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Adventitious roots induction of recalcitrant tropical woody plant, *Eurycoma longifolia*

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Abstract

Eurycoma longifolia is well known for its aphrodisiac and energy enhancing properties especially among the communities in Southeast Asia regions attributed to the bioactive compounds concentrated mainly in its tap root. Over-exploitation of the plants from the natural habitat has led to the shortage of the plant in the jungle. In this study, the effects of naphthaleneacetic acid (NAA), indole acetic acid (IAA) and indole butyric acid (IBA) at the concentrations of 0, 1, 3, 5 and 7 mg/L on adventitious roots induction from the leaf explants were tested on full strength Murashige and Skoog (MS) medium. The best auxin and the optimal concentration determined was NAA at 3 mg/L as measured in terms of percentage of explants forming roots and the number of roots formed per explant. In the study on sucrose concentrations, 3 mg/L of NAA-containing MS medium was supplemented with sucrose at 10, 30, 50 and 70 g/L. The results revealed that 50g/L sucrose that produced 3.2 roots per explant was better than 30g/L in inducing adventitious roots. Further studies using different carbon sources revealed that glucose recorded the highest rooting percentage (42.2%) while sucrose gave the highest root number (3.0) per explant. This study reported the first successful adventitious roots induction of E. longifolia, which promise a high potential of large scale commercial production in bioreactor for the pharmaceutical industries.

Keywords: adventitious root; aphrodisiac; carbon source; *Eurycoma longifolia*; plant growth regulators; tissue culture

Abbreviations: IAA, Indole acetic acid; IBA, Indole butyric acid; MS, Murashige and Skoog; NAA, Naphthaleneacetic acid.

Introduction

E. longifolia has a long history of medicinal usage in Asia. It is very popular traditionally for its aphrodisiac and anti-malaria properties, where the decoction of the root is taken orally to achieve these effects [1]. In addition, it has many other folk medicinal usages such as to lower high blood pressure, enhance energy levels and taken as a general tonic [2]. Nowadays, pharmacological studies have proven the aphrodisiac property, anti-malaria, anti-

ulcer, anti-microbial and cytotoxic activities of *E. longifolia* extracts [3]. Chemically active constituents such as quassinoids and cathine-6-one alkaloids are frequently extracted and isolated from the root of *E. longifolia* while some of them can be obtained from the leaves [4]. Quassinoids in the root have been proven to be as potent as aspirin against fever; anti-malaria activity against *Plasmodium falciparum*; cytotoxic activity against human cancer cell lines and anti-oxidant property in reducing cellular aging [5]. The medicinal value of *E. longifolia* especially its aphrodisiac property has led to high market demand of its plant products and hence their high commercial value in the market [6]. The most popular *E. longifolia* products in the market are health drinks, tablets and food supplements [7].

The demand for *E. longifolia* plant products keep increasing with every research and development study. For instance, the discovery on the ability of *E. longifolia* in increasing muscle strength has resulted in an added demand for this plant. Due to unplanned commercial harvesting and increasing market demand, especially in the pharmaceutical industry, the population of *E. longifolia* is diminishing from its natural habitat. Conventional propagation of *E. longifolia* by cutting and seed germination is insufficient to satiate the market demand today. This is because the plant takes at least five years to reach its mature age and the fruit yield is usually low. Thus, the reproduction of this plant through the conventional propagation is relatively low and slows [8]. As such, plant tissue culture techniques such as the production of root cultures can be used as an alternative in providing the raw materials of *E. longifolia* for the industries.

Adventitious roots are roots that are induced at unusual sites, such as roots forming on leaves, which then grow and branch rapidly [9]. The roots induced in this mode are considered to be genetically uniform and true-to-type, which give mass production of the desired pharmaceutical compounds [10]. A successful production of adventitious root culture of *Panax ginseng* with high amounts of ginsenosides in bioreactors has proven the ability of adventitious roots culture to be an alternative method for scale-up [11]. Although *in vitro* propagation is an alternative to produce *E. longifolia* for commercial and conservation purposes, studies on adventitious roots induction for this species are still scarce due to its woody nature. Thus, this study was carried out to determine the best medium formulation in terms of the types and concentrations of both auxins as well as the carbon source on the induction of adventitious roots from leaf explants of *E. longifolia*.

Materials and methods

Plant Materials

In this study, the *Eurycoma longifolia* plants were obtained from Sungai Buluh, Selangor, Malaysia. Only juvenile apical leaves were selected and used as the explants throughout this study.

