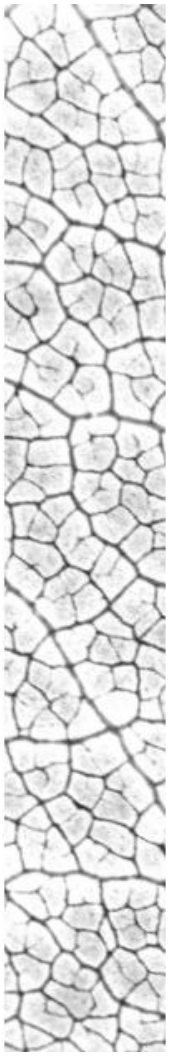

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Direct and indirect plantlet regeneration from explants of Albizia (*Albizzia falcataria*)

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Abstract
Background: The research described here developed plantlets through direct and indirect organogenesis of selected explants.
Materials and Methods: Sterile seeds were cultured in hormone-free Murashige & Skoog (MS) medium to generate explants. Nodal segments were cultured in MS basal medium for direct plantlet regeneration. Indirect plantlet regeneration through callus formation was done by culturing leaf, stem, and root explants. Explants from seeds were sterilized in 30% sodium hypochlorite for 20 minutes, 70% alcohol for 5 minutes, 20% sodium hypochlorite for 20 minutes, and 5% sodium hypochlorite.
Results: The best concentration of BAP for direct plantlet regeneration was 2 mgL^{-1}. Callus formation could be induced from the stem, leaf, and root explants; however, the largest amount of morphogenic callus was formed from stem explants. Albizia morphogenic callus had a friable and nodular structure, compact, shiny, with a yellowish color. This morphogenic callus regenerated into plantlets.
Conclusions: Complete plantlets from direct and indirect in vitro regeneration were ready for acclimatization.

Keywords: Albizia, *Albizzia falcataria*, callus, plantlets, regeneration

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INTRODUCTION

Paraserianthes falcataria (L.) Nielsen is formerly known as *Albizia falcataria* (L.) Fosberg (Soerianegara and Lemmens (1993) is an indigenous plant species of the eastern provinces of Indonesia. Albizia or sengon (the Indonesian name) is found scattered around South Sulawesi, Maluku, and Papua (Martawijaya et al. 1989). It can grow on a wide range of soils, including dry soils, damp soils, and even on salty to acid soils as long as drainage is adequate (Soerianegara and Lemmens 1993). Because of its nitrogen-fixing ability, this species is categorized as a pioneer plant and is commonly planted for reforestation and afforestation to improve soil fertility (Heyne 1987). Albizia plays an important role in both commercial and traditional farming systems. The main albizia cultivation areas are in Sumatra, Java, Bali, Flores, and Maluku (Charomaini and Suhaendi 1997). It is an important commercial timber species, used in both the pulp and paper industry and furniture. It is also a source of veneer and plywood, and is very suitable for the manufacture of particleboard, wood-wool board and hardboard, and has recently been used for blackboards.

Although albizia has many potential uses and is widely planted throughout the eastern part of Indonesia, and in Java, the tree improvement programs are still a slow process with regeneration from seed remaining the most common method of propagating this species. Therefore, it is important to develop alternative methods to improve the genetic quality of the planting stock and provide for fast propagation. *In vitro* propagation methods offer highly efficient tools that can be used for the mass multiplication of albizia.

Techniques, such as *in vitro* propagation, can be used to obtain offspring from parent trees that have genetic advantages (Zobel and Talbert 1984). Tissue culture technology is a breakthrough technology in cultivating biotechnology. The power of this technique is such that it is often associated with the birth of a second green revolution (Hartman et al. 1990). This is because it allows for the mass propagation of superior mother trees, increasing the quality of albizia timber and significantly increasing the efficiency of management because of high levels of homogeneity.

The *in vitro* regeneration procedure for albizia was successfully developed through direct organogenesis of axillary shoots (Widiyanto et al. 2008, Perveen et al. 2013). The current approach to *in vitro* regeneration and multiplication of *A. falcataria* was reported by axillary

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shoot organogenesis from young leaflet explant cultures (Ghosh et al. 2010). The use of hormones, such as cytokines (benzylaminopurine (BAP) and 6-furfurylaminopurine (kinetin), could be the critical factor in stimulating *in vitro* shoot development of albizia (Bon et al. 1998, Sasmitamihardja et al. 2005). The role of cytokines was also reported in axillary shoot development of node micro-cuttings of other leguminous tree species, such as *Pterocarpus marsupium* (Chand and Singh 2004), *Albizia odoratissima* (Rajeswari and Paliwal 2006), and *Albizia chinensis* (Sinha et al. 2000). Meanwhile, indirect organogenesis via callus formation was reported by Tomar and Gupta (Tomar and Gupta 1988). Callus was induced from hypocotyl explants in Gamborg B5 medium containing 10-7 - 10-5 M BAP. Callus formation for the induction of somatic embryogenesis in *Falcataria moluccana* was obtained from mature leaves (Sunandar et al. 2017).

