Short Communication
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Induction of shoot buds multiplication of eusideroxylon zwageri tesym. & binned (Borneo ironwood) by using nodal explants

Abstract

Eusideroxylon zwageri is a tree of the tropical rainforest which is belongs to a family of Lauraceae. This species is one of the hardest timber species in Southeast Asia and unfortunately endangered in some part of Southeast Asia. The objective of this study was to determine the optimal culture medium for the induction of shoot buds from nodal explants of *E. zwageri.* Different concentrations and combinations of BAP (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0mg/L) alone or either in combination with NAA (0.5mg/L) or IBA (0.5mg/L) were used in order to induce multiple shoot buds of this species. The nodal explants were collected from the healthy lateral branches of two to three years old of E. zwageri sapling. The finding in this multiple shoot buds study showed that MS medium which have been supplemented with 5.0mg/L of BAP alone or in combination with either 0.5mg/L of NAA or IBA had induced the highest mean number of shoots buds and mean number of leaves respectively. It can be recommended that studies on plantlet regeneration of this species should be conducted and field performance should be carried out on this regenerated species.

Keywords: borneo ironwood, micropropagation, eusiderin, recalcitrant seed, wood

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Introduction

Eusideroxylon zwageri is a tree of the tropical rainforest zone which belongs to a family of Lauraceae and is economically vital for a source of hardwood timber in Southeast Asia. This species is commonly known as Belian (Sabah and Sarawak), Tulian or Ulin (Indonesia), Borneo Ironwood (European Union) and Biliran, Sakilan or Tambulian (The Philippines). In Southeast Asia, *E. zwageri* is popularly known as the most durable timber and crucial for building material such as for power line poles, roof shingles, and furniture due to its strong physical characteristics in which it is not vulnerable to termites, fungus and tropical wood eating insects. As a result, many cutters are appealed to *E. zwageri* and this cause exploitation and destruction of this species. According to Peluso,[1] the introduction of chainsaws and extensive road systems by the timber companies and the alteration of forest to oil palm and timber estates has increases the exploitation of *E. zwageri*. The decline number of this species was first reported in 1955 in the regions such as Kalimantan, Sumatra, Sabah, Sarawak and The Philippines.[2] According to World Conservation Union and the IUCN Red List of threatened species,[3] Borneo Ironwood is classified as a "Vulnerable species" as per the criteria A1cd+2cd. According to IUCN,[4] any of a species under "Vulnerable" category is not critically endangered or endangered but this species may facing a high threat of extinction in the wild in the medium-term future.

Traditionally, *E. zwageri* was propagated by seed. The seed of this species are recalcitrant with strong tegument dormancy and these caused the germination rate of this species is slow. In the natural forests, the regenerated seedlings can be found under the mother trees but however due to over exploitation and shifting agriculture, the mother trees are cut off before they produced seeds and this limits the number of seed production of this species.[5] To date, this species has only been planted in a small scale as the supply of its seeds and seedlings is insufficient. Therefore, to prevent the extinction and derive the maximum benefits from this hardwood timber, it is necessary to preserve this species which was possible through the innovative and cost effective technologies. In this scenario, micropropagation by a mean of tissue culture such as through induction of shoot buds multiplication is one of the ideal way to overcome this problem. In this study, different concentrations and combinations of BAP (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0mg/L), NAA (0.5mg/L) and IBA (0.5mg/L) were used in order to induce multiple shoot buds of this species from the nodal segments.

Materials and methods

Plant material

In this study, the two to three years old of *E. zwageri* trees originally sapling from the forest were maintained in the pot culture outside Plant Tissue Culture Laboratory of Universiti Malaysia Sarawak (UNIMAS). The nodes were collected from the healthy lateral branches and used as the explants. Pre-sterilization procedure was carried out in which these explants were placed under running tap water for about one hour and soaked with 0.1% Benomyl for 30minutes before they were surface sterilized with 0.1% Mercuric chloride for 5minutes. After sterilization, these nodes were thoroughly washed three times with sterile distilled water. The nodal explants were cut into 1.0 to 2.0cm before they were cultured into culture

bottle containing MS medium without any addition of the plant growth regulators. All of these steps were carried out in the laminar flow cabinet. These cultures were incubated in the culture room at 25±2°C and kept under white fluorescent tubes providing irradiance of 50µmol m^2s^{-1} for 16hours photoperiod.