Surface Sterilization of Explants

The explants were briefly washed with detergent and rinsed several times with tap water followed by thorough washing under running tap water for 30 minutes. After that, the leaves were surface sterilized using 20% (v/v) Clorox® solution containing three drops of Tween-20 (Amresco, USA), with a continuous shaking for 15 minutes. The leaves were then rinsed three times with sterile distilled water for 5, 10 and 15 minutes, respectively. Then, the leaves were treated again with 20% Clorox® solution containing three drops of Tween-20 (Amresco, USA), with a continuous shaking for 15 minutes; rinsed three times with sterile distilled water for 5, 10 and 15 minutes, respectively. After sterilization, the leaves were cut into squares of 5 mm x 5 mm in size along the midrib and ready for culturing in full strength Murashige and Skoog (MS) medium [12] containing 5% (w/v) sucrose unless otherwise stated. The pH of the medium was adjusted to 5.8 ± 0.1.

Effects of Different Auxins

In the study on the effects of different auxins on adventitious roots induction, MS medium supplemented with different auxins: Indole butyric acid (IBA), Indole acetic acid (IAA) and Naphthaleneacetic acid (NAA) at the concentrations of 1.0, 3.0, 5.0 and 7.0 mg/L were used while the auxin free MS medium was used as the control in each treatment.

Effects of Concentrations of Sucrose

The surface sterilized leaf explants were cultured on full strength MS medium containing 3mg/L NAA and supplemented with 0, 10, 30, 50 and 70 g/L of sucrose.

Effects of Different Carbon Sources

The effectiveness of various carbon sources on adventitious roots induction was also examined by culturing the leaf explants on MS medium supplemented with 3 mg/L of NAA and different carbon sources at the same concentrations, which was 50g/L. These five carbon sources were sucrose, sorbitol, fructose, glucose and galactose.

Culture conditions

All the cultures were maintained at 25 ± 1ºC under 24 hours dark condition in the culture room throughout the eight weeks culture periods. Observation on adventitious roots and callus formation was carried out daily to determine the initial day of root and callus formation. The percentage of callus formation, degree of callus formation, percentage of roots formation, number of roots per explant, and morphological changes of the explants or roots were recorded at every two to three days interval, continuously for eight weeks.

Statistical Analysis

For all the treatments, five explants were cultured onto each Petri dish and three replicates were used for each treatment. The experiment was then repeated twice. The data were analyzed using Duncan Multiple Range Test with the significant differences between mean at $p<0.05$. All the statistical analysis was performed using SPSS Statistic 17.0.

Results and discussion

Effect of Auxins

The application of auxins is considered to play a major role in adventitious rooting process where numerous reports ascribed the initiation and division of adventitious roots to exogenous or endogenous auxins [13]. Bellamine et al. reported that auxin exerts the primary role in root formation by its involvement in successive and interdependent phases [14]. The researchers claimed that the formation of roots was completely inhibited when anti-auxins were applied. The differentiation of phloem ray parenchyma cells into root primordial depends upon the type and concentration of auxin because the differentiating cells require the most appropriate auxin to become competent to respond to the organogenic signal [15,16]. The process of differentiation and induction pathways in rooting can be triggered by the supplementation of specific auxins exogenously [17]. Further, it had been reported that the effectiveness of various auxins for induction of adventitious roots is variable for different species. For example, IBA was superior over IAA and NAA in the induction of adventitious roots from hypocotyl explants of *Psoralea coryfolia* [18]; IAA was reported to induce adventitious roots in *Helianthus annuus* [19] and *Antirrhinum majus* [20]

However, this study showed that NAA was more potent than IBA and IAA in triggering induction of adventitious roots from leaf explants of *E. longifolia*. All the NAA treatments (1, 3, 5 and 7 mg/L) successfully induced white hairy adventitious roots from the midrib or edge of the leaf explants of *E. longifolia* (Figure 1b). However, the day of roots formation varied in MS medium supplemented with different concentrations of NAA (Table 1). NAA has also been reported to be superior to IBA for adventitious roots production in

Cornus mas [21] and *Ulmus parvifolia* [22]. This study also revealed that medium supplemented with NAA at concentration higher or lower than 3mg/L showed decreased rooting percentage. This could be due to the higher concentration of auxin which induces the higher level of degrader metabolites in tissue, thus, blocking the regeneration process [23]. In general, the effect of an auxin on rooting is promotable at low concentrations and inhibitory at supra-optimal concentrations.

