



Regeneration of *Pogostemon cablin* Benth. 'Sidikalang' through Indirect Organogenesis and Shoot Multiplication for Production of True-to-Type Plant

Regenerasi Nilam Aceh 'Sidikalang' melalui Organogenesis tak Langsung dan Multiplikasi Tunas dalam Produksi Tanaman True-to-Type

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ABSTRACT

Patchouli *Pogostemon cablin* Benth. 'Sidikalang' (Acehnese Patchouli) is a member of the Lamiaceae, mint family. Aromatic oil known as patchouli oil can be produced from its leaves, which is highly valued in the perfumery and aromatherapy industry, because of its aromatic spicy fragrance. Patchouli oil also has various phytochemicals that have therapeutic effects including antimicrobial, antidepressant, anti-inflammatory, and antioxidant. This study aims to establish an efficient and reproducible protocol for indirect regeneration from leaf explants and multiple shoots from nodal explants. Indirect organogenesis was done using solid medium of MS (Murashige-Skoog) with some treatments, such as 0.5 mg/L NAA + 0.1 mg/L BAP, 1 mg/L NAA + 0.1 mg/L BAP, and 1.5 mg/L NAA + 0.1 mg/L BAP, whereas multiple shoots from nodal explants were cultured on MS medium with various concentration of BAP, such as 0.5 mg/L, 1.0 mg/L, and 1.5 mg/L. Then, for the rooting stage from shoots, the shoots were cultured on half-strength MS medium without Plant Growth Regulator (PGR) and MS medium with some treatment, such as without PGR, 0.5 mg/L IBA, and 0.5 mg/L NAA, respectively. Furthermore, the plantlets derived from the *in vitro* rooting stage treatment were acclimatized onto a combination of soil: compost (1:1). In addition, *in vitro* shoots were also planted directly as micro-shoot cuttings on combination soil: compost (1:1 ratio). The optimum treatment for indirect organogenesis was on MS medium supplemented with 1 mg/L NAA + 0.1 mg/L BAP. For multiple shoots from nodal explant, MS medium supplemented with 0.5 mg/L BAP was the optimum medium. Shoots were cultured in a half-strength MS medium for the rooting stage and grew to form plantlets with normal root morphology. Overall, patchouli plantlets were obtained more quickly by directly planting micro-shoot cuttings in *ex vitro* conditions rather than going through the *in vitro* rooting stage.

Keywords:

indirect organogenesis, multiple shoots from nodal explants, *Pogostemon cablin* Benth.

ABSTRAK

Kata Kunci:

Organogenesis tak langsung, tunas majemuk dari eksplan nodus, *Pogostemon cablin* Benth.

Nilam Aceh 'Sidikalang' (Acehnese Patchouli) merupakan salah satu anggota kelompok dari Lamiaceae, famili mint. Minyak aromatik yang biasa disebut juga dengan minyak patchouli dapat diproduksi dari daunnya, yang mana bernilai tinggi dalam industri parfum dan aromaterapi, karena wangi aromatik yang segar. Minyak patchouli juga memiliki variasi senyawa fitokimia yang memiliki efek terapeutik, seperti antimikroba, anti-depresan, anti-inflamasi, dan antioksidan. Penelitian ini bertujuan untuk menentukan protokol yang efisien dan reproduktibel untuk regenerasi tak langsung dari eksplan daun dan tunas majemuk dari eksplan nodus. Organogenesis tak langsung telah dilakukan menggunakan medium MS (Murashige-Skoog) dengan beberapa perlakuan, seperti NAA 0,5 mg/L + BAP 0,1 mg/L, NAA 1 mg/L + BAP 0,1 mg/L, dan NAA 1,5 mg/L + BAP 0,1 mg/L, sedangkan tunas majemuk yang dihasilkan dari eksplan nodus dikulturkan pada media MS dengan berbagai variasi konsentrasi BAP, yakni 0,5 mg/L, 1,0 mg/L, dan 1,5 mg/L. Untuk tahap perakaran dari tunas, tunas akan dikulturkan pada media ½ MS tanpa Zat Pengatur Tumbuh (ZPT) dan media MS dengan beberapa perlakuan, yakni tanpa Zat Pengatur Tumbuh (ZPT), IBA 0,5 mg/L, dan NAA 0,5 mg/L. Selanjutnya, planlet yang dihasilkan dari tahap perakaran secara *in vitro* akan diaklimatisasi pada kombinasi tanah : kompos (1:1). Sebagai tambahan, tunas *in vitro* juga akan ditanam secara langsung sebagai stek tunas mikro pada kombinasi tanah : kompos (1:1). Perlakuan optimal untuk organogenesis tak langsung menggunakan media MS dengan NAA 1 mg/L + BAP 0,1 mg/L. Dalam perbanyakan tunas majemuk dari eksplan nodus, media MS dengan BAP 0,5 mg/L merupakan media optimum. Tunas yang dikulturkan pada media ½ MS untuk tahap perakaran, tumbuh membentuk planlet dengan morfologi akar normal. Secara keseluruhan, planlet nilam lebih cepat terbentuk melalui penanaman langsung stek tunas mikro pada kondisi *ex vitro* dibandingkan melalui tahap perakaran *in vitro*.



