International Journal of Contemporary Research in Agriculture and Environmental Sciences
ISBN (eBook): 978-0-6481172-4-7
Year: 2019 , Volume: 01, Issue: 01

SPROUTING ABILITY IN MICRO PROPAGATION OF LABAN (*VITEX PUBESCENS*) : THE MEDICINALLY IMPORTANT TREE

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Abstract

Laban (*Vitex pubescens*), Indonesian native species, possesses diverse medicinal properties ranging from anti-inflammatory, anti-allergic, anti-microbial to anti-cancer and anti-HIV. Many potential bioactive molecules form an integral part, of which some are highly valued by multinational pharmaceutical companies. Unrestricted harvesting and high market demand of leban had drastically reduced the existing wild stocks. Micropropagation, a key tissue culture technique of medicinal trees has come forth to take up the challenge. The purpose of this research is to observe the sprouting ability of leban to get the highest nodes number as multiplication source, an important factor in micropropagation technique to produce high multiplication clones. We used 10 families of laban young seeds from Ketapang, West Kalimantan as mother plants. The in vitro shoots initiation were establishedon Murashige & Skoog (MS) mediumsupplemented with 1 g/LBAP (benzylaminopurin); 0.1 g/L kinetin and 0.01 g/LNAA (naphtalenaceticacid) (M1);2g/LBAP, 0.1 g/L kinetin and 0.01 g/LNAA (M2) and 3g/LBAP, 0.1 g/L kinetin and 0.01 g/LNAA (M3).All treatment shoots regenerated from explants tissue were transferred to a rooting medium consisting of half-strength MS medium supplemented with 0.5g/L IBA. After 6 months of shoots initiation subculture, the high average of shoots elongation was obtained fromfamily1(3.79 cm ± 1.06) and family 5 (3.47 cm ± 1.25)in M1 media. After 6 months of rooting subculture, the highest average of node number (8nodule in one shoot) was obtained from family 1. This family has the highest average of root elongation (5.36 cm ± 1.25). The best node number, highest shoot elongation and highest rooting elongation from *in vitro* regeneration were expecting the highest multiplication for mass micro propagation.

Keywords: Micropropagation, Node Number, Root Length, Shoot Length, *Vitex Pubescens*.

1. Introduction

The Republic of Indonesia is a culturally diverse archipelago located between Indochina and Australia and consists of over 13.000 islands including the “Spice Islands” (Gils & Cox 1994). Indonesia stretches across 5.100 km and encompasses a wide range of habitats (Whitten et al., 1996), and therefore, is blessed with a vast diversity of plants with medicinal value. According to the Indonesian Science Board (Lembaga Ilmu Pengetahuan Indonesia/LIPI), Indonesia is home to 30.000 out of 40.000 medicinal herbal plants in the world. It is only natural that in the jungles, forests, swamps, and even gardens of ordinary Indonesians, some plants with medicinal use could grow. There may even be some plants able to cure rare diseases just waiting to be found deep in the Indonesian ecosystem. Indonesia holds the potential to contribute its immense cabinet of plant-based-medicine and cosmetics to the world (Ministry of Trade, Republic of Indonesia, 2009).

Based on survey and research in 2012 and 2015 on medicinal plants by Medicinal Plant and Traditional Medicine Research and Development Center, in various ethnic groups in Indonesia, particularly in the East and North Kalimantan which recorded 38 species of plants used specifically for skin care/cosmetics and 16 of them were scientifically identified (Wahyono et al., 2016). The genus laban (*Vitex pubescens*),belonging to family Verbenaceae, possesses diverse medicinal properties ranging from anti-inflammatory, anti-allergic, anti-microbial to anti-cancer and anti-HIV (Venkateswarlu, 2012) was often found in the forest near the village in East and West Kalimantan. This species is one of the pioneer wood plant species, having properties of hard wood (Bratawinata, 1987) and have many potential bioactive molecules that form an integral part, which some are highly valued by multinational pharmaceutical companies (Venkateswarlu, 2012) and unfortunately, the scientific evidences about this medicinal tree have not been known yet.