Meanwhile, within the IAA treatments, adventitious roots were only induced in MS medium supplemented with 5 mg/L of IAA (Figure 1c). The roots formed were brown in colour with no hairy root was observed. The root structure was observed after 6.0 days of culture under dark condition. A total of 4.4% explants were recorded to induce an average of 0.7 roots. Callogenesis was observed in MS medium supplemented with 1 mg/L, 3 mg/L and 5 mg/L of IAA but not in 7 mg/L of IAA and the control medium. The percentage of callus formation recorded an increase from 6.7% to 8.9% when the concentration of IAA increased from 1 mg/L to 3 mg/L; whereas a decrease of 2.2% was recorded in 5 mg/L of IAA. On the other hand, there was no sign of adventitious roots induction from leaf explants of *E. longifolia* in MS medium supplemented with IBA. However, a high percentage of callus formation was recorded in all the treatments with IBA. Compact, white calli formed at the midrib or edge of the leaf explants cultured in MS medium supplemented with 1 mg/L IBA after 6.0 days of culture; followed by 5 mg/L (10.0 days) and 3 mg/L as well as 7 mg/L (11.0 days) of IBA. The calli developed into larger size and covered portions of the explants depended on the concentration of IBA added into the medium. The colour of the calli started to turn from white to yellowish in colour after 12 days of culture (Figure 1d). An increasing trend in the percentage of callus formation from 26.7% to 68.9% was recorded when the concentrations of IBA increased from 1 mg/L to 7 mg/L. In contrast to the treatments with NAA and IAA, the higher percentage of callusing was achieved with the higher concentration of IBA. An increase in concentrations (1 mg/L to 7 mg/L) also recorded an increase in the degree of callusing.

Table 1: Effects of different auxins at various concentrations on the adventitious root induction and callus formation from the leaf explants of *E. longifolia* under dark condition after 56 days of culture.

Treatments	Concentrations (mg/L)	Day of roots formation	Explants forming roots (%)	Number of roots per explant	Day of callus formation	Degree of callus formation
NAA	0	-^{a}	-^{a}	-^{a}	-^{a}	-
	1	22.0^{c}	6.7^{ab}	1.7^{ab}	10.0^{c}	+
	3	20.0^{c}	28.9^{c}	3.5^{b}	4.0^{b}	++
	5	21.0^{c}	6.7^{b}	1.3^{ab}	14.0^{c}	+
	7	7.0^{b}	2.2^{a}	1.0^{a}	4.0^{b}	+++
IAA	0	-^{a}	-^{a}	-^{a}	-^{a}	-
	1	-^{a}	-^{a}	-^{a}	3.0^{b}	+
	3	-^{a}	-^{a}	-^{a}	3.0^{b}	+
	5	6.0^{b}	4.4^{ab}	0.7^{a}	3.0^{b}	+
	7	-^{a}	-^{a}	-^{a}	-^{a}	-
IBA	0	-^{a}	-^{a}	-^{a}	-^{a}	-
	1	-^{a}	-^{a}	-^{a}	6.0^{b}	+
	3	-^{a}	-^{a}	-^{a}	11.0^{c}	++
	5	-^{a}	-^{a}	-^{a}	10.0^{c}	+++
	7	-^{a}	-^{a}	-^{a}	11.0^{c}	+++++

Means within the column having the same letter were not significantly different at P < 0.05 in Duncan Multiple Range Test. Degree of callus formation (cm): (-) no growth; (+) <0.2; (++) 0.2-0.4; (+++) 0.4-0.6; (++++) 0.6-0.8; (+++++) >0.8

