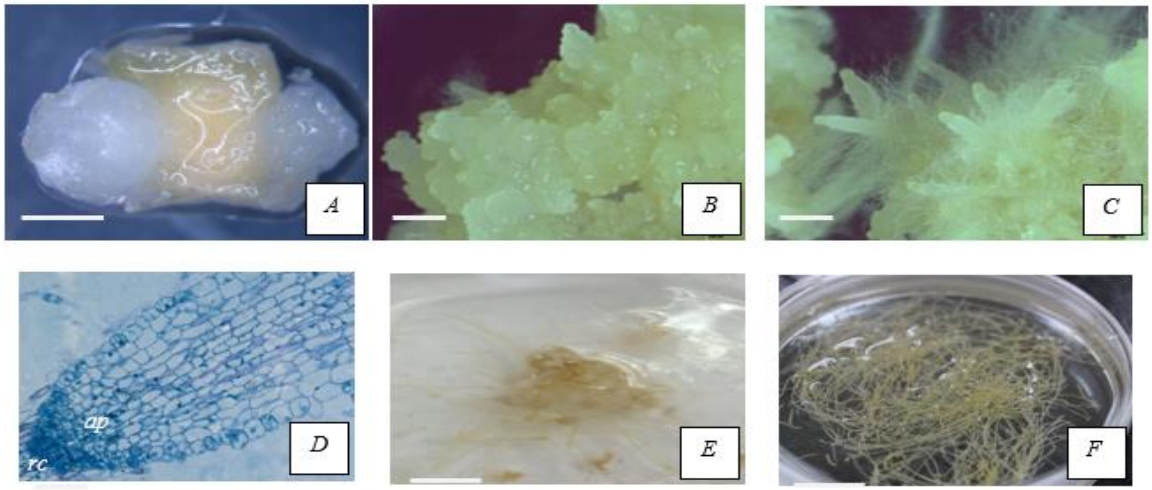

Fig. I. Initiation of adventitious root from indirect organogenesis (AdRId); *A*: callus initiated from shoot bud slice explant, *Bar: 1 mm*; *B*: callus multiplication, *Bar: 1 mm*; *C*: initiation of adventitious root (AdRId), *Bar: 2 cm*; *D*: histological examination of AdRId, *Bar: 100 µm*; *E*: initiation of AdRId suspension culture, *Bar: 2 cm*; *F*: multiplication of AdRId. *Bar: 2cm*. *rc*: root cap; *ap*: apical meristem.

Influence of different auxins on AdR induction

The AdRD and AdRId were transferred into culture medium containing different type and concentration of auxins to study their effects on AdR development. The highest frequency (100%) of AdR formation was observed in AdRId on explants cultured in MS medium supplemented with either 1 or 2 mg/l IBA or 3 mg/l NAA (Fig. III. A1). Only 88% of explants produced AdRD when cultured in 2 mg/l IBA (Fig. III. B1). High (5-9 mg/l) and low (0.5 mg/l) concentrations of IBA and NAA did not increase the AdR formation for both direct and indirect organogenesis. In this work, IBA was found to be more effective than NAA for AdR initiation.

Although IBA produced higher rooting response, the number of roots per explant was generally lower than NAA in both AdRD and AdRId. In AdRId, the highest number of AdR per explant (19.0) was achieved in medium supplemented with 3 mg/l NAA, whereas only 7.7 AdR per explant was recorded in medium containing 1 mg/l IBA (Fig. III. A2). Similar observation was found in AdRD, in which the highest number of AdR per explant in medium supplemented with 5 mg/l NAA or IBA was 16.6 and 11.0, respectively (Fig. III. B2). Low (0.5-2 mg/l) or high concentration (> 5 mg/l) of