Culture media and conditions

The preparation of MS basal media was based on the formulation and this MS media was added with 30g/L sucrose and solidified with 3.0g/L Gelrite. The pH of the medium was adjusted to 5.8 with 1 N KOH or 1 N HCl prior to sterilization by autoclaving at 121ºC for 20minutes. In each of the experiments, different concentrations and combination of various plant growth regulators such as BAP (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0mg/L), NAA (0.5mg/L) and IBA (0.5mg/L) were manipulated and added into sterilized medium and dispensed into the sterile glass bottles.

Statistical Analysis

The number of shoot buds and the number of leaves were recorded after cultured for three months. Data collected were analysed using one-way analysis of variance (ANOVA) followed by mean comparison carried out using Tukey Test at $p<0.05$ with SPSS Statistics Version 20.

Results

Effects of BAP on shoot buds proliferation

The shoot buds proliferation by using different concentrations of BAP did not showed any significance difference between each of the treatments. All culture medium supplemented with different concentrations of BAP in this sub-experiment were able to proliferate shoot buds but the mean number of shoot buds that have been proliferated were different. The nodal segments began to form shoot buds after three weeks of culture (Figure 1A). Based on the statistical analysis, MS medium with 5.0mg/L of BAP was the optimal culture medium for the proliferation of shoot buds from the nodal segments of *E. zwageri*. Similarly, there were no significant difference between each of the treatment on the number of leaves production. The mean number of the leaves for each of the treatments were varied from 4.40±0.00 to 8.60±1.34 (Table 1). Green and healthy leaves were produced in all culture medium with or without any addition of plant growth regulator, BAP (Figure 1B).

Effects of BAP in combination with IBA on shoot buds proliferation

There was no significance difference between each of the treatments using BAP in combination with 0.5mg/L of IBA on shoot buds proliferation from nodal explants of *E. zwageri*. The lowest mean number of shoot buds was 1.00±0.00 and this was recorded in a control treatment while the highest number of shoot buds was 2.80±0.45 and was recorded in a culture medium supplemented with 5.0mg/L of BAP and 0.5mg/L of IBA. Based on the result that have been analysed, MS medium that have been supplemented with 5.0mg/L of BAP in combination with 0.5mg/L of IBA was the optimal culture medium for shoot buds proliferation (Figure 2) in this sub-experiment. Also, there was no significance difference between each of the treatment using BAP in combination with 0.5mg/L of IBA on the number of leaves production (Table 2). Green and healthy leaves were produced in all culture medium with or without any addition of BAP and IBA.

Effects of BAP and NAA on shoot buds proliferation

There was no significance difference between each of the treatments using BAP in combination with NAA on shoot buds proliferation of *E. zwageri*. All treatments in this sub-experiment were able to produce shoot buds in which the mean number of shoot buds that have been recorded were varied from 1.00±0.00 to 2.60±0.55. In this sub-experiment, the optimal culture medium for shoot buds proliferation of from nodal segments of *E. zwageri* was MS medium supplemented with 5.0mg/L of BAP in combination with 0.5mg/L of NAA. Based on Tukey test, there was also no significance difference between each of the treatment using BAP in combination with NAA on the leaves production (Table 3). The highest mean number of leaves which was 8.80±1.10 was recorded in a culture medium that have been supplemented with 4.0mg/L of BAP in combination with 0.5mg/L of NAA and the lowest mean number of leaves which was 4.00±0.00 was recorded in a control culture medium. All of the *in-vitro* leaves in this sub-experiment were green and healthy (Figure 3).

Figure 1 Multiple shoot proliferation of nodal segments of *E. zwageri* in a culture medium with 5.0 mg/L of BAP. Arrow showed the initiation of shoot buds (A) Shoot buds proliferation during third week of culture; (B) Green and healthy leaves development after ten weeks of culture. (Bar =0.5cm).

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Table 1 Mean (±SE) number of shoot buds and leaves by using different concentration of BAP.

Concentrations of Plant Growth Regulator (PGR)	Mean (SE)	
BAP (mg/L)	No. of shoot buds	No. of leaves
0	1	4.4
	0	0
1	1.4	4.6
	0.55	0.55
2	1.4	5.4
	0.55	0.55
3	1.6	5.2
	0.89	1.1
4	1.8	8.6
	0.45	1.34
5	3	5.2
	0	1.3
6	2	5.2
	1	1.3

Mean along the column followed by the same alphabet are not significantly different at p≤0.05 (Tukey Test) Data represent mean of five replicat, each replicate consist of three explant.

Table 2 Mean (±SE) number of shoot buds and leaves by using different concentration of BAP in combination with 0.5mg/L of IBA.