The different effectiveness among the three auxins (NAA, IAA and IBA) might be affected by the differential affinity to the auxin receptor involved in rooting, the concentration of free auxin that reached the target competent cells, the amount of endogenous auxin and the metabolic stability of the auxins [24, 25]. The concentration of free auxin that reached the target competent cells depends on several factors such as uptake, transport and conversion of the added auxin [24]. The two main pathways of conversion are oxidation and conjugation. IAA and IBA might be inactivated irreversibly by oxidation but NAA is not oxidized [26]. Thus, NAA might be the preferable auxin in plants with high activity of auxin-oxidase [27]. Based on the high rooting ability with NAA but not IAA and IBA showed in this study, it could suggest that there might be high activity of auxin-oxidase in the leaf explants of *E. longifolia*. Furthermore, the uptake of the three auxins could be different in which NAA was taken up six times faster than IAA in tobacco explants [28] and IBA was four times faster than IAA in apple shoots [29]. Although all the three auxins might be conjugated due to wounding response, conjugation is a reversible inactivation as the free auxin might be released from the conjugates and taken up via cut surface [30]. IAA is rapidly oxidized or conjugated but conjugated IAA can be hydrolyzed in plants and hence yielding free auxin [26, 31]. This could possibly explain why IAA can induce adventitious roots in low percentage but only in medium supplemented with higher concentration of IAA (5 mg/L). De Klerk et al. also reported that a very high concentration of IAA has to be applied in *Malus* 'Jork 9' in order to obtain high number of roots [27].

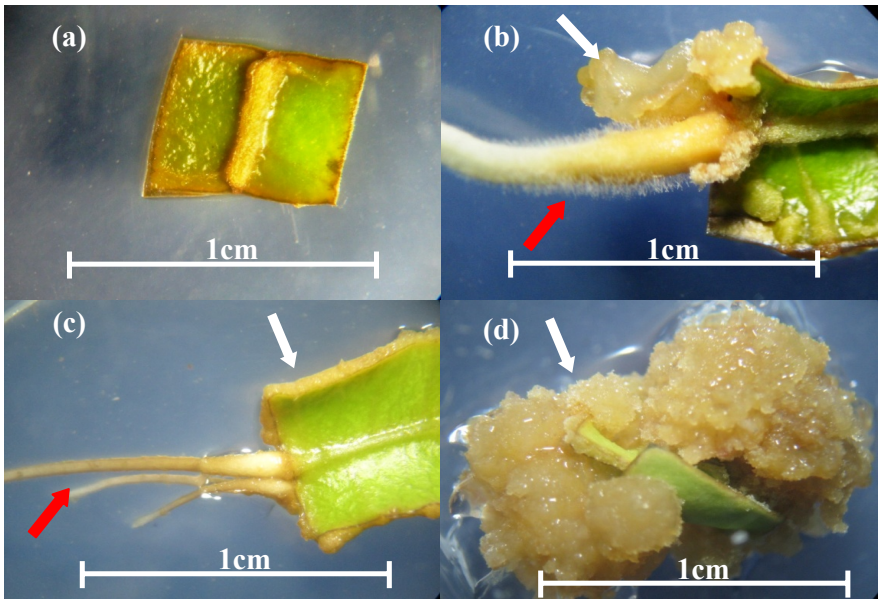

Figure 1: Effects of different auxins at various concentrations on the adventitious roots induction from the leaf explants of *E. longifolia* under dark condition after 56 days of culture. (a) control (b) 3 mg/L NAA (c) 5 mg/L IAA (d) 5 mg/L IBA. Black arrow shows the adventitious roots formed on the explant; white arrows show the callus formed on the explants.

Effects of Sucrose Concentrations

In the study to determine the effects of sucrose concentrations, only MS media supplemented with 30 and 50 g/L of sucrose had successfully induced adventitious roots from the leaf explants of *E. longifolia*. Sucrose at 50g/L was found to be superior to 30g/L of sucrose to induce roots from the leaf explants of *E. longifolia* in term of rooting percentage and number of roots induced. The root structures were observed in the treatments with 30 and 50 g/L of sucrose after 21.0 and 28.0 days of culture, respectively (Table 2). Although roots

were initiated in MS medium supplemented with 30g/L sucrose but further roots elongation was inhibited. White hairy roots were observed in 50g/L sucrose-supplemented medium. Similarly, the study of rooting from *Ceratonia siliqua* shoots reported that higher rooting frequency was observed in 50g/L sucrose-supplemented medium as compared to 30g/L of sucrose [32]. The number of roots induced from the shootlets of date palm was significantly higher in MS medium supplemented with 50g/L of sucrose [33]. In contrast, another study reported that among all sucrose concentrations tested including 50g/L sucrose, the best concentration to induce roots from leaf explants of *Orthosiphon stamineus* was 30g/L sucrose in terms of both rooting percentage and number of roots induced [34]. Studies on *Arabidopsis thaliana* found that sucrose at 5 to 20g/L was the most effective in inducing adventitious roots while at 50g/L, it suppressed roots induction [35]. Therefore, it was suggested that various species of plants might response differently to sucrose concentration.