The work described in this paper was the development of an albizia *in vitro* regeneration system for plantlet production. Both organogeneses via direct and indirect plantlet formation were described in this paper. Direct plantlet production involved Murashige & Skoog (MS) basal medium supplemented by BAP at various concentrations. Indirect plantlet regeneration was achieved through the induction of morphogenic callus. The response of various explants of albazia was explored in induction callus media and their callus characteristics were examined based on morphological parameters.

MATERIALS AND METHODS

Sterile explants from selected phenotypically superior seed

Seeds were collected from albizia mother trees. Selection of mother trees was based on the growth and quality of wood, with the following criteria: (a) rapid growth, (b) large diameter and solid structure, (c) straight stems, and (d) resistance to disease. Seeds were selected from pods of mature physiological age, that were uniform, sunk when placed in water and had a percentage of germination > 80%. This latter capability was evaluated using a Petri dish germination assay on paper. The albazia seeds were soaked overnight in tap water and drained, before being soaked in a 2 gL^{-1} fungicide solution for 1 hour. The seeds were then sterilized using one of 3 protocols: (Protocol I) 70% alcohol for 5 minutes, 10% sodium hypochlorite (commercial bleach) for 10 minutes, 15% sodium hypochlorite for 20 minutes, and 2% sodium hypochlorite; (Protocol II) 30% sodium hypochlorite for 20 minutes, 70% alcohol for 5 minutes, 20% sodium hypochlorite for 20 minutes, and 5% sodium hypochlorite; and (Protocol III) 25% sodium hypochlorite for 10 minutes, 70% alcohol for 5 minutes, 20% sodium hypochlorite for 20 minutes, and 2% sodium hypochlorite. Experiments were designed based on a completely randomized design (CRD). Each treatment was repeated 3 times and there were 10 bottles of culture for each replication.

Direct plantlet regeneration of albizia in media containing BAP

Sterile *in vitro* grown seedling 3 - 5 cm in height and about 7 - 10 days old were cut into node segments. Each piece was planted in MS medium containing 30 gL^{-1} sucrose, 7.5 gL^{-1} agar, and BAP (1, 2, or 3 mgL^{-1}). Media was sterilized at a temperature of 121 °C for 15 minutes and adjusted to pH 5.6 after sterilization. Each treatment, consisting of 5 cultures, was repeated 3 times. Cultures were prepared following CRD and incubated at 25 °C with cyles of 16 hours of light and 8 hours in dark. Once bud formation had taken place, cultures were transferred to MS basal medium containing 1.0 mgL^{-1} naphthalene acetic acid (NAA) for shoot elongation and root formation. Data were examined by analysis of variances (ANOVA) using SAS program version 6:12. If there was a treatment effect, the analysis was continued using a test of Least Significant Difference (LSD) at a 5% significance level to distinguish the average value of the mean.

Indirect plantlet regeneration of albizia via morphogenic callus formation

Callus induction

A sterile albizia seedling 3 - 5 cm was cut into leaf, stem, and root segments. Each piece was planted in an induction callus medium containing 30 gL^{-1} sucrose, 7.5 gL^{-1} agar, and 2 mgL^{-1} 2,4-dichlrophenoxyacetic acid (2,4-D). Media were sterilized at a temperature of 121 °C for 15 minutes and adjusted to pH 5.6 after sterilization. Each treatment, consisting of 5 cultures, was repeated 3 times. Cultures were prepared following CRD and incubated at 25 °C with cyles of 16 hours of light and 8 hours in dark. Subculturing was performed every 4 weeks for callus multiplication. Treatments were evaluated by measuring the percentage of callus formation. Morphology of callus was examined using a digital microscope NTSC System- DC2-456 connected to a computer.