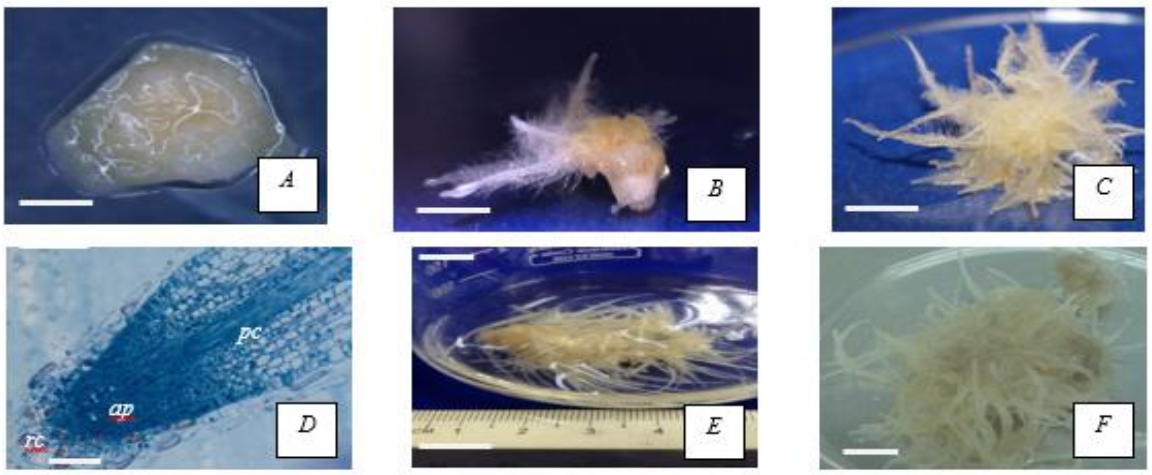

Fig.II. Initiation of adventitious root from direct organogenesis (AdRD); A: shoot bud slice explant. Bar: 1mm; B : initiation of AdRD. Bar : 1 cm; C : elongation of AdRD. Bar: 2 cm; D : histology of AdRD. Bar: 100 µm; E : AdRD suspension culture. Bar: 2 cm;

auxin produced a smaller number of AdR. In this study, we observed that longer roots were formed in IBA than NAA treatment, especially in AdRId. Maximum root length in AdRId and AdRD was recorded in medium containing 5 mg/l IBA (Figs. III. A3 and III. B3).

Multiplication of AdR and ZER production

culture medium to determine their biomass and ZER production. We found that the growth of AdR suspension culture was influenced by initial inoculum. AdRId produced the highest biomass of 39.2 g FW (4.4 g DW) after two months of culture in MS medium supplemented with 3 mg/l NAA under 16:8 h/day light regime (Fig. VI. A1). However, the highest ZER production (713 µg/g