Propagation of high secondary metabolite producing clones and sustains the genetic material of laban needs vegetative techniques to conserve or maintain the superior traits. Unrestricted harvesting and high market demand of many medicinal plants including laban had drastically reduced the existing wild stocks, a concern that needs attention. Such practices necessitate the intervention of biotechnological techniques such as tissue culture for vegetative mass propagation of this valuable species. High sprouting ability of laban plantlet in vitro was expected to get a large amount of nodes in auxiliary shoot of tissue culture technique for mass propagation. Controlled environment in vitro provides ex situ effects with high availability of nutrients for explant sprouting ability. In a natural environment, sprouting is a common response to tissue damage for woody plants and is a source of regeneration that contributes to the composition and development of forest ecosystems (Bond & Midgley 2001, Del Tredici 2001). Sprout production varies among species and in relation to parent tree size, tree age, and site productivity (Dey et al. 1996a, Bellingham & Sparrow 2000, Weigel & Peng, 2002). This paper studies tissue culture of ten families as mother plant sources of laban from traditional community's forest in Central Kalimantan for in vitro sprouting ability selection to get the highest nodes as multiplication source. The role of different factors of plant growth regulators and their dosages in developing an efficient sprouting regeneration system for the laban tissue culture were observed. The results of this paper are expected to contribute information for science, forest community and industry in developing technology for the utilization of laban for medicinal trees micro propagation.

2. Research Problem

Even though there are an estimated 707 plant species known as Vitex sp. worldwide, 230 species have a taxonomically accepted name, 455 names are synonyms and 22 names are unresolved. Protocols for the micro propagation of only six species have been established, and most have been dedicated to *V. negundo* (27 articles from a total of 46 papers dedicated to the genus) and *V. trifolia* (9 articles). The only four reports that exist for *V. glabrata* are related to the production of secondary metabolites (Sinlaparaya et al., 2007; Chamnipa et al., 2012; Thanonkeo et al., 2011) but a micro propagation protocol is not available for this species yet (da Silva et al., 2016).Likewise no information is available for laban (*V. pubescens*).

3. Review of The Relevant Literature

Fifteen Vitex species (*V. acunae Borh.* & *Muniz, V. ajugaeflora Dop., V. amaniensis W. Piep, V. cooperi Standl., V. evoluta Da¨ niker, V. gaumeri Greenm., V. heptaphylla A. Juss., V. keniensis Turrill, V. kuylenii Standl., V. lehmbachii Gu¨ rke, V. longisepala King & Gamble, V. parviflora Juss., V. urceolata C.B. Clarke, V. yaundensis Gu¨ rke, and V. zanzibarensis Vatke)* are included in the IUCN Red Data list (IUCN, 2016)for various reasons, but all are related to unsustainable harvesting. The other Vitex species has not yet been studied. Thus, urgent attention is needed to conserve Vitex species. Tissue culture provides a viable solution for the

largescale propagation of medicinally important plants (Kher et al., 2016; da Silva et al., 2015; Nataraj et al., 2016), including cryoconservation and genetic transformation (da Silva et al., 2015). The micropropagation of three Vitex species (*V. negundo, V. agnus-castus* and *V. trifolia*) from 29 published reports of Vitex sp. has recently been reviewed Ahmad et al., (2015). However, that review lacks vital details about disinfection methods, temperature, light source and intensity, photoperiod, basal medium, plant growth regulator type and concentrations, medium pH, carbon source type and concentrations for culture initiation, multiplication and rooting, all of which are essential factors that influence the outcome of the tissue culture protocol for Vitex species (da Silva et al., 2016).