Table 2: Effects of various concentrations of sucrose on the adventitious root induction and callus formation from the leaf explants of *E. longifolia* under dark condition after 56 days of culture.

Concentrations (mg/L)	Day of roots formation	Explants forming roots (%)	Number of roots per explant	Day of callus formation	Degree of callus formation
0	-[a]	-[a]	-[a]	-[a]	-
1	-[a]	-[a]	-[a]	4.0[b]	+
3	21.0[b]	11.1[b]	1.7[ab]	4.0[b]	+
5	28.0[b]	31.1[c]	3.2[b]	11.0[c]	+
7	-[a]	-[a]	-[a]	4.0[b]	+

Means within the column having the same letter were not significantly different at P < 0.05 in Duncan Multiple Range Test. Degree of callus formation (cm): (-) no growth; (+) <0.2; (++) 0.2-0.4; (+++) 0.4-0.6; (++++) 0.6-0.8; (+++++) >0.8

Apart from that, the study of adventitious roots formation in apple showed the influence of sucrose concentration on the number of roots induced [36]. Although the effect on rooting was small at a broad range of sucrose concentrations (1 to 90g/L) but at extreme concentrations (0 to 110 g/L), relatively low percentage of rooting was recorded. Similar effect was observed in this study whereby the inhibitory effect on rooting was recorded in the MS medium without sucrose. The deficiency of exogenous sugar supply in dark condition caused inability to root on the leaf explants. Exogenously supply of sugar in culture medium for plants growth had been considered to balance the low or negative net of photosynthetic rate of *in vitro* culture [37]. The inability to photosynthesize caused the explants unable to synthesis starch; hence, they relied on the use of exogenous sugar as carbon source for growth [38]. This might explain why exogenous sucrose was required to induce adventitious roots from leaf explants of *E. longifolia* under total darkness. Takahashi et al. had proven the important of exogenous sugar in adventitious roots induction under dark condition [35]. They found that the hypocotyls of *A. thaliana* must be in contact with the sugar-supplemented medium to uptake sucrose in order to induce roots in total darkness. There was no root induced when the hypocotyls were not in contact with the medium. Besides, even if the hypocotyls were in contact with the medium, there was no root induced if the medium was not supplemented with sucrose.

Moreover, the inability to root in the MS medium supplemented with 10 and 70g/L of sucrose was possibility due to the osmotic pressure [36]. The osmotic pressure of 10g/L sucrose-supplemented medium was probably too low to act osmotically between the medium and the leaf explants of *E. longifolia*. The concentration higher than optimal, 70g/L of sucrose, inhibited rooting, probably due to the rather high osmotic pressure in the medium

that caused water deficit of the leaf explants in this study. Water supply has been proven to be essential for root regeneration in the study demonstrated by Klebs in 1903 and later works had further confirmed the study [39,40].

Effects of Carbon Sources

Among five carbon sources tested (sucrose, glucose, fructose, galactose and sorbitol), only sucrose and glucose showed the ability to induce adventitious roots from the leaf explants of *E. longifolia*. An early root structure was observed in MS medium supplemented with sucrose after 28.0 days of culture under dark condition while the root was induced in glucose-supplemented medium after 32.0 days of culture (Table 3). White hairy roots were observed in both media supplemented with sucrose and glucose. Although glucose took a longer time for root initiation, a higher percentage of explants forming roots were recorded in MS medium supplemented with glucose. The percentage of rooting for glucose was 42.2% whereas 35.5% of rooting in the sucrose-supplemented medium.

Table 3: Effects of different carbon sources on the adventitious root induction and callus formation from the leaf explants of *E. longifolia* under dark condition after 56 days of culture.

Treatments	Day of roots formation	Explants forming roots (%)	Number of roots per explant	Day of callus formation	Degree of callus formation
Sucrose	28.0[b]	35.5[b]	3.0[b]	11.0[b]	+
Glucose	32.0[b]	42.2[b]	2.0[b]	15.0[b]	+
Fructose	-[a]	-[a]	-[a]	12.0[b]	++
Galactose	-[a]	-[a]	-[a]	17.0[b]	+
Sorbitol	-[a]	-[a]	-[a]	-[a]	-

Means within the column having the same letter were not significantly different at P < 0.05 in Duncan Multiple Range Test. Degree of callus formation (cm): (-) no growth; (+) <0.2; (++) 0.2-0.4; (+++) 0.4-0.6; (++++) 0.6-0.8; (+++++) >0.8