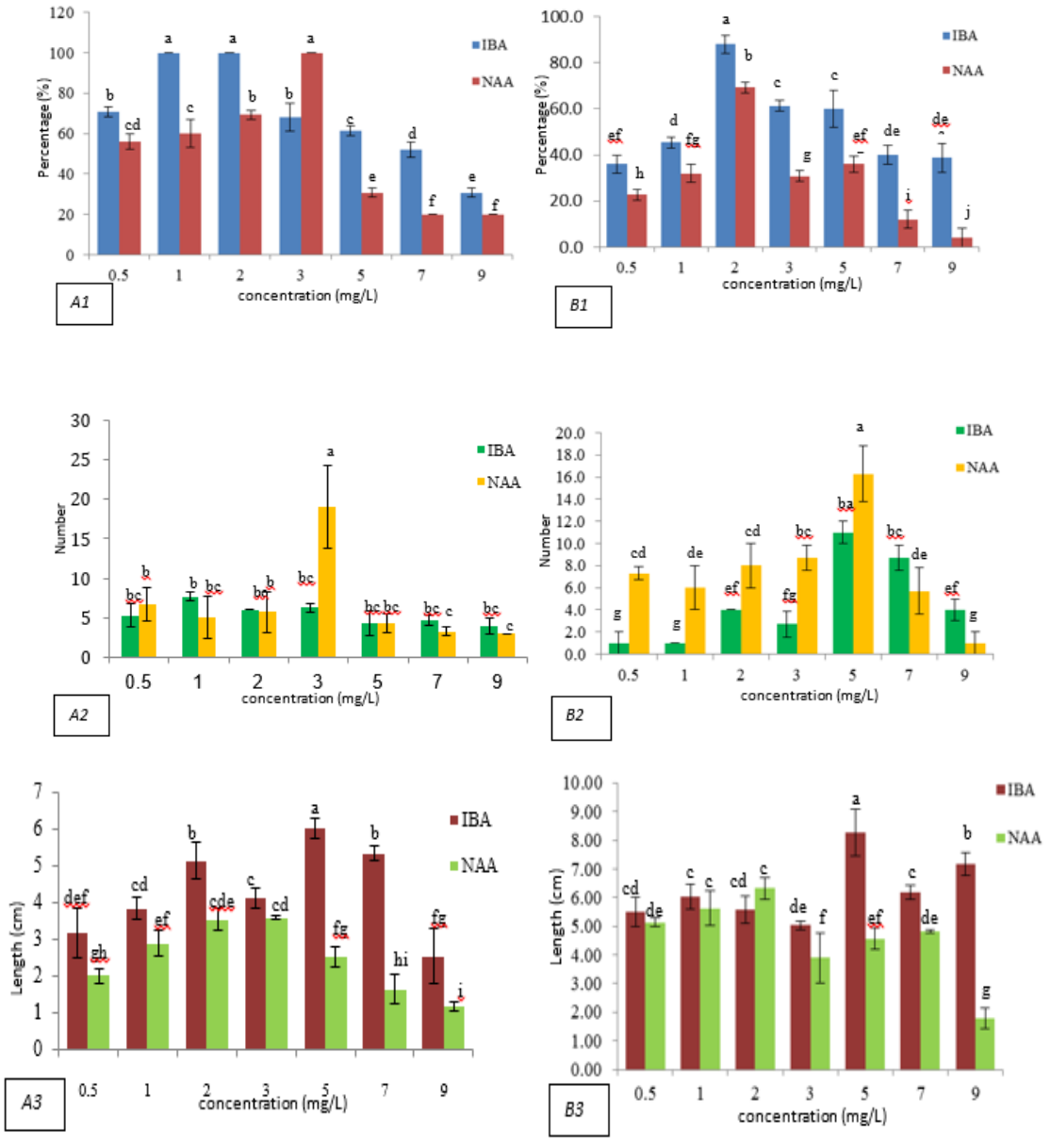

Fig.III. Percentage, number and length of AdR initiated through A: direct and B: indirect organogenesis in different concentrations of auxin. A1 & B1: Percentage; A2 & B2: Number; A3 & B3: Length

AdRs obtained from both direct and indirect organogenesis were further optimized in liquid

DW) was achieved in MS medium containing 7 mg/l IBA in the dark condition (Fig. IV. B1). In