*V. pubescens*is are employed for the healing of gastrointestinal symptoms, such as diarrhea, and even for scorpion stings (Meena et al., 2011. The glue of its leaves is helpful for abrasions(Ong & Nordiana, 1999) and is also used for fever as an antipyretic (Batubara et al., 2009). In addition, it has anti-dysentery, anti-inflammatory, analgesic, anti-fungal and anti-tumor activities (Meena et al., 2011; Oramahi & Yoshimura, 2013). The gastro protective efficiency of *V. pubescens* leaves against ethanol-induced gastric hemorrhagic laceration in rats was observed by Al-Wajeeh et al., 2016. Gastric homogenates revealed a remarkable increase in endogenous antioxidant enzyme activities and a decrease in the lipid peroxidation level in animals pre-treated with *V. pubescens* extract compared with the ulcer control group. Tissue culture of *V. pubescens* has been observed from one mother plant seeds for acid tolerant clones in Murashige & Skoog adjusted at low pH (Putri, 2010). The medicinal properties of medicinal plants are derived from the presence of single or multiple secondary metabolites (Gandhi et al., 2015). Based on some previous studies, *V. glabrata* species were intensively researched on secondary metabolites related to tissue culture. *V. glabrata* produces sterols like 7-dehydrocholesterol (7-DHC), a-ecdysone and 20-HES (Werawattanametin, 1986). 20-HES has multiple uses, including as an insecticide (Dhadialla et al., 1998), an anabolic steroid in sports and bodybuilding, and as a tonic supplement for male and female reproductive systems (Dinan & Lafont, 2006). Tissue culture from callus material was used as culture for experimental study in 20-HES production of *V. glabrata* (Sinlaparaya et al., 2007; Thanonkeo et al., 2011; Chamnipa et al., 2012).

4. Material and Methods

4.1. Study Site

The literature study, identification field site, material exploration and laboratory research has been conducted fromJune2010 to September 2015 and then it had been deepened in 2016. The laban seeds were collected from non-cultivated grass land sites beside Pawan river areas in Ketapang regency, south province of West Kalimantan, on Borneo, Indonesia (0° 19’ 00” - 3° 05’ 00” N and 108° 42’ 00” E).

4.2. Plant Materials

Fresh, young seeds were collected from 10 families of 10-15 years old of laban, naturally regenerated as pioneer native species. The 100 seeds per family were soaked in distilled water overnight. After washing with distilled water containing detergent for 1 min, they were rinsed thoroughly with running tap water for 10 min, and then soaked in hot water (80~90°C) for 5 min to break down dormancy of seeds. After that, they were surface-sterilized with fungicide and bactericide 1% (v/v) for 15 min in outside lamina air flow (LAF). Subsequently, surface sterilization was carried out with 3% (v/v) sodium hypochlorite aqueous solution containing a few drops of Tween 80 (Wako Pure Chemical Industries, Japan) for 10 min and washed 3 times with sterile distilled water. Then, they were sterilization with 70% alcohol solution for 3 min and washed 3 times with sterile distilled water again in inside LAF. Surface-sterilized seeds were cultured on MS (Murashige & Skoog 1962) medium with 3% sucrose and 0.7% agar, whose pH was adjusted to 5.7 ± 0.1. They were cultured in MS with no hormones added (MS0) until

plantlet regeneration as explants source for shoot and root initiation. The all treatment were cultured under a 16-hour photoperiod (50~70 µmol.m-2.s-1) at temperatures 20°C for 4 weeks.

4.3. Bud Break and Shoots Initiation

Two nodes from plantlets were isolated and cultured in MS media supplemented with 1 g/LBAP (benzylaminopurin); 0.1 g/L kinetin and 0.01 g/LNAA (naphtalenaceticacid) (M1);2g/LBAP, 0.1 g/L kinetin and 0.01 g/LNAA (M2) and 3g/LBAP, 0.1 g/L kinetin and 0.01 g/LNAA (M3) for 3 months incubation in culture room .Bud break initiation were establishedby sub-culturing at 4-week intervals in culture tubes, followed by a passage on growth regulator free medium to encourage their elongation for 3 months incubation. Sprouting ability was assessed as number of node per shoot.

4.4. Rooting

All treatment shoots regenerated from explants tissue were transferred to a rooting medium consisting of half-strength MS medium supplemented with 0.5g/L IBA. After 3 months of culture in rooting medium to get a plantlets, rooting was assessed as roots elongation.