INTRODUCTION

Nowadays, patchouli oil from Acehese patchouli (*Pogostemon cablin* Benth. 'Sidikalang') leaves is at the highest consumption level of essential oil. Patchouli oil plays a significant role within the cosmetic and perfume industry since it has fixative properties (scent binders) and therapeutic materials. So, patchouli oil is used as the main raw material for those products. The higher quality of patchouli oil can be produced from Acehese patchouli, which can help to expand the utilization of essential oils as industrial raw materials. In this manner, the need for patchouli oil in the world market will increase in cosmetic and fragrance products.

The propagation of patchouli presents several problems which affect the yield of essential oil and biomass. The susceptibility of plants to various pathogens such as viruses, fungi, and bacteria can cause the low production of essential oils. Hidayah et al., (2021), compared the content of patchouli oil from patchouli leaves grown under *ex vitro* and *in vitro* conditions. The results proved that Aceh patchouli leaves under *in vitro* conditions were able to produce twice as much patchouli oil (40 mL) than Aceh patchouli leaves in *ex vitro* conditions (26 mL). Furthermore, the good quality of patchouli oil depends on the propagation strategy, which is in this case, the plant tissue culture technique as micropropagation method used to obtain uniform patchouli leaves for commercial purposes (Jin et al., 2014; Kusuma & Mahfud, 2017; Nuryani, 2006; Van Beek & Joulain, 2018).

Thus, *in vitro* propagation (micropropagation) is an alternative method that can be applied to obtain pathogen-free plants and allows it to be carried out on a large scale in a relatively short time, so it is more effective for the rapid multiplication of plant species. The

addition of exogenous growth regulators is commonly used in plant tissue culture to increase the production of secondary metabolites and biomass. The response to plant growth regulators depends on the type of explant tissues and plant species. For example, auxin is very ideal for callus induction (indirect organogenesis) and a low concentration of cytokinin can promote shoot differentiation from callus (Mayerni, 2020; Nakasha et al., 2016; Saha et al., 2020). Several previous studies indicated that the production of true-to-type Acehese patchouli was initiated by regeneration through indirect organogenesis with growth regulators, by adding various concentrations of Naphthalene Acetic Acid (NAA) as auxin and 6-BenzylAmino Purine (BAP) as cytokinin in solid Murashige-Skoog (MS) medium. Then, Acehese patchouli shoot explants from callus were transferred to MS medium for rooting induction with the addition of Indole-3-Butyric Acid (IBA) and NAA as auxin, also full strength and half-strength MS medium without auxin. IBA is an auxin used to initiate callus and somatic embryogenesis stimulants, but in a lesser extent (Ipekci & Gozukirmizi, 2004; Nakasha et al., 2016; Rai et al., 2007; Rathore et al., 2011).

There are two types of organogenesis, i.e direct and indirect organogenesis. Growth of *in vitro* plant cells through callus formation by utilizing various types of tissues sources as explants is indirect organogenesis, while direct organogenesis is *in vitro* approach without the callus stage (Lalthafamkimi et al., 2021). This study aims to find out the most appropriate media for growing callus and root of Acehese patchouli through indirect and direct organogenesis, respectively.

Patchouli is commonly propagated by *ex vitro* stem cuttings or from directly acclimatized *in vitro* plantlets. Hence, this study was also carried out to determine

whether *in vitro* shoot cuttings explants were able to grow when transferred directly into *ex vitro* conditions (without roots).

