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# Sterilisation Protocols and *in vitro* Shoot and Root Induction of *Zingiber officinale* var. Rosc. using 6-Benzylaminopurine (BAP) and Naphthalene Acetic Acid (NAA)

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## *ABSTRACT*

Bentong ginger is the most well-known variety of *Zingiber officinale* in Malaysia, owing to its extensive use in traditional medicine and as a culinary spice. It is vegetatively propagated, and large quantities of rhizomes are required as initial planting materials. In addition, a variety of different soil-borne diseases are associated with the vegetative propagation of ginger using its rhizomes; hence, producing them on a massive scale requires a lot of time. Plant tissue culture techniques have utilised many plant species to produce disease-free planting materials. Therefore, the study aimed to determine the effects of ethanol on the surface sterilisation of shoot bud explants as well as the optimisation of 6-Benzylaminopurine (BAP) and Naphthalene acetic acid (NAA) on shoot and root propagation. The shoot bud explant was successfully sterilised using 20% (v/v) of Clorox and Tween20 for 30 minutes and 95% (v/v) of ethanol for 1 minute, resulting in a 58% contamination-free explant. The highest number of shoots and roots were initiated from shoot bud explants of *Z. officinale* on MS solid medium supplemented with 4.0 mg/l BAP and 1.5 mg/L NAA, respectively. MS medium incorporated with a combination of 3.0 mg/l BAP and 1.5 mg/l NAA produced the highest length of shoot. These results suggested that the protocols used can be adopted for large-scale propagation of disease-free planting materials of *Zingiber officinale* var Rosc.

**Keywords:** Micropropagation, tissue culture, halia bentong, *Zingiber officinale*, Zingiberaceae

## INTRODUCTION

*Zingiber officinale* Rosc., also known as Halia Bentong among the locals, is a member of the *Zingiberaceae* family. It is widely used in the production of spices and medicines. Bentong ginger has been shown to be beneficial in the treatment of cancer (Zhang et al., 2021), diabetes (Said et al., 2020), asthma (Choi et al., 2018), and cardiovascular diseases (Ghafoor et al., 2020). This plant's polyphenol and flavonoid content have been identified as an important factor in its pharmacological effects (Ghasemzadeh et al., 2010).

Ginger is propagated vegetatively, and conventional ginger reproduction by mature rhizome segments resulted in low multiplication rates as well as virus accumulation in the germplasm (Legg et al., 2015; Abbas et al., 2018). The *in vitro* culture technique enables the rapid micropropagation of virus-free ginger plants (Sharma and Singh, 1997). Various *in vitro* ginger shoot regeneration had been demonstrated using a variety of plant growth regulators (PGRs) such as 6-Benzylaminopurine (BAP), Naphthalene Acetic Acid (NAA), and Thidiazuron (TDZ) (Kambaska and Santilata, 2009; Abbas et al., 2011; David et al., 2016; Wang et al., 2021; Zahid et al., 2021; Zhou et al., 2022).

The aseptic culture establishment, shoot regeneration capacity, rooting, and acclimatisation are all critical to the success of the *in vitro* technique. Shoot buds, which are frequently used as explant sources in *Zingiberaceae*, have been shown to be more responsive. However, the initial establishment of a contamination-free culture is difficult due to rhizome exposure to various soil pathogens (Thakur et al., 2018). Surface sterilisation of the explants is required to eliminate these pathogens. To control contamination by surface sterilisation of the explant, the disinfectants' type, concentration, and exposure period must be carefully determined to prevent noxious impacts on plant tissues.