4.5. Micro Propagation

Multiplication was regenerated from plantlets and cultured in media which have the best supplement treatment for shoot initiation, and transferred again to a rooting medium for re-multiplication.

5. Data Analysis

All experiments were set up in completely randomized design (Gomez & Gomez, 1984) and repeated thrice. Twenty-five axenic (no contamination) cultures were used per treatment per family for shoot regeneration from one node explants. Data pertaining to mean percentage of bud break, cultures showing shoot induction response in terms of average number of shoots per explant (sprouting ability), root length were recorded and analyzed and average of sprout number per nodule. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences ($P<0.05$) among the treatment means.

6. Result and Discussion

Laban seedlings (Fig. 1a) from young seeds of laban (green seeds) (Fig. 1b), obtained from mother plants in the field (Fig. 1c) inoculated on MS solid medium as explants source (Fig. 2a). Auxiliary shoots as explants were induced directly on MS medium supplemented with varying levels of hormone combinations M1, M2 and M3.

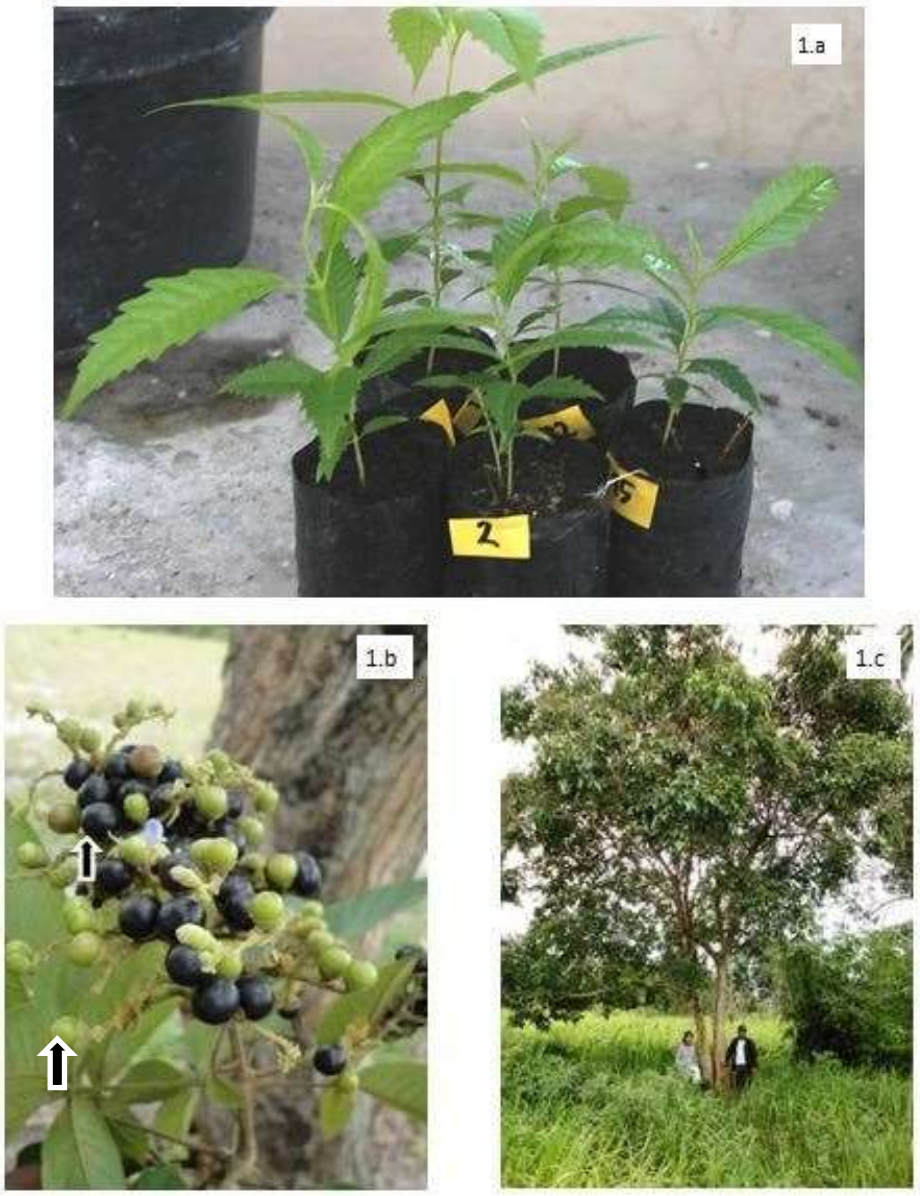

Figure 1. Explant sources for tissue culture propagation: auxiliary shoot from seedlings (1.a); mature seeds for seedling material (1.b); mother plant of medicinal pioneer tree of laban from non-cultivated grass land sites in Ketapang regency, West Kalimantan.