Concentrations of PGR BAP+IBA (mg/L)	Mean (±SE)	
	No. of shoot buds	No. of leaves
0+0	1.00±0.00	3.80±0.45
0.0+0.5	1.20±0.45	4.60±0.89
0.0+0.5	2.00±0.00	5.80±0.45
2.0+0.5	1.80±0.84	6.00±0.00
3.0+0.5	1.80±0.45	6.20±0.84
4.0+0.5	1.80±0.84	8.40±1.82
5.0+0.5	2.80±0.45	5.20±1.30
6.0+0.5	2.00±1.00	6.00±0.71

Table 3 Mean (±SE) number of shoot buds and leaves by using different concentration of BAP in combination with NAA

Concentrations of PGR BAP+NAA (mg/L)	Mean (±SE)	
	No. of shoot buds	No. of leaves
0+0	1.20±0.45	4.00±0.00
0+0.5	1.00±0.00	4.40±1.14
1.0+0.5	1.40±0.55	5.50±0.71
0.0+0.5	1.60±0.55	4.80±1.92
1.0+0.5	2.20±0.84	7.00±1.00
4.0+0.5	1.80±1.10	8.80±1.10
5.0+0.5	2.60±0.55	5.80±0.84
6.0+0.5	1.40±0.55	4.80±1.10

Mean along the column followed by the same alphabet are not significantly different at p≤ 0.05 (Tukey Test) Data represent mean of five replicates, each replicate consist of three explants.

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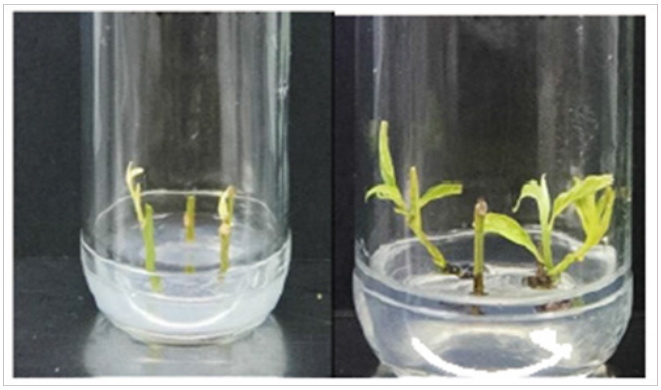

Figure 2 Multiple shoot proliferation of nodal segments of *E. zwageri* in a culture medium with 5.0mg/L of BAP in combination with 0.5mg/L of IBA after ten weeks of culture. (Bar=0.5cm).

Figure 3 Multiple shoot proliferation of nodal segments of E. zwageri in a culture medium with 5.0 mg/L of BAP in combination with 0.5 mg/L of NAA. Arrow showed the initiation of shoot buds (A) Shoot buds proliferation during third week of culture; (B) Green and healthy leaves development after ten weeks of culture. (Bar=0.5cm).

Discussion

In this study of using BAP alone for shoot buds multiplication, the optimal MS medium for the multiple shoot buds proliferation of the nodal explants of *E. zwageri* was the MS medium that have been supplemented with 5.0mg/L of BAP alone without any of combination with other plant growth regulators. This result was further concurred with the finding of Pelegrini et al.,[6] for the multiple shoots proliferation using nodal segments of one of Lauraceae species, *O. porosa* in which the use of BAP alone with concentration of 5.0mg/L was successfully induced the highest numbers of shoot buds induction of this species. Shah et al.,[7] also reported the same result on shoot multiplication of *L. glutinosa* in which the MS medium with 5.0mg/L of BAP induced the highest numbers of shoot multiplication. Similar effect of BAP on shoot multiplication was observed by previously Ramesh et al.,[8] on a micropropagation of *T. bellirica*, one of a tropical hardwood tree species in which the use of BAP alone with concentration of 5.0mg/L without any combination with other PGRs have induced the maximum number of shoots induction of *T. bellirica* from the nodal segments with 82% response as compared to when BAP was combined with other PGRs. Another study on a tropical hardwood tree species of *M. alternifolia* by Yohana et al.,[9] also found that the culture medium that have been supplemented with single BAP induced the highest numbers of shoots induction per nodal explant as compared to when BAP was used in combination with other PGRs such as NAA and IAA.

According to Shah et al.,[7] BAP was superior compare to others types of Cytokinin such as Kinetin, TDZ and Zeatin for the differentiation and growth of the new shoots and this was because of that BAP was much closely related to a natural Cytokinins. The majority of the research on tropical woody tree species such as *D. sissoo*,[10] *Simmondsia chinensis*,[11] *Casssia angustifolia*[12] and *M. azadirachta*[13] has reported that BAP was the most active Cytokinin for shoot multiplication of woody trees. According to Barciszewski et al.,[14] BAP singly was commonly used in plant tissue culture system as Cytokinins such as BAP is one of the N6-subtituted adenines which are functioning in most of the growth regulatory activities in plants that was useful for the plants cell division as well as for cell differentiation and therefore added to the culture medium as they induce division and cell organogenesis in plants.