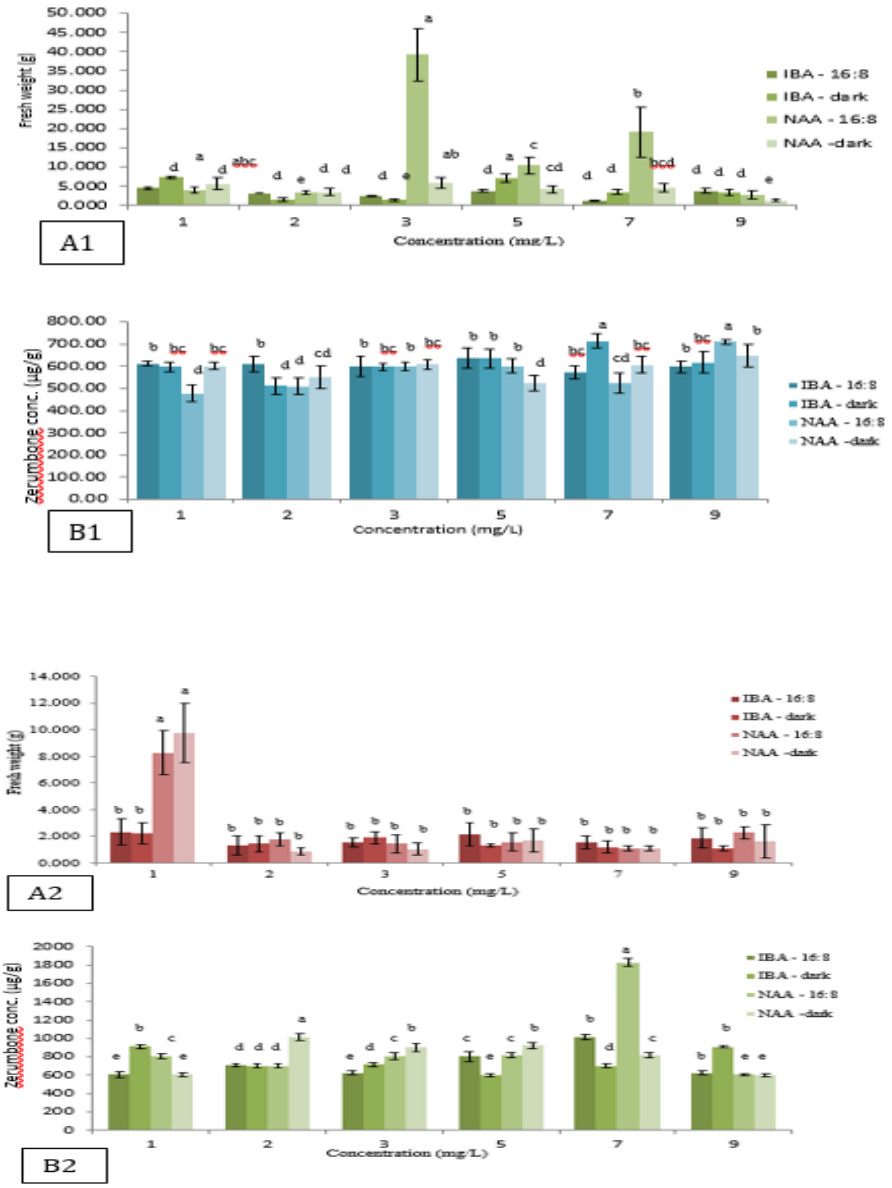

Fig.IV. Biomass of AdR suspension culture and zerumbone production from AdRD in different concentrations of auxins and light regime. A1: Fresh biomass of AdR suspension culture initiated through AdRId; B1: Zerumbone production from AdRId suspension culture; A2: Fresh biomass of AdR suspension culture initiated through AdRD; B2: Zerumbone production from AdRD suspension culture

comparison, lower biomass of 9.7 g FW (1.1 g DW) was recorded in AdRD when cultured in MS medium supplemented with 1 mg/l NAA (dark condition) after two months of culture (Fig. IV. A2). Similar to AdRId, ZER was highest (1,826 µg/g) in medium supplemented with 7 mg/l NAA when cultured under 16 h/d light regime (Figure IV.B2). Our results indicated that high concentration of auxin (7 mg/l) increased the ZER production but negatively affected the biomass accumulation, whereas lower concentrations of auxins promoted biomass accumulation. Taken together, MS medium supplemented with 1 mg/l NAA was selected for further optimization for AdRD because the average ZER production and high biomass accumulation was eight-fold compared to 7 mg/l NAA. Higher ZER production could be achieved by using other strategies such as elicitation.