Bud breaks is one sign of explants response by hormone supplementation (Fig. 2b). The highest percentage of bud break was obtained on family 3 (98.20 %) (Table 1). However, there was no significant difference between the all media composition with respect to *in vitro* bud break, suggesting that endogenous compound of explants sources of laban has succeeded in providing nutrient and cytokine (BAP) for bud break induction. Juvenile tissues, usually embryos or explants taken from young seedlings, have been demonstrated to form large number of adventitious buds in culture when treated with cytokines (Tranvan, 1979). The development of bud break can be achieved by culturing the tissues firstly on medium supplemented with BAP to initiate the buds (6 months after transfer explants) followed by a passage on growth regulator free medium to encourage their elongation.

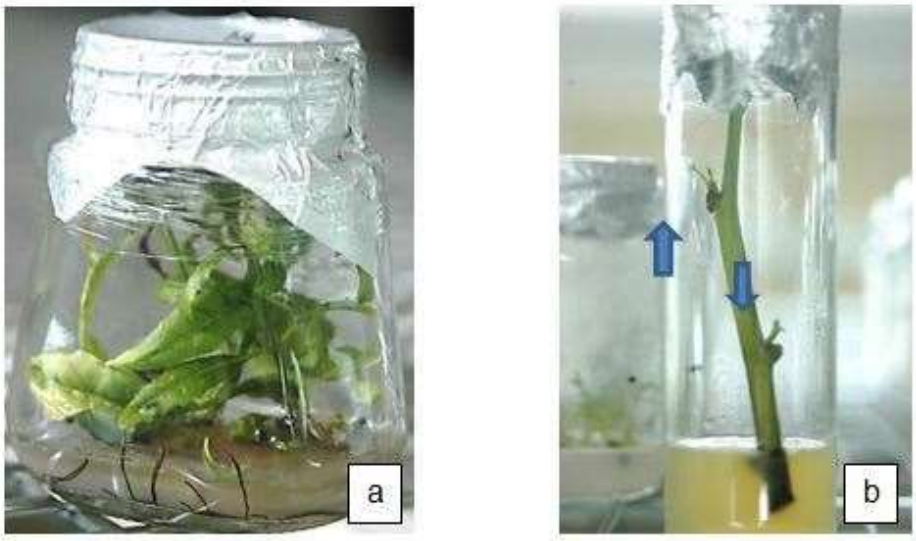

Figure 2. Laban plantlet as explants source (a) and buds break phase in explants initiation (arrows) (b).

No recent reports have described the regeneration of laban species. For first time, here, we report the direct regeneration of shoots from node segment of explant without intervening callus phase. The high average of shoots length were obtained in family 1 and family 5 at an optimal concentration of 1 g/L BAP on completion of 6 months cultured period at M1 medium (Table 1). Shoots grew after bud break in 100 % of explants node at every growth regulator treatments and control. It has been concerned that the regeneration potential of shoots elongation is influenced by the endogenous cytokines of explants BAP and Kinetine, however shoots length per explant could be increase by employing the balance of exogenous cytokines in the medium.