Shoot regeneration capacity, on the other hand, is determined by culture media formulation and plant growth regulators, primarily cytokinins. Murashige and Skoog (MS) medium (Murashige and Skoog., 1962) supplemented with BAP is more commonly used for ginger shoot multiplication (Zuraida et al., 2016; Mehaboob et al., 2019). Since BAP is actively involved in natural organogenesis when added to *in-vitro* propagation conditions, its application at the proper concentration can effectively promote shoot multiplication (Zainal et al., 2023). Furthermore, a developed root system is an important phase in plant vegetative propagation, which guarantees an excellent survival rate of micropropagated plantlets during the acclimatisation stage (Miri, 2020). Auxin supplementation in the culture medium improves ginger root induction *in vitro* (Mehaboob et al., 2019). Different auxin types and concentrations influence *in vitro* root induction in various *Zingiberaceae* species (Jualang et al., 2015).

Further improvements in *in vitro* shoot regeneration would facilitate wider applications of biotechnology in ginger. Since studies on *in vitro* shoot regeneration of Bentong ginger were quite limited; therefore, development in *in vitro* shoot regeneration protocols for Bentong ginger variety were of significance. The aim of this study was to determine the duration of ethanol exposure for explant surface sterilisation of Bentong ginger. This study also aimed to determine the best concentration of BAP and NAA for shoot and root induction of Bentong ginger.

## MATERIALS AND METHODS

### Plant material and location of study

This research was carried out at the Tissue Culture Laboratory, Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA (UiTM), Cawangan Melaka, Kampus Jasin, Melaka, Malaysia. The plant materials

used in this study were purchased from a local farmer in Bentong, Pahang. Two-week-old sprouted shoot buds from the rhizomes of *Z. officinale* Rosc. were used as explants.

**Establishment of aseptic explants**

The buds were collected and immersed in 20% (v/v) Clorox and Tween20 for 30 minutes in a laminar airflow (Yusuf et al., 2007). Then, the shoot buds were soaked in 95% (v/v) ethanol for 1 minute and 10 minutes to determine the appropriate exposure time to reduce the contamination rate. After the surface sterilisation process, the buds were rinsed three times in sterile distilled water. The external leaves of the shoot buds were then removed and trimmed until they were between 1 and 3 cm in size. They were then inoculated onto solid MS medium for shoot and root induction for four weeks. All of these buds were used as explants, and the procedures outlined above were carried out within the laminar airflow.

**Preparation of shoot and root induction medium**

The solid MS media containing 4.4 g/l MS powder, 30 g/l sucrose, 2.5 gelrite, and different concentrations of NAA (1.0 to 2.0 mg/l) and/or BAP (2.0 to 4.0 mg/l) were used (Table 2). MS medium without PGRs was used as a control treatment. The pH of the solution medium was adjusted to 5.8 by using the pH metre before autoclaved at 121°C for 21 minutes. Next, under aseptic conditions, the autoclaved media was poured into the vials and labelled accordingly. The aseptic explants were inoculated on the treatment media for shoot and root multiplication for four weeks. Each treatment was replicated three times. The induction cultures were maintained in the culture room with a 16/8 h photoperiod (light/dark) at 25 ± 2°C (Yusuf et al., 2011).

**Scoring and analysis of data**

The cultures were observed weekly for four weeks. The effects of different concentrations and combinations of hormones were observed and recorded for the number of shoots, number of roots, and length of the shoot. The data for each parameter was analysed using a one-way ANOVA followed by a mean comparison with Duncan at $p \leq 0.05$ using SPSS version 27.

## RESULTS AND DISCUSSION

**Establishment of aseptic explant**

Increasing the exposure time of 95% (v/v) ethanol from 1 to 10 minutes decreased the production percentage of aseptic cultures. The long exposure (10 minutes) to ethanol was not effective in eliminating the contamination and resulted in 14% aseptic cultures. On the other hand, the exposure of 1 minute to ethanol reduced contamination and resulted in 58% aseptic cultures (Table 1). The higher concentration of alcohol, which was more than 95%, killed the explants if they were immersed for a long time. According to previous studies, low concentrations of ethanol in the range of 70% to 80% were actually preferable for disinfection as it contained more water, which made it easier to permeate cells, dissolve more gradually, and kill bacteria (Swinder, 2022). Furthermore, alcohols were reported to become more quickly in bactericidal than bacteriostatic when it comes to vegetative forms of bacteria, but they do not eliminate bacterial spores (Gross, 1987). Besides, only 2% of the browning observed in this study might be related to the long exposure time to ethanol.