METHODOLOGY

Materials and Methods

This research was carried out at the University of Surabaya, Jalan Kalirungkut. Some tools were used in this research, such as Laminar Air Flow (LAF) Cabinet, beaker glass, measuring cylinder glass, culture bottle, microwave, precision scale, pH meter, tweezers, scissors, lighter, and Bunsen burner, whereas the materials used in this research were leaves explants of Acehese Patchouli (*Pogostemon cablin* Benth.) var. Sidikalang, MS medium, phytigel, sucrose, Naphthalene Acetic Acid (NAA), Indole-3-Butyric Acid (IBA), 6-BenzylAminoPurine (BAP), 96% alcohol, soil, and compost.

Indirect Organogenesis

Explant of Acehese patchouli leaves *ex vitro* were cultured in solid Murashige-Skoog (MS) media containing 30 g/L sucrose and 2.8 g/L phytigel. Three types combination of growth regulators were added in the medium at different concentrations, such as 0.5 mg/L NAA + 0.1 mg/L BAP, 1 mg/L NAA + 0.1 mg/L BAP, and 1.5 mg/L NAA + 0.1 mg/L BAP. Each treatment medium was repeated five times with five explants in each culture bottle. After shoots formation emerged, they were transferred onto the shoot multiplication medium. Then, the roots were induced under *in vitro* conditions. After that, the plantlets were transferred onto *ex vitro* conditions for acclimatization.

Shoots Multiplication and Rooting

Acehese patchouli shoot explants from callus were transferred and cultured in a solid Murashige-Skoog (MS) medium containing 30 g/L sucrose and 2.8 g/L phytigel. MS medium was fortified with two types of auxins such as IBA and NAA at varied levels (0.5 ppm IBA and 0.5 ppm

NAA). Moreover, shoot explants were also cultured in solid media, such as full-strength MS PGR-free medium (MS₀) and half-strength MS PGR-free medium (¹/₂MS₀). Therefore, there were four types of media used for shoot multiplication repeated ten times containing three shoots explants in each culture bottle. There were two observed parameters, which are plantlet root morphological feature and mean fresh weight (g).

Microshoot Cuttings

Plantlets of Acehese patchouli *in vitro* were collected six weeks after initiation, and the micro-cuttings were transferred onto soil: compost (1:1 ratio) mix and vermiculite medium under *ex vitro* conditions. In both media, three replications were carried out with one shoot in each pot. The number or percentage of rooting and growing micro-shoots indicate the success of the growth of micro-shoot cuttings under *ex vitro* conditions.

Data Statistics Analysis

One-way Analysis of Variance (ANOVA) was used in data analysis using the SPSS program. Differences between treatments were identified by Fisher's Least Significant Difference (LSD) test at 5% significance level.

RESULTS AND DISCUSSION

Effects of PGRs on Shoot through Indirect Organogenesis from Leaves Explant of Acehese Patchouli (*Pogostemon cablin* Benth.) var. Sidikalang

Effects of PGRs on the shoot through indirect organogenesis from leaves explant of Acehese Patchouli (*Pogostemon cablin* Benth.) var. Sidikalang is shown in Figure 1 and Table 1 below.

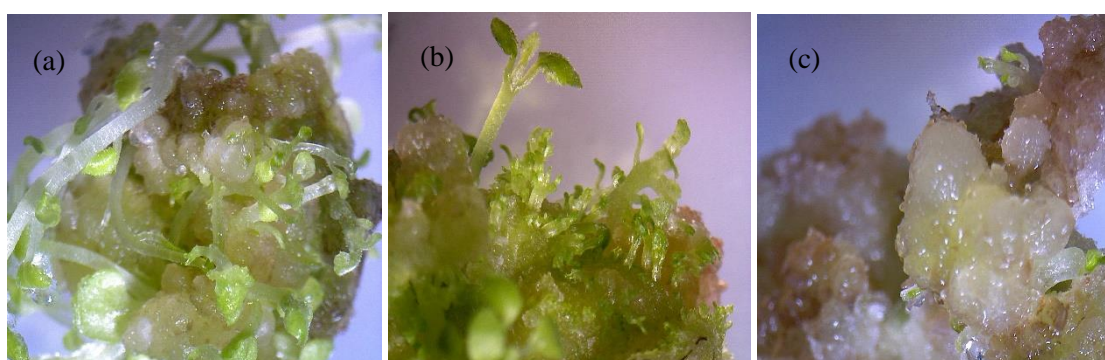


Figure 1. Indirect organogenesis of Acehese patchouli (*Pogostemon cablin* Benth.) var. Sidikalang in MS medium supplemented with: (a) 0.5 mg/L NAA + 0.1 mg/L BAP; (b) 1 mg/L NAA + 0.1 mg/L BAP; (c) 1.5 mg/L NAA + 0.1 mg/L BAP on 35 days after planting