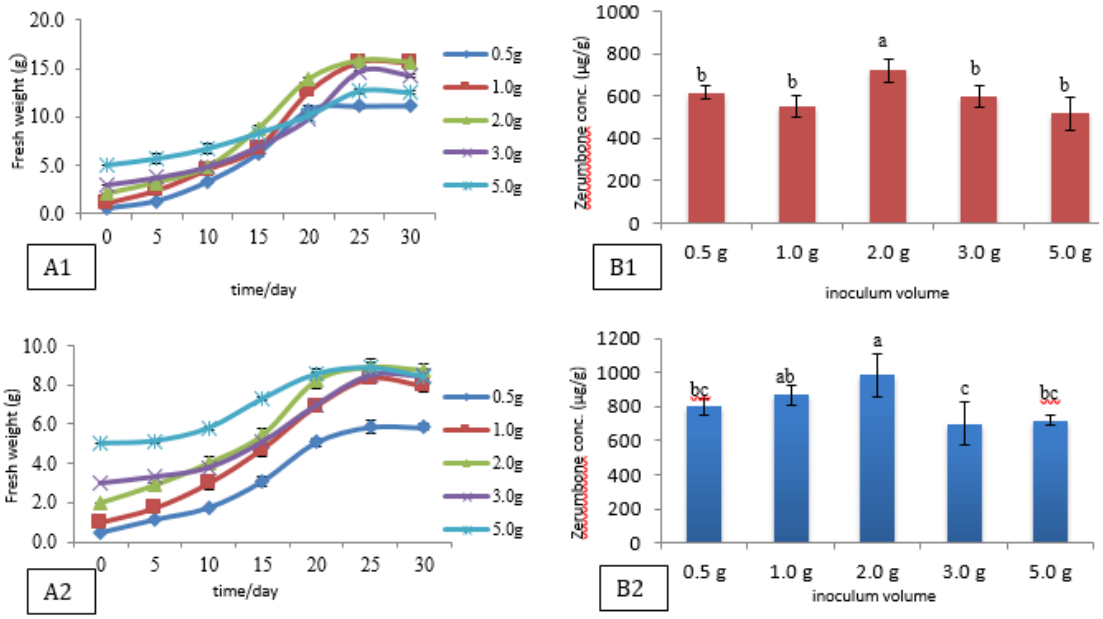

Fig.V. Growth rate and zerumbone compound production in of AdR in different inoculum volumes. A1: Growth rate of AdRId B1: Zerumbone compound production in of AdRId A2: Growth rate of AdRD B2: Zerumbone compound production in of AdRD

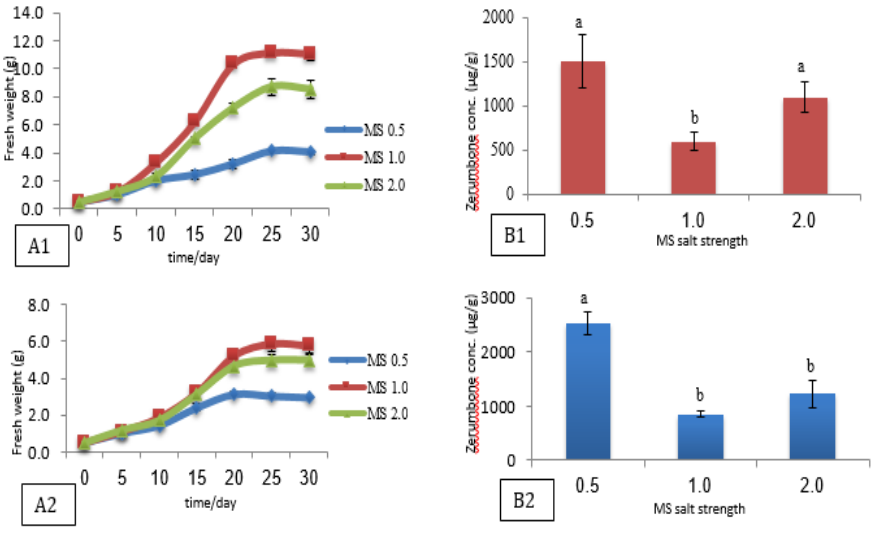

Fig.VI. Growth rate and zerumbone compound production in of AdR in salt strengths. *A1*: Growth rate of AdRId *B1*: Zerumbone compound production in of AdRId *A2*: Growth rate of AdRD *B2*: Zerumbone compound production in of AdRD

Initial inoculum density in biomass accumulation and ZER production

The established AdR suspension culture was further optimized to determine the suitable initial inoculum density. Our results showed that the growth of AdR suspension culture initiated from