Callus regeneration

Morphogenic callus was transferred into MS medium containing 30 gL^{-1} sucrose, 7.5 gL^{-1} agar, and 3 mgL^{-1} BAP. Subculturing was done every 4 weeks in the same media. Data were analyzed with ANOVA using SAS program version 6:12. If there was a treatment effect, the analysis continued by using a test of the Least Significant Difference (LSD) at a 5% significance level to distinguish the average value of the mean.

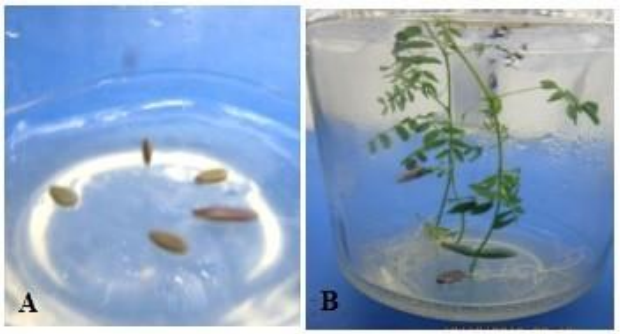

Fig. 1. (A) Sterile seed and (B) Seedling explants of albizia established from selected plant material

Table 1. Percentage of sterile albizia explants and percentage of contamination in MS medium containing seeds from different sterilization protocols

Treatments	% of sterile and viable explants	% contamination
Proto I: Al 70%, Sh 10%, Sh 15%, Sh 2%	68.41 a*	29.59
Proto II: Sh 30%, Al 70%, Sh 20%, Sh 5%	98.00 b	0.00
Proto III: Sh 25%, Al 70%, Sh 20%, Sh 2 %	90.86 b	7.14

Table 2. Single column table on top of a column

BAP Concentration	% Explants grown	Mean number of shoots per explant
1.0 mgL^{-1}	56.25a*	2
2.0 mgL^{-1}	74.99b	7
3.0 mgL^{-1}	72.22b	5

Means followed by the same letter in the same column were not significantly different at a 5% Least Significant Difference (LSD)

RESULTS AND DISCUSSION

Establishment of sterile explants

Sterile seedlings provided alternative explants for the mass production of albizia plantlets **(Fig. 1)**. Seed sterilization was more successful and easier compared to sterilizing leaves, buds, or stems, especially for woody trees. The most important thing is that sterile seedlings provided a juvenile source of an explant from all organs (roots, cotyledons, hypocotyls, node segments, leaves). Of the 3 methods of seed sterilization, protocol II was the most effective technique. Using Protocol II, no explants were contaminated, and about 98% of the explants remained sterile and viable (**Table 1**). By comparison, Protocol I resulted in 29.59% contamination and only 68.41% of explants remained sterile and viable. Protocol III, a variation on Protocol II gave 90.86% sterile explants. Protocol II was the best protocol for establishing sterile explants of albizia from seeds. Protocol II contained a higher concentration of sodium hypochlorite than the protocol I or protocol III. Tomaszewska-Sowa and Figas (2011) found that among the tested methods of sterilization in *Silphium perfoliatum* L., 30% ACE commercial bleach, of which a component is sodium hypochlorite) proved to be the most effective for sterilization.

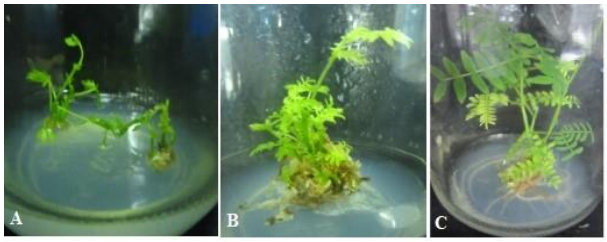

Fig. 2. Plantlets of albizia: (A) Plantlet from nodal segments, (B) Rooting plantlet, (C) Complete plantlet ready for acclimatisation

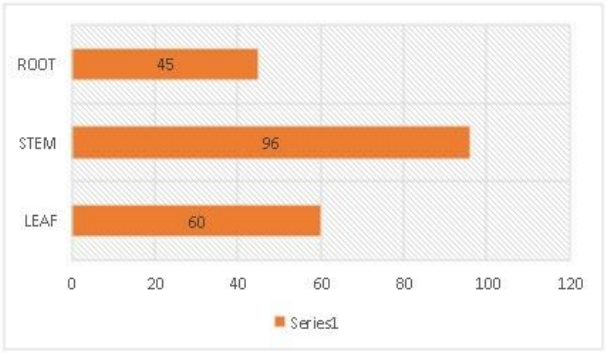

Fig. 3. Percentage of albizia callus formation from different sources of explant (leaf, stem, and root)