Comparison of bud break, shoot length, root length and sprout number in tissue culture regeneration media were shown in Table 1.Inclusion of low BAP (1 g/L) in the culture M1 of family 1 along with the optimum concentration of kinetin (0.1 g/L) and NAA (0.01 g/L) promoted a highest average of shoot elongation (3.79 cm ± 1.06) and promoted a highest average of roots elongation (5.36 cm ± 1.25) after transferred in half-strength MS medium supplemented with 0.5 g/L IBA. The application of this exogenous IBA promote100 % of explants have rooting initiation (Fig. 3). However, roots elongation development hamper by no exogenous IBA added in control culture medium. Auxine not only induces an increase in rooting percentage itself, but it requires a low concentration for improving root quality, avoiding bud dormancy. The absence of bud dormancy is essential for acclimatization and subsequent sprouting ability in shoots development phase (De Gyves et all., 2007).

Parameter	Fam. 1	Fam. 2	Fam. 3	Fam. 4	Fam. 5	Fam. 6	Fam. 7	Fam.8	Fam. 9	Fam. 10
Media M1										
Bud break (%)	96.33^{ns}	93.26^{ns}	**98.20^{ns}**	88.97^{ns}	90.87^{ns}	89.93^{ns}	89.38^{ns}	93.29^{ns}	88.98^{ns}	96.29^{ns}
Average shoot length (cm)	**3.79 ± 1.06***	2.54± 1.35*	2.44± 1.32*	2.14± 1.51*	**3.47± 1.25** *	1.89± 1.04*	2.08± 1.30*	2.27± 1.27*	2.51± 1.42*	2.20± 1.22*
Average root length(cm)	**5.36 ± 1.25***	3.88± 1.03*	3.73± 1.36*	3.90± 1.33*	4.88± 1.27*	3.59± 1.41*	4.10± 1.03*	3.31± 1.25*	3.28± 1.33*	3.62± 1.61*
Average node number per shoot	**8** *	5.8 *	6.7 *	6.5 *	6 *	6 *	6.2 *	5.8 *	5 *	6 *
Media M2										
Bud break (%)	89.87^{ns}	93.90^{ns}	86.98^{ns}	88.87^{ns}	90.34^{ns}	95.87^{ns}	92.65^{ns}	89.77^{ns}	97.54^{ns}	87.55^{ns}
Average shoot length(cm)	2.89± 1.65*	2.67± 1.33*	2.55± 1.03^{ns}	3.02± 1.42*	2.77± 1.16*	2.14± 1.22^{ns}	2.20± 1.32^{ns}	3.32± 1.67*	3.16± 1.54^{ns}	3.75± 1.65^{ns}
Average root length(cm)	3.98± 0.68^{ns}	3.26± 1.43^{ns}	4.17± 1.87^{ns}	3.49± 1.48^{ns}	2.87± 0.64^{ns}	3.31± 1.53^{ns}	2.74± 1.48^{ns}	2.89± 1.55^{ns}	3.21± 1.26^{ns}	4.52± 1.85^{ns}
Average node number per shoot	5.7^{ns}	5.3*	6.2^{ns}	5.7^{ns}	5.3^{ns}	5.0*	4.2^{ns}	5.3^{ns}	4.3^{ns}	5.0^{ns}
Media M3										
Bud break(%)	90.56^{ns}	94.40^{ns}	93.32^{ns}	90.75^{ns}	87.87^{ns}	90.87^{ns}	95.34^{ns}	90.34^{ns}	88.94^{ns}	92.63^{ns}
Average shoot length (cm)	3.10± 1.54^{ns}	3.53± 1.06^{ns}	2.89± 1.40^{ns}	2.58± 1.32*	2.52± 1.58^{ns}	3.51± 0.43^{ns}	3.21± 1.32^{ns}	2.60± 1.46^{ns}	2.50± 1.22^{ns}	2.48± 1.44^{ns}
Average root length(cm)	4.12± 1.55*	2.83± 1.52^{ns}	2.51± 1.17^{ns}	2.69± 1.30^{ns}	3.68± 1.42^{ns}	3.52± 1.04*	2.74± 1.58^{ns}	2.55± 1.14^{ns}	3.60± 1.33^{ns}	3.15± 1.00^{ns}
Average node number per shoot	5.2^{ns}	5.4*	5.0^{ns}	5.7^{ns}	5.0^{ns}	5.1^{ns}	4.8^{ns}	5.4^{ns}	4.8^{ns}	5.7^{ns}

Table 1. Comparison of bud break, shoot length, root length and node number in tissue culture regeneration media (M1, M2, M3), (n = axenic culture on every treatment).

