RESEARCH ARTICLE

IN VITRO SHOOT PROLIFERATION OF IRONWOOD (*EUSIDEROXYLON ZWAGERI* TEIJSM. & BINNED)

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Received-08.01.2022, Revised-22.01.2022, Accepted-29.01.2022

Abstract: The over exploitation of natural resource such as tropical forest for deforestation and urbanization have threatened the population of endangered tropical timber tree species such as *Eusideroxylon zwageri*. The present study reported the results of a number of experiments aimed at optimizing shoot proliferation protocol for the mass propagation of *E. zwageri*. The effect of different combinations and concentrations of plant growth regulators (PGR) such as BAP, IBA and NAA on the shoot proliferation and elongation were evaluated. The findings showed that the best treatment for nodal segment multiplication in terms of the number of shoots and leaves per explant were obtained in the half strength MS medium supplemented with either 5.0 mg/L BAP alone or 5.0 mg/L BAP in combination with 0.5 mg/L IBA. Healthy shoots treated with 300 mg/L IBA for 20 min followed by transfer to another half MS medium without PGR resulted in 80.3% of shoots producing roots.

Keywords: Conservation, Timber, Borneo Ironwood, In vitro culture, BAP, Shoot elongation

INTRODUCTION

The environmental degradation poses the greatest threat to plant species. The natural forest products including timber are continuously disappeared due to the deforestation and urbanization (Tzafira et al., 2010). The IUCN Red List has classified one of the hardest timber tree species in Southeast Asia known as *Eusideroxylon zwageri* as “Vulnerable” and facing a high threat of extinction in the wild in the medium-term future. Despite renowned as Ironwood due to its durability and rot resistance, this timber species from Lauraceae family is facing a regeneration challenge as its produced recalcitrant seed with strong tegument dormancy which is hard to germinate (Irawan and Gruber, 2004). According to Hidayat (2007), the seed germination is usually taking around 9 to 12 months and it requires around 15 to 20 years for the species to start producing flowers. Another bottleneck arises after seed germination where the seedling of *E. zwageri* is always attack by pathogens and animals.

To date, *E. zwageri* has only been planted in a small scale in Malaysia and Indonesia as the supply of its seeds and seedlings is insufficient (Hidayat, 2007). In accordance to Irawan (2003), this species can be regenerated through sprouting and cutting but the regeneration rate is slow and low. Hence, it is necessary to mass propagate *E. zwageri* to prevent the further extinction of this species. *E. zwageri* requires active conservation intervention and in vitro culture technique by shoot proliferation could represent a very useful tool for the mass propagation of this species. The conservation of this species is crucial for the ecosystem services as it provides habitat for the pollinator and beneficial species that act as the natural pest control, reduced downstream effect from nutrient run off and improved nutrient availability for the growth and development of other species (Hidayat, 2007).

Thus far, only several in vitro culture studies have been conducted on this timber tree species (Entuni and Edward, 2016) studied that the addition of Auxin such as Indole-3-acetic acid (NAA) and Indole-3-butyric acid (IBA) and Cytokinin such as 6-Benzylaminopurine (BAP) into MS culture medium have promoted shoot bud multiplication in *E. zwageri*. BAP alone at the concentration of 5.0 mg/L or in combination with either 0.5 mg/L NAA or IBA successfully induced the maximum average number of shoots buds and leaves respectively. The addition of low concentration of Auxin to Cytokinin has been reported of promoting optimal shoot proliferation in other woody plant species. In Andaman Redwood (*Pterocarpus marsupium*) (Husain et al., 2004), the multiple shoot proliferation was obtained in MS medium fortified with higher concentration of BAP at 4.0 mg/L to IAA at 0.5 mg/L. Pandey et al. (2016) reported that shoot multiplication of Arjuna tree (*Terminalia arjuna*) was achieved on MS medium with 4.0 mg/L BAP and 0.5 mg/L NAA. In tea tree oil (*Melaleuca alternifolia*), BAP alone at concentration of 5.0 and 10.0 mg/L of BAP was added to MS medium the optimal induction of multiple shooting (Yohana et al., 2010). Besides, in other woody trees such as Indian rosewood (*Dalbergia sissoo*) (Gulaiti and Jawal, 1996), Jojoba (*Simmondsia chinensis*) (Agrawal et al., 2002) and Senna (*Casssia angustifolia*) (Agrawal and Sadar, 2003).