Direct plantlet regeneration

Induction of buds from node segment explants of albizia required cytokines of a specific concentration. BAP at 2.0 mgL-1 was the best concentration for direct shoot formation, with about 75% explants growing and producing 7 shoots per explant (**Table 2**). Concentrations of BAP < 2.0 mgL-1 were less effective in inducing shoot formation and growth of explants. At this concentration even though buds were induced on more than 50% explants, the total number of shoot per explant was very few. Most explants produced roots after 8 weeks in MS media containing 1.0 mgL-11-naphthaleneacetic acid (NAA). Complete plantlets with adequate roots and shoots (**Fig. 2**) were ready for acclimatization after 10 - 12 weeks.

Indirect plantlet regeneration

Callus of albizia could be induced from explants of leaves, stems, and roots in MS basal medium containing 2 mgL^{-1} 2,4-D. However, callus induction in albizia was influenced by the source of the explant. Stem explants from sterile seedlings were the most successful in forming callus, about 96% of stem explants were able to form a callus. The poorest explant was rooting, only 45% of explants grew into callus; while 60% of leaf explants showed callus formation (**Fig. 3**). Explants started to enlarge after 1-2 weeks of culture and formed callus (initial callus; **Fig. 4A**) at 3-4 weeks of culture.

The callus continued growing and showed active cell division and proliferation, leading to nodular callus formation (**Fig. 4D**). Further growth of the callus which

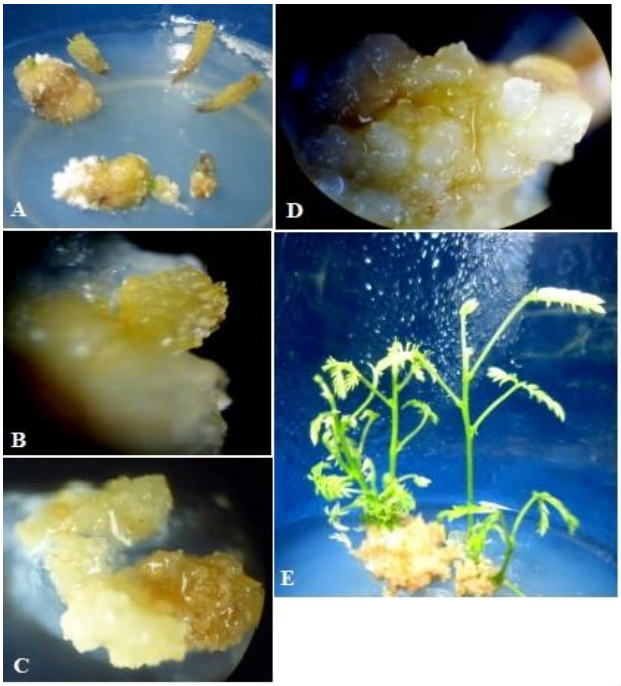

Fig. 4. Callus development of albizia stem explants: (A) explant enlarged leading to callus formation, (B-D) morphogenic callus, (E) morphogenic callus with shoots

was yellowish in color, friable, nodular, rough, shiny, and transparent led to organogenesis (**Fig. 4B-D**). This kind of callus was described as a morphogenic or organogenic callus.

Morphogenic callus after being transferred to regeneration medium, supplemented with 3 mgL^{-1} BAP, underwent bud formation and produced shoots after 2-3 weeks (**Fig. 4E**). Roots emerged from the basal surface of shoots, with or without NAA addition, resulting in complete plantlets.

Clonal propagation through *in vitro* technology in woody trees has been intensively investigated recently. *In vitro* shoot, culture protocols have been developed by Rathore et al. (2004) for several trees, such as *Balanites aegyptiaca* (Balanitaceae), Hingota; *Citrus limon* (Rutaceae), Nimbu; and *Syzygium cuminii* (Myrtaceae), Jamun. In the case of albizia (A. falcataria or P. falcataria) there were reports of induced shoot proliferation from leaflets, node segments, and cotyledon explants in *in vitro* culture with cytokines and auxin (Sinha and Mallick 1993, Sasmitamihardja et al. 2005, Widiyanto et al. 2008, Ghosh et al. 2010, Kim et al. 2015). Most recently Sunandar et al. (2017) reported on *in vitro* culture of albizia via somatic embryogenesis. *In vitro* technology has become a promising plant regeneration technique for the mass production of woody plant seedlings, including albizia.