* = significantly different at the 0.05 probability level; ns = not significant; the bold number is a high value in several treatment; ns = not significant.

Table 2. Analysis of variance summary for the effects of media on node number initiation.

Source	Degree of freedom	Sum of squares	Mean square	F value	Significant
Media	2	111.50	55.75	240.07*	0.01
Explants	72	16.72	0.23		
Total	74	128.22	55.75		

* = significant *F*-value at the 0.005 level

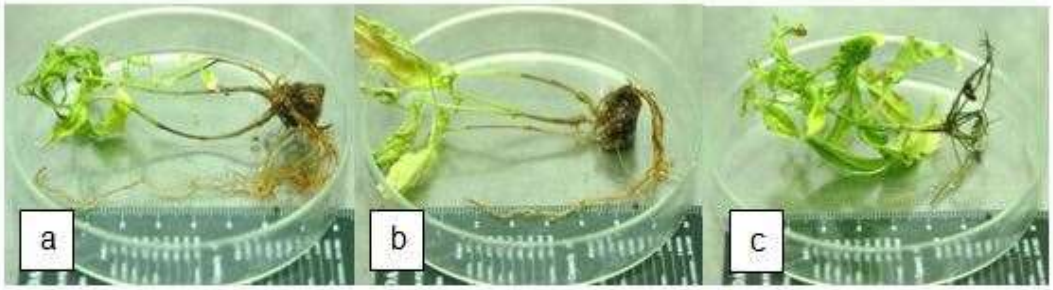

Figure 3. Rooting ability of plantlets after 6 months of roots initiation subculture, the high average of roots elongation was obtained from family 1 (5.36 cm ± 1.25) (a) and family 5 (4.67 cm ± 1.16) (b) in M1 media compared with in no hormone media (1,67 ± 1.00 (c)

Sprouting of laban *in vitro* have a low ability, all media treatment can only induce one shoot in all trees families, with various number of nodes. However, ability to regenerate 4-8 nodes in one shoots is high enough as multiplication source, its mean at least 4 times more plantlets from one explant to be examined *in vitro*. The highest average node number was in family 1 (8 nodes)at M1 medium (Table 1). Influence of BAP to sprouting ability was shown in Figure 4. Shoot number from one nodule of all control explants were high in medium without exogenous BAP, but no nodule in every shoot. This phenomenon shows that BAP do not only contribute to the shoots elongation, but also to the formation of nodules, more important in micro propagation. Analysis of variance summary for the effects of media on node number initiation were shown in Table 2. Medium M1, M2 and M3 have significant difference on node number initiation, M1 have best composition. Highest two periods moving average of node number was M1 (red line), than M2 (blue line) and M3 (green line).

Figure 4. Node number after 6 months of shoots initiation subculture, the highest average of nodes number was obtained from family 1 in M1 media (8 nodes from 1 shoot) compared with family 3 (6.2 nodes from 1 shoot) and no node from 3 shoots in control