Figure 1 shows indirect organogenesis methods for micropropagation of Acehese patchouli. This method used *ex vitro* Acehese patchouli leaves as the explants to produce callus in MS medium supplemented with various concentrations of 0.5 mg/L, 1 mg/L, and 1.5 mg/L of NAA, respectively, in combination with 0.1 mg/L BAP. The calli produced in those three medium formulations were yellowish and compact. The combination of plant growth regulators (PGRs) and type of explant gave a massive impact on the response of callus

induction. According to Jin et al. (2014), the type of explants such as leaf segments and nodal stem segments were the most responsive with the highest shoot regeneration frequency reaching up to 100%. Visually, based on Figure 1, MS medium supplemented with the combination of 1 mg/L NAA and 0.1 mg/L BAP was the best medium because it produced morphogenic callus (greenish, organogenic, and compact calluses) indicated by the initiation growth of shoots at the medium.

Table 1. Effect of PGR on the shoot through indirect organogenesis from leaves explant

MS + PGR	% calli	% shoots	Number of shoots (per explant)	Shoot length (cm)
0.5 mg/L NAA + 0.1 mg/L BAP	100	100	21±2.77 ^b	10.2±0.14 ^b
1 mg/L NAA + 0.1 mg/L BAP	100	100	36±3.59 ^c	7.5±0.63 ^c
1.5 mg/L NAA + 0.1 mg/L BAP	100	100	10±1.62 ^a	3.6±0.71 ^a

Different letter notations indicate that the data is significantly different based on the Least Significance Difference (LSD) test at $\alpha=0.05$

Shoot multiplication of Acehnese patchouli (*Pogostemon cablin* Benth.) var. Sidikalang is shown in Figure 2.

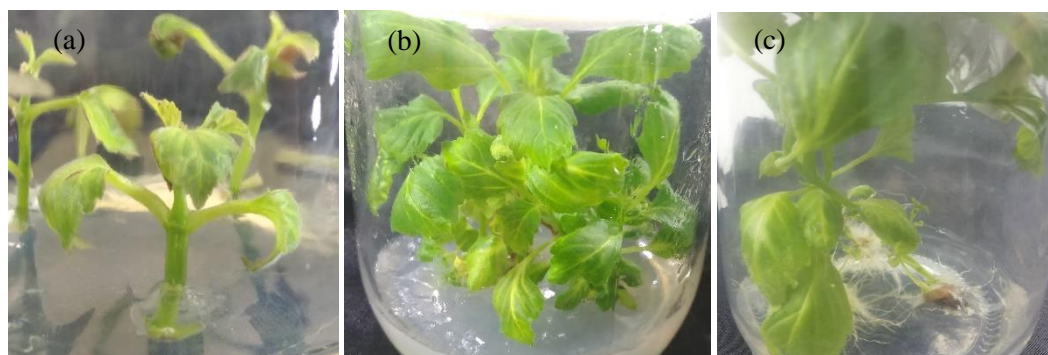


Figure 2. Shoots multiplication of Acehnese patchouli (*Pogostemon cablin* Benth.) var. Sidikalang : (a) single nodus, (b) multiple shoots, (c) plantlet

Effect of BAP on shoot proliferation Acehnese patchouli (*Pogostemon cablin* Benth.) var. Sidikalang 35 days after planting is shown in Table 2.

Table 2. Effects of BAP on shoot proliferation 35 days after initiation

MS + PGR	Number of shoots (per explant)	Shoot length (cm)
0.5 mg/L BAP	26±1.98	6.81±0.85
1.0 mg/L BAP	38±2.87	2.46±0.97
1.5 mg/L BAP	47±1.65	1.8±0.15

Effects of various concentrations of BAP on shoot proliferation of Acehnese patchouli (*Pogostemon cablin* Benth.) var. Sidikalang 35 days after planting is shown in Figure 3.