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After its proliferation, shoot elongation is necessary prior to rooting in *E. zwageri*. The shoot multiplication and elongation processes in tissue culture are usually conducted separately. However, to reduce the culture time and cost for plantlet production, the processes were combined using the optimal combination and concentration of PGRs such as Cytokinin and Gibberellic acid in this study. Thus, this study was conducted with the aim to develop a rapid and efficient protocol using nodal segment for Ironwood.

MATERIALS AND METHODS

Materials

The nodal segments for culture establishment were collected from three years old seedlings of *E. zwageri* originated from the primary forest at Lubok Antu, Sarawak, Malaysia located at 1°02'25.7"N 111°50'08.4" E.

Preparation of culture media

The shoot proliferation cultures were initiated using half strength MS (Murashige and Skoog, 1965) basal media. The preparation of half strength MS media was according to the formulation. The media was added with 30 g/L Sucrose and solidified with 3.0 g/L Gelrite. The pH of the media was then adjusted to 5.8 with 1 N KOH before autoclaved at 121ºC for 20 minutes. The autoclaved media was then dispensed into glass bottles (65 mm in diameter × 120 mm in height) and supplemented with different concentration of BAP alone or BAP in combination with either IBA and NAA. The half strength MS media without BAP was used as a control.

Surface sterilization

Only the healthy nodes from the lateral branches of *E. zwageri*'sseedlings were selected and used as the explants. The nodal segments were first pre-sterilized under running tap water for one hour and subsequently soaked with 0.1% systemic fungicide known as Benomyl (Benex, Malaysia) for 30 minutes. The nodal segments were further sterilized with 20% Clorox® (Bleach, Malaysia) with addition of three drops of Tween® 20 (Sigma-Aldrich, Inc.) for 5 minutes. After that, the explants were rinsed four times with sterilized distilled water and air dried in the laminar flow before culture process.

Multiple shoot proliferation using nodal segments

The nodal segments of *E. zwageri* were cut into 1.0 to 2.0 cm in size before cultured into the glass bottles. The glass bottles contained half strength MS medium that have been added with BAP alone at concentration of 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg/L, BAP at 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg/L in combination with NAA at 0.5 mg/L and BAP at 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg/L in combination with IBA at 0.5 mg/L. Each treatment consisted of five replicates with three nodal explants per replicates. The cultures were inoculated into glass bottle and incubated in the culture room at temperature of about 25±2°C and kept under white fluorescent tubes providing irradiance of 50 µmol m^2s^{-1} for 16 hours photoperiod. After 4 weeks, the mean number of shoots bud and leaf induced per explant were measured in each treatment. Four sub-culturing were conducted and at the regular intervals of one week.

Rooting

For rooting, the in vitro shoots of 3.0 cm in height were subjected to treatment with IBA solution (0, 100, 300, and 500 mg/L) for 30 minutes before transferred into half-strength basal MS medium without PGR. After 30 days, the percentage of rooted plants were measured in each treatment.

Statistical analysis

The data for the number of shoot buds, number of leaves and rooting percentage were recorded and analysed using one-way analysis of variance (ANOVA). The mean separations were conducted using Tukey Test at $p < 0.05$ to determine the optimal medium for shoot proliferation and rooting. All the statistical data were analyzed with SPSS Version 28.0.1.

RESULTS

Multiple shoot proliferation using nodal segments

The nodal segments began to form shoot buds after three weeks from culture initiation (Figure 1a). The shoot proliferation using single BAP concentration did not showed any significance difference between treatments. All culture medium augmented with different concentrations of BAP were able to proliferate shoot buds but the mean number of shoot buds proliferated and number of leaves produced were different. Based on the findings (Table 1), half strength MS basal medium with BAP alone at concentration of 5.0 mg/L gave the higher mean number of shoot buds (6.80±0.45) and number of leaves (12.60±1.24) productions for shoot bud proliferation of *E. zwageri*.