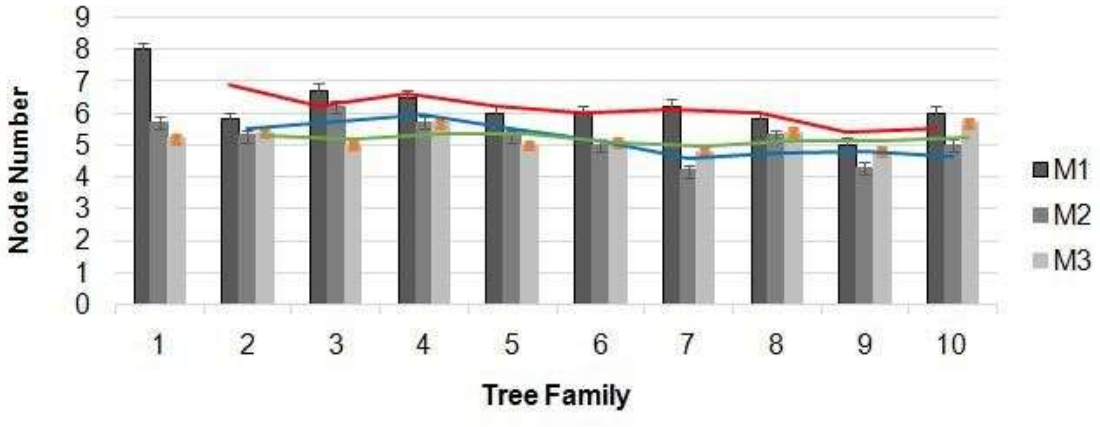

Figure 5. Nodes number of laban family after 6 months initiation incubation in media 1 (M1), media 2 (M2) and media 3 (M3), two periods moving average of M1 (red line), M2 (blue line) and M3 (green line)

Well grown healthy shoots and roots of laban plantlet, obtained on completion of 12 months culture period, have ready for next *in vitro*re-multiplication regeneration in the best medium for shoot and root initiation at previous experiment (Fig. 5).

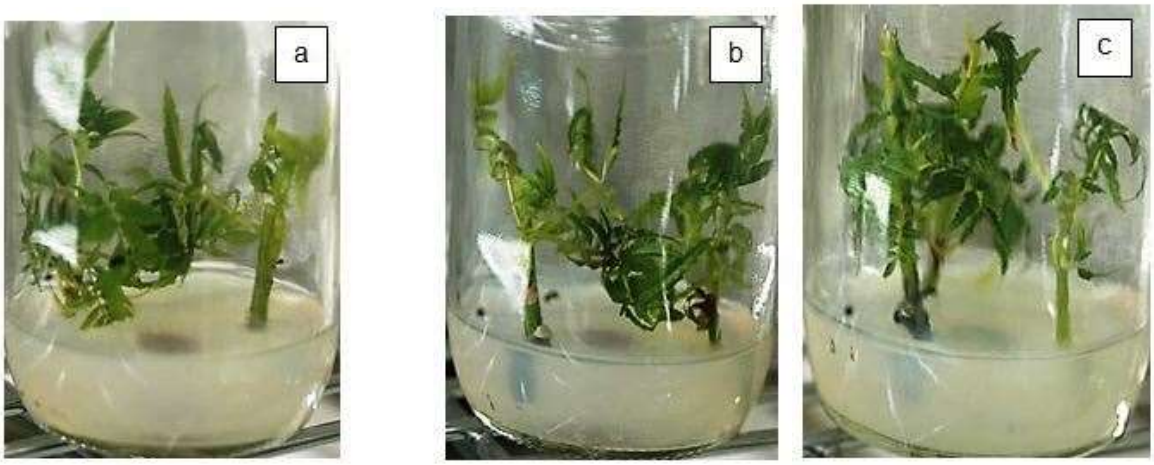

Figure 5. Re-multiplication of laban micropropagation from family 1: eight explants (a, b and c) were obtained from one sprout source.

Conclusion

The study revealed a part of micro propagation technique findings of laban (*Vitex pubescen)*, a medicinally important tree. The research conducted to date has demonstrated that balancing of exogenous-endogenous cytokine was important factor in high number of nodule as explant source multiplication for well grown healthy shoots and roots of laban. Low sprouting ability of laban *in vitro* is thought to affect the higher number acquisition of nodule, thus physiological reaction deserves further evaluation to provide alternative propagation technique for high quality of laban clones.

Acknowledgments

The authors are very grateful to Toni Herawan, Nur Syamsifor providing encouragement and help in carrying out this work, also sincerely thankful to Suprihati, Endin Izudin and Rudi Hartono for laboratory assistance.

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