**Table 1.** Effects of different time exposures of 95% ethanol as a sterilant agent on explant surface sterilisation

| Time Exposure of 95% Ethanol | Browning (%) | Aseptic Culture (%) |
|---|---|---|
| 1 min | 0 | 58 |
| 10 min | 2 | 14 |

n=50

**Shoot and root induction**

The aseptic shoots of *Z. officinale* cultured on MS medium supplemented with different concentrations of BAP (2.0 - 4.0) mg/l in combination with NAA (1.0 - 2.0) mg/l proceeded with their growth and produced shoots and roots simultaneously (Table 2 and Figure 1). Despite the use of various explants, shoot buds have been chosen in this study, and it was found to be a very efficient material in Curcuma micropropagation (Brijesh and Ajjappala, 2023). Several plant regeneration studies were successfully attempted in Curcuma and Boesenbergia species, which belong to the Zingiberaceae family (Yusuf et al., 2007; Yusuf et al., 2011; Jena et al., 2020; Gurav et al., 2020; Shantala et al., 2020). These results indicated that, irrespective of the species studied, various explants cultured in MS medium supplemented with BAP and with or without other phytohormones or vitamins favoured callus induction and multiple shoot production.

**Table 2.** Effects of different concentrations and combinations of NAA and BAP on the shoot and root multiplication of shoot buds of *Zingiber officinale* Rosc.

| BAP (mg/l) | NAA (mg/l) | Means Number of Shoot | Means Height of Shoot (cm) | Means Number of Root |
|---|---|---|---|---|
| 0 | 0 | $1.33 \pm 0.33^{bc}$ | $3.93 \pm 1.95^{a}$ | $7.67 \pm 2.40^{b}$ |
| 0 | 1.0 | $1.00 \pm 0.00^{c}$ | $0.87 \pm 0.32^{a}$ | $2.67 \pm 1.20^{cd}$ |
| 0 | 1.5 | $3.33 \pm 1.45^{abc}$ | $1.30 \pm 0.31^{a}$ | $17.33 \pm 0.67^{a}$ |
| 0 | 2.0 | $1.00 \pm 0.00^{c}$ | $2.80 \pm 2.10^{a}$ | $3.67 \pm 1.45^{bcd}$ |
| 2.0 | 0 | $3.67 \pm 0.33^{abc}$ | $1.45 \pm 0.41^{a}$ | $6.00 \pm 1.00^{bcd}$ |
| 2.0 | 1.5 | $3.00 \pm 1.00^{abc}$ | $2.00 \pm 0.76^{a}$ | $4.67 \pm 0.33^{bcd}$ |
| 3.0 | 0 | $4.00 \pm 1.53^{ab}$ | $1.87 \pm 1.27^{a}$ | $2.33 \pm 1.33^{cd}$ |
| 3.0 | 1.5 | $2.67 \pm 0.88^{abc}$ | $4.30 \pm 0.70^{a}$ | $3.33 \pm 1.67^{bcd}$ |
| 4.0 | 0 | $4.33 \pm 0.88^{a}$ | $2.02 \pm 0.64^{a}$ | $1.67 \pm 1.20^{d}$ |
| 4.0 | 1.5 | $2.00 \pm 0.58^{abc}$ | $0.73 \pm 0.31^{a}$ | $6.67 \pm 0.88^{bc}$ |