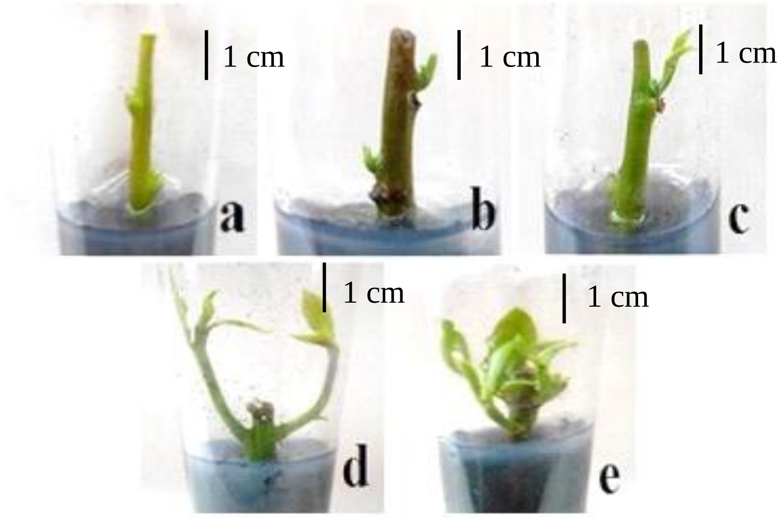

Figure 1. The shoot proliferation progresses from nodal segments of *E.zwageri* in half strength MS basal medium supplemented with 5.0 mg/L BAP alone from (a) shoot initiation to (e) multiple shoot elongations and leaves production.

Table 1. The mean (±SD) number of shoot buds and leaves productions in the different concentrations of BAP at 4 weeks from culture initiation.

Concentrations of PGR	Mean (±SD)	
BAP (mg/L)	**No. of shoot buds**	**No. of leaves**
0	3.00±0[a]	5.00±0[a]
1.0	4.40±0.45[a]	6.50±0.45[a]
2.0	4.40±0.55[a]	6.40±0.55[a]
3.0	4.20±0.45[a]	7.20±1.10[a]
4.0	5.80±0.45[a]	9.40±1.14[a]
5.0	6.80±0.45[a]	12.60±1.24[a]
6.0	4.00±1.00[a]	5.20±1.30[a]

Mean along the column followed by the same alphabet are not significantly different at $p \leq 0.05$ (Tukey Test)

When BAP was combined with IBA, the higher mean number of shoot bud multiplication (4.40±0.45) and leaves productions (10.20±1.40) were observed in a culture medium supplemented with 5.0 mg/L of BAP and 0.5 mg/L of IBA (Table 2).

Table 2. The mean (±SD) number of shoot buds and leaves production in the different concentrations of BAP in combination with 0.5 mg/L of IBA at 4 weeks from culture initiation.

Concentrations of PGR	Mean (±SD)	
BAP + IBA (mg/L)	**No. of shoot buds**	**No. of leaves**
0 + 0	2.00±0.45[a]	4.80±0.45[a]
0.0 + 0.5	3.40±0.55[a]	4.60±0.45[a]
1.0 + 0.5	3.80±0.45[a]	5.80±0.45[a]
2.0 + 0.5	3.80±0.84[a]	6.00±0.45[a]
3.0 + 0.5	3.80±0.45[a]	6.20±0.84[a]
4.0 + 0.5	3.40±0.84[a]	8.40±1.82[a]
5.0 + 0.5	4.40±0.45[a]	10.20±1.40[a]
6.0 + 0.5	3.00±1.00[a]	4.00±0.71[a]

Mean along the column followed by the same alphabet are not significantly different at $p \leq 0.05$ (Tukey Test)

In the culture medium with BAP and NAA, the higher mean number of shoot bud proliferation and leaves production were obtained in a medium supplemented with 5.0 mg/L of BAP and 0.5 mg/L of NAA (Table 3). The mean number of shoot buds obtained was 4.60±0.55 and mean number of leaves was 8.80±1.10.

Table 3. The mean (±SD) number of shoot buds and leaves productions the different concentrations of BAP in combination with 0.5 mg/L of NAA at 4 weeks from culture initiation.