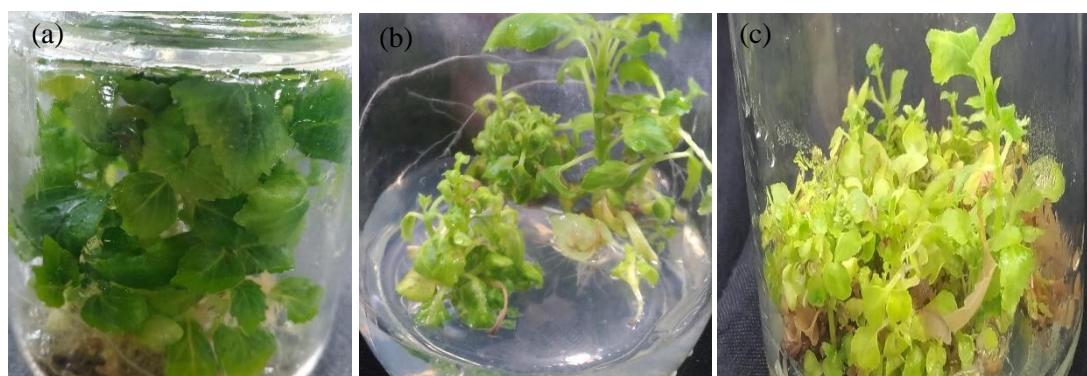


Figure 3. Shoot proliferation at various concentrations of BAP 35 days after planting: (a) 0.5 mg/L BAP, (b) 1.0 mg/L BAP, (c) 1.5 mg/L BAP

According to Table 1, the highest number of shoots (per explant) was in MS medium supplemented with 1 mg/L NAA and 0.1 mg/L BAP, whereas the longest of shoots emerged at the shoots were cultured at MS medium supplemented with 0.5 mg/L NAA and 0.1 mg/L BAP. Meanwhile, Figure 2 shows the other one

of micropropagation method of Acehese patchouli through *in vitro* shoots multiplication. This method would produce Acehese patchouli plantlet faster than indirect organogenesis. In contrast, previous studies reported that *in vitro* callus induction or shoots multiplication and successive plantlet regeneration were influenced by the presence of proper amount and different concentrations of auxins and cytokinins combined or singly in an MS medium (Hesami & Daneshvar, 2018b, 2018a; Islam & Alam, 2018; Shekhawat et al., 2015a; Zayova et al., 2020).

Data in Table 2 were collected 35 days after planting. Based on these data, we come to one hypothesis, that there is a relation between BAP concentration and the number of shoots. The number of shoots (per explant) as BAP concentration increased. The number of shoots (per explant) on MS medium supplemented

with 0.5, 1.0, and 1.5 mg/L BAP were 26 ± 1.98 , 38 ± 2.87 , and 47 ± 1.65 respectively. However, the opposite result was recorded on the shoot length. The increase in BAP concentration shortened the shoot length, which was recorded at 6.81 ± 0.85 cm, 2.46 ± 0.97 cm, and 1.8 ± 0.15 cm in MS medium supplemented with 0.5, 1.0, and 1.5 mg/L BAP, respectively. Figure 3 shows shoot proliferation at various concentrations of BAP at 35 days after initiation. MS medium supplemented with 0.5 mg/L BAP resulted in the lower number of shoots (per explant), but the longest shoot, whereas MS medium supplemented with 1.5 mg/L BAP resulted in the highest number of shoots (per explant) but all of the shoots were dwarf (Figure 3). According to George et al. (2008), BAP is one of the cytokinins types with the most effective in enhancing shoot multiplication and triggering shoot elongation.

Effects of PGRs on Dry Weight and Ratio of Root Dry Weight/Total Dry Weight of Acehese Patchouli (*Pogostemon cablin* Benth.) var. Sidikalang (4 weeks after Initiation)

Table 3. Dry weight and ratio of root dry weight/total dry weight Acehese patchouli (*Pogostemon cablin* Benth.) var. Sidikalang (4 weeks after initiation)

Rooting Medium	Average of total dry weight (g)	Average of root dry weight (g)	Average number of root dry weight to total dry weight ratio
MS + 0.5 ppm IBA	0.0277 ± 0.00211	0.0042 ± 0.0002	$15.16^a \pm 0.600$
MS + 0.5 ppm NAA	0.0357 ± 0.00176	0.0086 ± 0.0006	$24.09^c \pm 0.720$
MS ₀	0.0489 ± 0.02532	0.0071 ± 0.0032	$13.57^a \pm 1.520$
½MS ₀	0.0404 ± 0.00497	0.0078 ± 0.0012	$19.20^{bc} \pm 1.652$