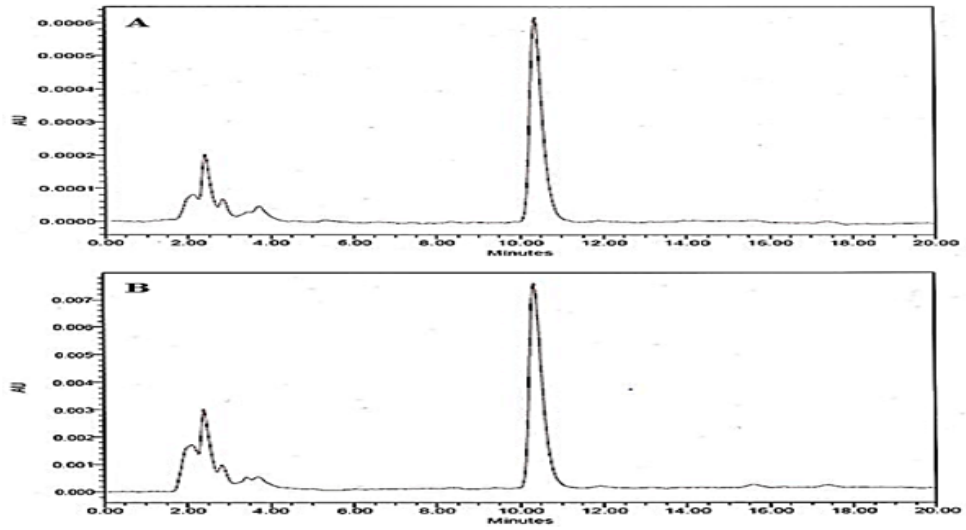

Fig.VII. Adventitious root cultures of Zingiber zerumbet were extracted and analyzed to produce zerumbone; retention time: 10.5 minutes; A: control, B: elicited roots.

AdRId was superior to AdRD suspension cultures. The maximum specific growth rate ($10.8 \times 10^{-2}\mu$) was achieved at 0.5 g of initial inoculum density in AdRId, whereas lower specific growth rate of $8.4 \times 10^{-2}\mu$ was observed at 1 g of initial inoculum density (Figs. V. & A1 V. A1). As for the bioactive compound, the initial inoculum of 2.0 g produced 720 and 983 μg/g ZER in AdRId and AdRD, respectively (Figs. V. B1 & V. B2).

Effects of medium strength on biomass accumulation and ZER production

In this study, we tested half-, full-, and double medium strengths in producing root biomass and ZER. We found that full strength MS medium favored the biomass accumulation in both AdRId and AdRD, while double salts strength media showed contrary root growth (Figs. V1.A1 & V1. A2).

In contrast to the biomass, ZER production was highest in half strength medium for both AdRId and AdRD with a mean amount of 1,500 μg/g and 2,520 μg/g, respectively (Figs. V1.B1 & VI. B2).

Elicitation of AdRs by using elicitor

Adventitious root culture in optimized root growth media was elicited using methyl jasmonate (MeJA). HPLC analysis showed that ZER concentration was elevated ten times (9500 μg/g DW) in comparison to control or non-treated roots (800 μg/g DW) (Fig. VII).

Discussion

Different growth rates were observed between AdRId and AdRD in this study, as short and tiny roots in AdRD and AdRId were generated after one and two weeks of culture, respectively. Similar observation was reported in *Gynura procumbens* and *Castilleja tenuiflora* Benth (Saiman et al., 2012; Sivanesen and Jeong, 2009).

Histological examination of the roots showed that both types of roots possessed root cap with similar root anatomy. On the other hand, AdRId had less meristematic cells and no vascular bundle in comparison with AdRD in which the apical and elongation regions contained dense cells and defined vascular bundle. The observed different growth rates may be attributed to the idea that the typical root structure in AdRD could absorb more nutrient in the culture media than AdRId which accounted for better growth.

Supplementing MS medium with IBA was found to be more effective than NAA for AdR initiation. This was in accordance with the finding of Baque et al. (2010) in which the culture medium containing IBA showed superior effect than NAA in inducing AdR from leaf segments of *Morinda citrifolia*. Similar results have been reported in *Podophyllum peltatum*, *Echinacea angustifolia*, and *Raphanus sativus* (cv. Peking Koushin) (Anbazhagan et al., 2008; Wu et al., 2006; Betsui et al., 2004). In contrast, NAA was found to be in favor of AdR induction in *Andrographis paniculata* (Praveen et al., 2009), *Castilleja tenuiflora* Benth (Gómez-Aguirre et al., 2012) and *Gynura procumbens* (Saiman et al., 2012). Zhang et al. (2013) reported a synergistic and promotive effect of NAA and IBA in enhancing the regeneration of AdR in *Psammosilene tunicoides*.