Concentrations of PGR BAP + NAA (mg/L)	Mean (±SD)	
	No. of shoot buds	No. of leaves
0 + 0	2.20±0.45[a]	4.00±0.45[a]
0 + 0.5	2.00±0[a]	4.40±1.14[a]
1.0 + 0.5	2.40±0.55[a]	5.00±0.71[a]
2.0 + 0.5	2.60±0.55[a]	4.80±1.92[a]
3.0 + 0.5	2.20±0.84[a]	7.00±1.00[a]
4.0 + 0.5	3.80±1.10[a]	8.80±1.10[a]
5.0 + 0.5	4.60±0.55[a]	5.80±0.84[a]
6.0 + 0.5	2.40±0.55[a]	3.80±1.10[a]

Mean along the column followed by the same alphabet are not significantly different at $p \leq 0.05$ (Tukey Test)

Rooting

To promote rooting (Figure 2), the healthy in vitro shoots of *E. zwageri* with height averaged 2 to 3 cm were treated with different concentrations of IBA for 30 minutes before transferring to half strength MS basal medium without PGR. The in vitro shoots of *E. zwageri* that have been treated with IBA at concentration of 300 mg/L recorded the highest mean rooting percentage (80.3%) compared with those treated with 0, 100 and 500 mg/L IBA (Figure 3).

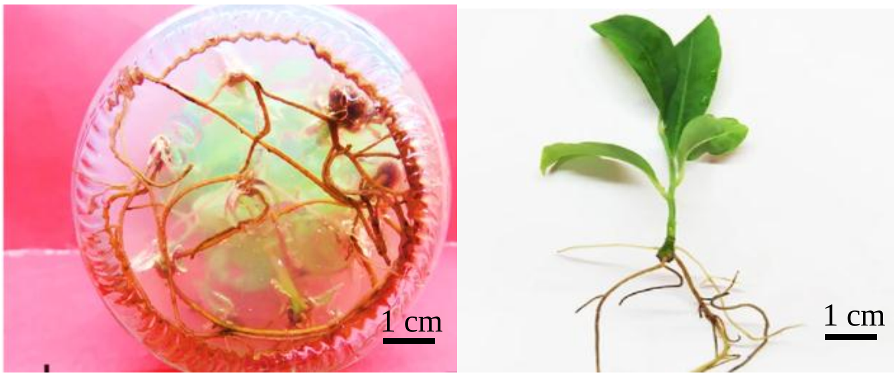

Figure 2. The regenerated plantlets of *E. zwageri* with the well- developed roots.

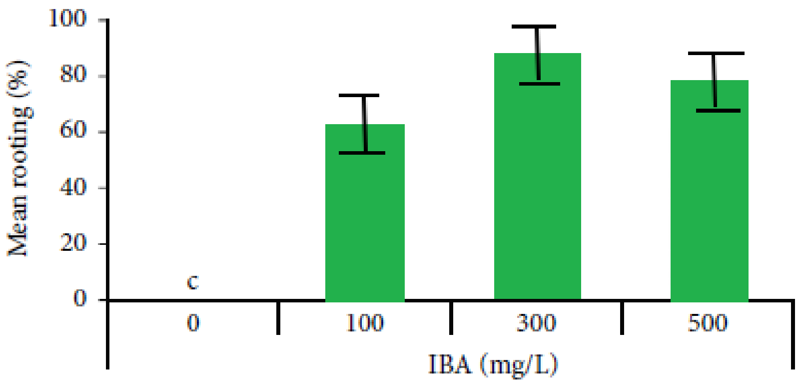

Figure 3. The effect of dipping in different concentrations of IBA on percentage of rooted shoots.

DISCUSSION

Multiple shoot proliferation using nodal segments

Based on the findings, half strength MS medium with BAP alone at concentration of 5.0 mg/L were the better medium than other treatments for the initiation of shoot bud proliferation of *E. zwageri* in term of mean number of shoot buds (6.80±0.45) and number of leaves (12.60±1.24) productions. The study on shoot proliferation of *E. zwageri* also revealed the similar result but the number of shoot buds and leaves were lowest than observed in this present study. The possible cause for such variation is different in the strength of MS media where the MS media strength was reduced to half in this study. Thus, this is the first report demonstrating the possible effect of different MS media strength on the shoot proliferation of *E. zwageri*.

The use of comparable concentration of 5.0 mg/L BAP alone was also reported by Pelegrini *et al*. (2011) for the optimum shoot proliferation and leaves production of *Ocotea porosa* and Shah et al. (2013) for *Litsea glutinosa*. Compared with other Cytokinin including Kinetin, Thidiazuron and Zeatin, BAP was superior for the differentiation and growth of the new shoots as it is closely related to a natural Cytokinins (Pelegrini et al., 2011). Barciszewski et al. (1999) stated that BAP alone was commonly used in plant tissue culture as the N6-subtituted adenines which functioning for the vital growth regulatory activities such as cell division and differentiation.