n=3

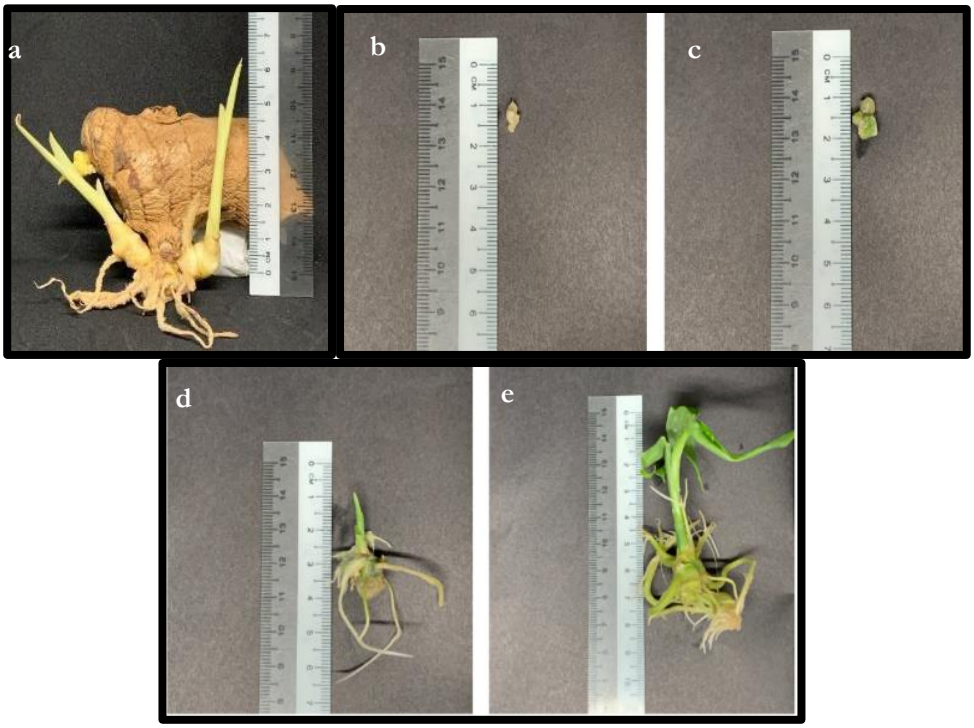



**Fig. 1.** *In vitro* shoot induction of *Zingiber officinale* var. Rosc. from shoot bud explants. (a) The shoot buds germinated from rhizomes were used as explants (2-5 cm); (b) the explant growth after a week of culture induction (1.5 cm); (c) the explant growth after 2 weeks of culture induction (2 cm); (d) the explant growth after 3 weeks of culture induction (4 cm); and (e) the shoot and root growth after 4 weeks of culture induction (7 cm).

**Multiplication of shoots**

The concentration of BAP ranging from 2.0 - 4.0 mg/l BAP alone or in combination with 1.5 mg/l NAA produced multiple shoots (Table 2). The mean number of shoots showed significant differences among treatments where the MS medium supplemented with 4.0 mg/l BAP showed the highest number of shoots at 4.33 ± 0.88. PGRs under the cytokinin family play a role in stimulating the formation of the shoot, promoting cell differentiation that acts in the formation of shoots, and also in the apical dominance (Yuniastuti et al., 2018). The research of Zainal et al. (2023) showed that the higher concentration of BAP produced the highest number of shoots. One of the key success criteria in shoot multiplication is the quantity of shoots; if more shoots form, the multiplication can yield a high number of new shoots. Typically, shoots that grow in the leaf axils produce two shoots; however, due to shoot multiplication, some explants can create additional shoots that emerge from the base of the axillary shoots (Zainal et al., 2023). Similarly, Yusuf et al. (2011) found that when high concentrations of BAP and low amounts of NAA were tested on shoot bud explants, numerous shoots were developed.