In the research reported in this paper, direct organogenesis of albizia through shoot induction was achieved from node segment explants taken from sterile seed-derived seedlings of selected mother plants. Direct organogenesis without callus intervention maintained the genetic stability of offspring and preserved the parental genetic background. The highest shoot proliferation of albizia was obtained from medium supplemented with 2 mgL^{-1} BAP. The effectiveness of BAP in inducing shoot proliferation was reported in many plant tree species, such as in other species of the genus *Albizia* (Bon et al. 1998, Sinha et al. 2000, Rajeswari and Paliwal 2006, Borthakur et al. 2011), in *Acacia mangium* Willd (Bon et al. 1998), in *Pyrus pyrifolia* Nakai (Kadota and Niimi 2003), in *Melia azedarach* L. (Vila et al. 2004), and in *Premma serratifolia* L. (Singh et al. 2011). The influence of BAP on shoot proliferation of albizia or other woody plants depended on its concentration and the type of explant. For example, in *Albizzia odoratissima* (L.f.) Benth the effective concentration of BAP was 0.75 mgL^{-1} to induce shoot proliferation from apical bud explants (Borthakur et al. 2011). In Melia azedarach L. 1.0 mgL^{-1} BAP was the best concentration for direct shoot regeneration from leaf explants (Vila et al. 2004). However, Premma serratifolia L. required 3 mgL^{-1} BAP combined with 0.15 mgL^{-1} kinetin to induce adventitious buds from stem explants (Singh et al. 2011).

Even though direct plant regeneration from albizia is a useful method to produce plantlets, the concern is that with this technique they cannot be produced in a large enough quantity. Aside from somatic embryogenesis, indirect plant regeneration via callus formation is still preferable for mass production. This method also produces less genetic variability compared to somatic embryogenesis. Callus formation was initiated from various explants of albazia and placed in MS medium with 2 mgL^{-1} 2,4-D. Callus initiation of albazia occurred on the surface of explants. Observation showed that the callus of albazia was of hard consistency, cream or yellowish in color, friable, shiny, and with a nodular structure (**Fig. 4B-D**). Active cell proliferation led to the development of substantial volumes of callus. This callus could regenerate and become complete plantlets.

Quality of callus is believed to be related to the morphogenic or embryogenic capability. In some plants, the morphogenic callus was characterized by its appearance, such as white-yellowish, compact, nodular, and friable (Gill et al. 2004, Noor et al. 2005, Sumaryono et al. 2008). Tiwari and Tripathi (2005) described organogenic callus in *Glycine max* as a mixture of creamish and light green calli which was dense and glossy in texture. While, Schiff et al. (2009) reported that one of the callus types in *Foeniculum vulgare* was green, dark coloured, compact, and highly morphogenic. Gatz and Kowalski (2011) found that the morphological features of callus in pepper were smooth surfaced, compact (large degree of cell cohesion), and green color.

Despite growth regulators playing a major role in callus formation, induction of albazia callus was also dependent on the source of the explants. Root explants,

for example, produced less callus compare to stem explants (**Fig.3**). Leaf explants were also responsive to callus formation. However, the most suitable explant was from the stem. Sakpere et al. (2014) found that stem and leaf explants produced a higher percentage and larger size of callus in *Telfairia occidentalis* Hook F. compared to nodal segments.

CONCLUSION

This research demonstrated an efficient method for the establishment of sterile explant material of albizia (*Albizzia falcataria*) from superior mother plants. Direct plantlet regeneration was established from explants of nodal segments and indirect plantlet regeneration was achieved from explants of stem, leaf, and root. The best method of sterilization for albizia explant material was 30% sodium hypochlorite for 20 minutes, 70% alcohol for 5 minutes, 20% sodium hypochlorite for 20 minutes and 5% sodium hypochlorite. Robust direct shoot regeneration was obtained by culturing explants of nodal segments in MS medium contained 2 mgL^{-1} BAP with subsequent culturing in MS medium with 1.0 mgL^{-1} NAA for rooting. Indirect plant regeneration was successfully achieved through induction of morphogenic callus, which had the following characteristics: nodular and friable structure, compact, shiny with yellowish colour. The highest production of morphogenic callus was from stem explants in MS medium supplemented with 2 mgL^{-1} 2,4-D. Morphogenic callus had the capacity to regenerate and become complete plantlets. Considering albizia is a very important timber plant, a supply of superior seedlings in adequate numbers is required. Future research on mass propagation using morphogenic callus for somatic embryogenesis, and optimization of the acclimatization phase, is highly recommended.

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