The growth of AdR suspension culture initiated from AdRId was found to be more than that of AdRD suspension cultures. As for the effect of initial inoculum density on biomass accumulation and ZER production of *Zingiber zerumbet* Smith, higher inoculum density (>2 g) resulted in lower growth ratio of AdR, probably due to the competition of nutrients. Initial inoculum density is one of the important parameters that can influence the cell growth. Cui et al. (2010) reported that the root biomass of *Hypericum perforatum* was increased with elevated inoculum densities, but the growth ratio greatly decreased. The authors demonstrated that inoculum size at 8 and 10 g/l FW produced high number of lateral roots but reduced the growth ratio to 31.8 and 44%, respectively (Cui et al., 2010). This might be limited by several factors, such as reduction of nutrients and oxygen in the culture medium (Zhang et al., 2013).

Initial inoculum density is also important in the production of plants' secondary metabolites (Chin et al., 2014). The initial inoculum of 2.0 g in the present study produced 720 and 983 µg/g ZER in AdRId and AdRD, respectively. Moderate initial inoculum density has been shown to produce higher secondary metabolites accumulation than low or high initial inoculum density (Zhang et al., 2002). In medicinal ginger, low initial inoculation volume resulted in low flavonoid yield, probably due to insufficient critical mass of surviving cells in the culture medium (Yusuf et al., 2013).

The composition of the medium is not only crucial for cell growth, but also to ensure the maximum production of secondary metabolites (Ramachandra and Ravishankar, 2002). In this study, we tested half-, full-, and double medium strengths in producing root biomass and ZER. Full strength MS medium in this study was found to better support the biomass accumulation in both AdRId and AdRD. This agreed with the findings of the study carried out by Nagella and Murthy (2010), in which cell suspension cultures of *Withania somnifera* produced maximal biomass cultured in full strength MS medium. However, in this work double salts strength media showed contrary root growth. This could be due to high nutrient concentration which might cause an osmotic stress to the cell cultures and thus affect the nutrient uptake (Ata el al., 2015). Similar observation was found to inhibit root growth of *Hyperium perforatum* (Cui et al., 2010). Whereas half strength MS salts and vitamins provide inadequate nutrients causing least root growth than the others. Cui et al. (2010) reported that roots cultured in ¼ MS were aging rapidly and did not achieve a desired amount of biomass.

Higher ZER production was recorded in half strength medium for both AdRId and AdRD organogenesis. Several studies reported similar results on the metabolite production in numerous plant species, including *Dature metel* (Cusido et al., 1999), *Podophyllum peltatum* (Anbazhagan et al., 2008) and *Eleutherococcus koreanum* Nakai (Lee and Paek, 2012). Lee and Paek (2012) found that the production of targeted compounds from *Eleutherococcus koreanum* Nakai AdR achieved their maximum production in a bioreactor at half strength MS medium. Single and double strength MS, however, decreased the production of their targeted compounds suggesting that the medium salt strength influenced the metabolite production.

Finally, supplementation of the growth media with methyl jasmonate highly increased ZER concentration in this study. This is in line with Chodisetti et al. (2015) who showed MeJA yielded the maximum gymnemic acid content. The

effectiveness of using elicitors in enhancing the bioactive compounds was also reported by Kannan et al. (2020) on anthraquinone and phenolic compound enhancement in AdR of *Morinda coreia* Buck. This suggests that the enhancement of secondary metabolites could be achieved by using elicitor. Thus, MeJA is the potential elicitor for scaling up ZER production in future studies.

Conclusions

No correlation was found between growth of roots and zerumbone production in the adventitious root cultures of *Zingiber zerumbet* in the optimized media. The use of optimized media for adventitious root cultures that supported growth is recommended since the zerumbone production was shown to increase with elicitation.

Acknowledgments

The authors would like to acknowledge the University of Malaya for the UMRG Grant RP003/2012A, and Ministry of Education, Malaysia, for the financial support.

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