The mean value for length of shoots showed no significant differences among treatments. The longest shoot length was observed from a combination between 3.0 mg/l BAP and 1.5 mg/l NAA, which was 4.30 ± 0.70 cm but the shortest shoot was from the combined concentration of hormones of 4.0 mg/l BAP and 1.5 mg/l NAA, which only produced 0.73 ± 0.31 cm. In many aspects of plant development and growth, auxin and cytokinin play rather significant roles, and this is also due to each cell's capacity for receiving a unique PGR response and having an ideal limit. Auxin and cytokinin interactions are crucial for controlling a few developmental processes, such as the growth and maintenance of meristems, which are crucial for establishing

the entire plant body (Su et al., 2011). The regulation of cell size appears to be linked to the regulation of cell proliferation in leaf morphogenesis (Tsukaya, 2003). Leaf growth is a process of differentiation that is promoted in shoot buds with the help of two plant growth regulators, auxin and cytokinin. Early cell division and growth, at the beginning of shoot expansion, are called periclinal divisions. The activation of the cleavage anticlinal will increase leaf surface area (Salisbury and Ross, 1995). Due to the improvement of protein in the leaves, the process of prolonging the leaves takes place. This is because cytokinin inhibits the activity of these enzymes, protein life extends, and the ageing process in leaves is caused by the protease enzyme that converts proteins into amino acids. The function of cytokinin is to prevent protein breakdown and postpone the ageing process of leaves (Santoso and Nursandi, 2002). This concept is supported by the fact that external cytokinin signal input which is caused by cytokinin signalling, aids in shoot regeneration.

Other than that, the auxin receptor TIR1 and the response gene ARF3 are also necessary for the development of de novo shoots (Cheng et al., 2013; Qiao et al., 2012). This shows that endogenous auxin signalling may also play a role in the effects of exogenous cytokinin on shoot induction, at least to some extent (Ying and Xian, 2014). Hence, the results in Table 2 showed that even though the hormone was used for the development of the shoot or root, it could still develop both of these plant parts because it was a self-produced hormone to stimulate the shoot and root.

**Multiplication of Root**

Plant growth also depends significantly on their roots. The Bentong ginger has a well-positioned growth medium, and it has a long root system. The root cell developmental phases and distance from the apical meristem are approximately associated (Birnbaum et al., 2003). The presence of auxin in the media speeds up the process of root elongation. Cells that are generated while the embryo is still growing in the mother plant cause elongation (Salisbury and Ross, 1995). Therefore, based on the results in Table 2, the concentration that had the highest number of roots was NAA at 1.5 mg/l, which was 17.33 ± 0.67. The concentration of hormone that produced the lower number of roots was 4.0 mg/l of BAP of 1.67 ± 1.20 roots after 4 weeks.

The development of pre-existing roots, lateral root initiation or root branching, and adventitious root production are all regulated by auxins (Singh, 2021). Furthermore, producing the adventitious rooting system takes a lot of energy and a larger food supply for root initiation. This system uses cell division, in which pre-set cells undergo morphogenetic pathways to become mother cells for the root primordia (Aeschbacher et al., 1994). Auxins such as NAA hormone, in addition to reserve food sources, are crucial for adventitious roots, plant growth, and morphological variation (Eun et al., 2006). The added auxin boosts endogenous auxin levels and builds up in the cuttings' basal region, where it functions as a metabolising agent to trigger the rooting signal (Husen, 2008).

## CONCLUSION

The study revealed that shoot bud explants of Bentong ginger were successfully established through *in vitro* culture after surface sterilization in 20% (v/v) of Clorox and Tween20 for 30 minutes in a laminar airflow, followed by 1 minute exposure in 95% (v/v) ethanol, resulting in 58% aseptic culture. The concentration of BAP optimised shoot multiplication of Bentong ginger, with 4.0 mg/l BAP alone producing a 4.33 ± 0.88 mean number of shoots per explant, while 1.5mg/l NAA significantly produced the highest number of roots at 17.33 ± 0.67. This demonstrated that MS medium can produce shoots and roots and has a positive effect on shoot height when combined with the growth regulator BAP alone or in combination with NAA. The BAP growth regulator is one of the cytokinin groups that can stimulate and induce shoots, whereas NAA is the auxin group to promote the root formation. However, the sort and concentration of these plant growth regulators needed to increase the shoot and root formation vary depending on the type of plants. These findings indicated that

the methods might be applied to the large-scale propagation of *Zingiber officinale* var. Rosc disease-free planting materials.


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