Different letter notations indicate that the data is significantly different based on Least Significance Different (LSD) test at $\alpha=0.05$

Based on the data shown in Table 3, MS medium supplemented with 0.5 ppm NAA produced the highest average number of root dry weight and total dry weight ratio (24.09 ± 0.72). In general, NAA played a role in inducing callus and shoot regeneration into plantlets and was not effective enough for root induction, but in this study, it turned out that NAA was also

well-induced roots. However, the average total dry weight (0.0357 ± 0.00176 g) was higher than the average root dry weight (0.0086 ± 0.0006 g). Accordingly, Rahman et al. (2015), found that the highest mean of shoot length (3.7 cm) was obtained on the MS medium containing 1.0 mg/L NAA. Different concentrations of NAA added to MS medium singly or in combination with



another PGR affected shoot proliferation rate, the average length of the shoots, and the number of shoots produced. Similarly, Yudhanto & Wiendi (2015) explained that 1 mg/L NAA added to MS medium singly had given the most number of shoots (5.2 shoots) within 10 weeks after initiation.

In addition, the average number of root dry weight is because NAA can induce rooting during the tissue culture process, and this exogenous auxin can interact with internal auxin which cannot synthesize itself due to limited and small tissues/organs (Akbar M et al., 2017).

Abdulmalik et al. (2013), proved that MS medium supplemented with 1 mg/L NAA was found to be effective for root induction of micro-shoots of *Arachis hypogaea* L. as it produced the highest number of roots (9.25) and root induction frequency (82.42%). Similarly, Gil et al. (2020) found that high-intensity blue light with NAA treatment efficiently induced adventitious root formation and improved root formation from poor stems of *Chrysanthemum* ‘Baekma’ single leaf-bud cutting.

Effects of PGRs on Root Morphology of Acehnese Patchouli (*Pogostemon cablin* Benth.) var. Sidikalang (4 weeks after Planting)

Table 4. Root morphology of Acehnese patchouli (*Pogostemon cablin* Benth.) var. Sidikalang 6 days after planting.

Rooting Medium	Root Morphology
MS + 0.5 ppm IBA	Short roots (not fine fibers), the base of the stem swells and grows callus (% rooted culture = 60%)
MS + 0.5 ppm NAA	Roots grew a lot in the scion and form fine fibrous roots (% rooted culture = 90%)
MS ₀	Roots were short and grew more than in MS medium + 0.5 ppm IBA (% rooted culture = 70%)
1/2MS ₀	Roots grew (% rooted culture= 100%)

Statistically, 1/2MS₀ medium had no significant differences ($\alpha=0.05$) with MS medium supplemented with 0.5 ppm NAA (Table 3). The root of Acehnese patchouli on 1/2MS₀ medium can even grow well morphologically and the percentage of rooted culture reached up to 100%. Meanwhile, Acehnese patchouli cultured on MS supplemented with 0.5 ppm NAA medium gave about 90% of rooted culture but formed a lot of hairy roots, which was also quite good (Table 4). MS medium itself already contains the appropriate micronutrients and macronutrients which can meet plant growth and development needs. Abdulmalik et al. (2013), reported that a PGR-free medium could be effectively

used for root elongation of micro-shoots of *Arachis hypogaea* L. as it produced the longest root length (7.35 cm). Accordingly, based on the report of Satish et al. (2015), rooting occurred immediately when the elongated shoots of *Eleusine coracana* (L.) Gaertn. genotype ‘CO(Ra)-14 were transferred to half-strength MS medium with or without IAA, then the rooted shoots were transferred to greenhouse condition and all in-vitro regenerated plants grew well. Similarly, Shekhawat et al. (2015) reported that half-strength MS medium supplemented with 2.5 mg/L proved best for *in vitro* root induction from shoots of *Passiflora foetida* (67%).

The addition of external hormones allows nutrient uptake to be slower because

the composition of the media becomes more complex, hence plants need to break down the components of the media into simple ones to make it easier to uptake. Plants may require hormones such as auxin for root induction, but at higher concentrations, it could inhibit root growth. Moreover, the addition of excessive plant growth regulators can actually inhibit plant growth. This is caused by the difference in the concentration of endogenous hormones

of the plant itself. The response that appears depends on the ability of explants to absorb and use existing endogenous growth regulators and exogenous PGRs that are absorbed from the growth media. Commercially, half-strength PGR-free MS ($1/2MS_0$) was chosen because the required operational costs would be less than full-strength MS medium supplemented with various PGRs (Abdulmalik et al., 2013; Mulia et al., 2020; Tuhuteru et al., 2018).