In the culture medium with combined BAP and IBA, the higher mean number of shoot bud multiplication (4.40±0.45) and leaves productions (10.20±1.40) were observed in a culture medium supplemented with 5.0 mg/L of BAP and 0.5 mg/L of IBA. Similar with the present finding, the combination of higher concentration of BAP to lower concentration of IBA have been used for the shoot bud proliferation of Camphor tree (*Cinnamomum camphora*) (Du et al., 2015) and Neem tree (*Azadirachta indica*) (Arora et al., 2010). Murashige and Skoog (1962) also studied the successful regeneration of tobacco plant using higher concentration of Cytokinin such as BAP to Auxin such as IBA. A suitable Auxin concentration is critical in conjunction with higher concentration of Cytokinin for the shoot bud multiplication in many tropical hardwood tree species (Pijut et al., 2012). This is because the proper concentration of Auxin will promote a direct induction of shoot without callus formation such as in this study on the shoot bud multiplication of *E. zwageri*.

When BAP was combined with NAA, the higher mean number of shoot bud proliferation and leaves productions were found in a medium with 5.0 mg/L of BAP and 0.5 mg/L of NAA. The mean number of shoot buds obtained was 4.60±0.55 and mean number of leaves was 8.80±1.10. The comparable use of higher concentration of BAP (2.5 mg/L) as opposed to NAA (0.5 mg/L) was also reported on shoot proliferation of *Dehaasia incrassate*, a timber tree species from the similar family of Lauraceae with *E. zwageri*. The present finding demonstrated that when BAP is combined with IBA, the better shoot bud proliferation medium was those with BAP at concentration of 5.0 mg/L and IBA at concentration of 0.5 mg/L.

Thus, this suggested that higher concentration of Cytokinin as opposed to Auxin will help to enhance and increase shoots proliferation and leaves productions from the nodal explants of *E. zwageri*. However, at the higher concentration, BAP of 6.0 mg/L and NAA of 0.5 mg/L, was less effective for the proliferation of shoot and leaves from the nodal segment of *E. zwageri*.

Rooting

To encourage rooting, the healthy in vitro shoots of *E. zwageri* with height averaged 2 to 3 cm were treated with various concentrations of IBA for 30 minutes before cultured to half strength MS basal medium without PGR. The in vitro shoots of *E. zwageri* which have been treated with 300 mg/L of IBA observed with the highest mean rooting percentage (80.3%) than those treated with 0, 100 and 500 mg/L IBA. In accordance to Cerdas and Guzman (2004) and Pijut et al. (2012), treating the microshoots at the cut end with higher concentrations of IBA have successfully promoting root regeneration in various hardwood tree species. Compared with other PGRs, IBA was commonly used for rooting in many tissues culture protocol because it inhibits the primary root elongation and stimulates lateral root formation such as in this study.

CONCLUSION

This study established a rapid and efficient in vitro shoot proliferation's protocol for the ex-situ conservation of tropical hardwood timber tree species, *E.zwageri*. The half strength MS medium added with different concentration of BAP alone or BAP in combination with 0.5 mg/L NAA or 0.5 mg/L IBA were able to promote shoot proliferation from nodal segment of *E. zwageri*. However, BAP alone at concentration of 5.0 mg/L produced both higher mean number of shoot proliferation and leaves productions. One of the important outcomes from this study is that this was a first study that reported the successful rooting of in vitro shoot of *E. zwageri* using IBA at higher concentration such as 300 mg/L. All findings in this study is helpful for the commercial production of the micropropagated plantlets of *E. zwageri* and other tropical hardwood timber tree species.

ACKNOWLEDGEMENTS

The authors thank the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak for providing instruments.

AUTHOR CONTRIBUTIONS

Gibson Entuni assisted in conducting the experiments, performed the data collections, data visualization, statistical analysis and wrote the manuscript. Rebicca Edward and Chyntia Jaby Entuni designed the experiments and wrote the manuscript. All authors have read and approved of the final manuscript.

CONFLICT OF INTEREST

The authors declare that they hold no competing interests.

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