Root initiation and development are high energy processes which require the expense of available metabolic substrates such as sugars [32]. The inadequate photosynthesis in *in vitro* culture results in the explants must uptake exogenous sugar as carbon sources. These carbon sources play an important role as energy sources and also for maintaining osmoticum [41]. Sucrose has a role in controlling the expression of several enzymes and proteins such as stimulate plant growth by inhibit proteinase; hence, growing and differentiating tissues are often found in the presence of high level of sucrose and invertase [33]. Sucrose is the most commonly found sugar in phloem sap of angiosperms, so, it is usually added into the medium of *in vitro* culture [32]. The efficiency of sucrose uptake across the plasma membrane is also one of the reasons to consider sucrose as the common carbon source used for plant tissue culture works. The presence of sucrose in the medium had been reported to increase the intracellular sucrose concentration in sugarcane [42]. However, sucrose is not the immediate carbon substrate in plant tissues. It is usually hydrolyzed into its constituent monosaccharides (glucose and fructose) by the cell wall-bound invertase before it can be utilised in metabolic processes [43]. The hydrolysis of sucrose in the culture medium during root induction phase of *Rosa multiflora* had been reported by Riek et al.[44]. Therefore, the explants are usually exposed to a mixture of sucrose, glucose and fructose.

The type of carbon sources has proven to affect the adventitious roots induction in many plant species. Thompson and Thorpe claimed that the different in carbohydrate requirements are depending upon the stage of culture and plant species [45]. The frequency

and quality of roots can be improved by amending different types of carbohydrates in the culture medium [46]. The beneficial effects of sucrose on rooting had been reported in apple and *Gladiolus hybridus* [36,47]. Although sucrose is used commonly in culture medium for rooting, few reports had showed that glucose or fructose could be a better carbon source. In this study, a higher percentage of explants forming roots were recorded in the MS medium supplemented with glucose. Thus, it was suggested that direct uptake of glucose instead of sucrose could induce higher rooting frequency. This was in accordance with the study carried out by Romano et al., who reported that glucose was the most effective carbon source in promoting rooting for cork oak followed by sucrose and filter-sterilized fructose [48]. Another study also reported that glucose induced roots up to 89 % in difficult-to-root *Eucalyptus globules* [49]. This proved that glucose is a regulatory factor for rooting. Correa et al. also claimed that more cells were recruited for root induction with the presence of glucose in the induction phase and hence, increased the rooting frequency [49]. Glucose may induce higher mitotic activity because it is a faster and effective metaboliser as compared to sucrose [50]. Moreover, glucose was found to have growth hormone-like activities and it interacts positively with auxin signaling, thereby, inducing adventitious root initiation [50, 51].

The fact that fructose was unable to stimulate rooting in this study might due to the phytotoxicity of furfural formed from fructose after medium autoclaving. This was supported by the completely ineffectively on root induction in cork oak when autoclaved fructose was tested whilst roots were induced in the medium with filter-sterilized fructose [48]. Thus, it was suggested that the rooting response induced by fructose was dependent on the sterilizing procedure. Besides, the inability to root in the sorbitol containing medium found in this study was also observed in the rooting of cork oak. In contrast, Yaseen et al. reported that sorbitol was the best carbon source in inducing roots from apple rootstock M 9 and M 26 [52]. This could be due to the high motility of boron (rooting cofactor) in *Malus*, which is immobile in higher plants and forms boron-sorbitol complexes only in sorbitol rich species [53]. Based on the failure of rooting by sorbitol in this study, it can be concluded that boron was probably immobile in *E. longifolia* plant and this plant might not be a sorbitol rich species. As for galactose, it is a monosaccharide which should be more easily decomposed but its utilization in morphogenesis may depend on the endogenous levels of galactokinase [54]. There was no sign of root development from the leaf explants of *E. longifolia* in the medium supplemented with galactose, but roots were induced by galactose in chrysanthemum [55].

Conclusions

The first successful adventitious root induction from *E. longifolia* promises various future studies on mass production of the root cultures in the bioreactor system in order to meet the high and continuous market demand. Apart from that, the identification and quantification of the active compounds such as eurycomanone in the adventitious roots of *E. longifolia* cultivated in bioreactors can also be studied with the purpose of providing desirable useful compounds for the pharmaceutical industries.

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