Effect Type of Explant on Percentage Survival Plantlets at Acclimatization Stage

Table 5. Effects of explant types on the Number of Survived Plantlets and Survival Percentage

Type of explant	Number of Explant	Survived Plantlet	Percentage of Survival (%)
Microshoot cuttings	20	20	100
Plantlet	20	20	100

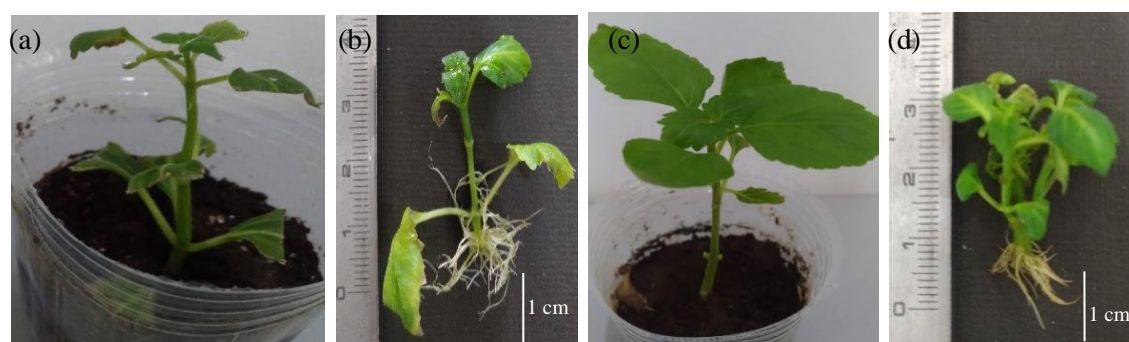


Figure 4. Acehnese patchouli (*Pogostemon cablin* Benth.) var. Sidikalang under *ex vitro* conditions 3 weeks after planting: (a) micro-shoot cuttings, (b) root of micro-shoot cuttings (2 weeks) after planting, (c) plantlet, (d) root of plantlet

According to Figure 4, the micro-shoot cuttings and plantlet of Acehnese patchouli were transferred to greenhouse conditions into the mix of soil: compost (1:1) medium. The micro-shoots rooted and grew vigorously, as well as plantlets on *ex vitro* conditions. This result proved that the transfer of Acehnese patchouli to *ex vitro* condition not only can be done after the explant has developed into a plantlet, but it could also be done directly through micro-shoot cuttings, and it could be done in a shorter time (Table 5). Both plantlets

and micro-shoot cuttings were 100% successful in acclimatization.

CONCLUSION

The optimum treatment for indirect organogenesis was on MS medium supplemented with 1 mg/L NAA + 0.1 mg/L BAP. For multiple shoots from nodal explant, MS medium supplemented with 0.5 mg/L BAP was the optimum medium.

The half-strength PGR-free MS ($1/2MS_0$) could be commercially promising for root induction of *in vitro* shoot of

Pogostemon cablin Benth. var. Sidikalang. However, micro-shoot cuttings are also easy to root when directly planted in *ex vitro* conditions without *in vitro* rooting stage. Thus, patchouli plantlets were obtained more quickly through acclimatization of micro-shoot cuttings.

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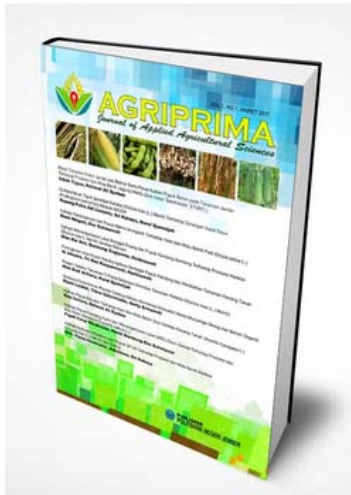




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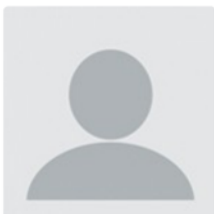
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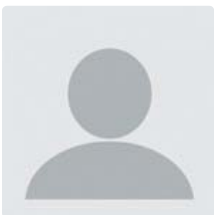
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