In the other study of using BAP in combination with 0.5mg/L of IBA, the highest mean number of shoots buds that have been induced was in the MS medium supplemented with 5.0mg/L of BAP and 0.5mg/L of IBA with mean number of shoot buds of 2.80±0.00. This result was supported by Li Du et al.,[15] on shoot buds multiplication of *C. camphora* where the combinations of 5.0mg/L of BAP and 0.5mg/L of IBA successfully induced the highest mean number of shoot buds proliferation of this species with 85% success rate. This relationship between the combinations of these two plant growth regulators, the BAP and IBA in which the higher the concentration of BAP was concurs with the generally accepted theory in plant tissue culture by research of Murashige et al.,[16] which stated that the higher level of Cytokinin to Auxin was more conducive to the development of *in vitro* stem shoots of tobacco plant. A low level of Auxin is crucial in conjunction with high level of Cytokinin when shoot multiplication is required but however it is important to choose a suitable Auxin concentration that will promote induction of shoots without inducing callus formation such as in this experiment of *in vitro* shoot buds multiplication of *E. zwageri*.

In the study of using different concentrations of BAP in combination with 0.5mg/L of NAA, the highest number of shoot buds proliferations was obtained in the MS medium with 5.0mg/L of BAP in combination with 0.5mg/L of NAA. The similar application of higher concentration of BAP to NAA was also used by Su et al., (2006) on *in vitro* shoot regeneration of Lauraceae species, the *D. incrassate* in which the culture medium that have been fortified with 2.5mg/L of BAP in combination with 0.5mg/L of NAA induced the highest number of shoot multiplication of the species. This result of sub-experiment was similar to when BAP in combination with 0.5mg/L of IBA was used, the optimal shoot buds induction medium was the MS medium supplemented with 5.0mg/L of BAP in combination with 0.5mg/L of IBA. In this study, it was proved that the higher concentrations of Cytokinin as opposed to Auxin will promote the increase number of shoot buds from nodal explants of *E. zwageri*. However, when the concentration of BAP was increased to

6.0mg/L in combination with 0.5mg/L of NAA, the mean number of shoots buds was decreased. According to Barcelo et al.,[17] BAP was used for avocado shoot regeneration and elongation, but however if this plant growth regulator was used at high concentration in either multiplication or maintaining medium, BAP may inhibited and reduced the growth of newly shoots. In this study, among various concentrations of BAP that have been tested for the induction of shoot buds from nodal explants of *E. zwageri*, the maximum number of shoot buds per explants induced was observed in MS medium supplemented with 5.0mg/L of BAP alone or in combination with IBA or NAA. The range of different concentrations of Auxin in combination with Cytokinin have been used in many woody trees species in which the addition of low concentrations of Auxin to the high concentrations of Cytokinin was vital to increase shoot numbers in many woody plant species such as *S. sebiferum*,[18] *G. arborea*,[19] and *Tectona grandis*.[20]

In this study on the induction of shoot buds from the nodal segments of *E. zwageri*, proved that MS medium without any addition of plant growth regulator still able to induce multiple shoot buds of *E. zwageri*. This present finding have been found in agreement with previous report on micropropagation of *P. marsupium,* in which the MS basal media without any addition of plant growth regulators was still able to induced shoot buds of this tropical hardwood tree species. According to George,[21] this was due to the presence of high nitrate ammonium (39.4mM nitrate and 20.61mM ammonium) content in the basic MS media composition in which this elements were responsible for the nitrogen uptake and pH regulation during culture of the induction of shoot buds.[22]

Conclusion

This is the first report on micropropagation of *E. zwageri* through induction of shoots multiplication by using nodal explants. The protocol for the induction of shoot buds multiplication of *E. zwageri* has been successfully established in MS medium fortified with different concentrations and combinations of BAP (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0mg/L), NAA (0.5mg/L) and IBA (0.5mg/L). The results in this multiple shoot buds study, showed that the presence of BAP in the culture medium was important for the induction of shoot buds in which MS medium supplemented with 5.0mg/L of BAP alone or in combination with either 0.5mg/L of NAA or IBA had induced the highest mean number of shoots buds and mean number of leaves respectively. The protocol established in this study could be useful in multiplying an elite stock of this species within a limited time by using nodal explants collected from two to three years old seedling.

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None.

Conflict of interest

The author declares no conflict of interest.

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