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Research Paper

Polyisoprenoid Distribution in Stems and Leaves of *Pinus Merkusii* Strains

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Abstract
Living things produce secondary metabolites, one of which is abundant in plants, namely polyisoprenoid alcohol compounds. Polyisoprenoids are also found in plants with different chain lengths. The distribution of polyisoprenoid compounds (dolichol and polyprenol) was found in the leaf and stem tissues of *Pinus merkusii* strains derived from Aceh, Tapanuli and Kerinci. The extracted samples were analyzed by the two-dimensional thin-layer chromatography (2D-TLC) method. The distribution of dolichol and polyprenol compounds on the stems and leaves of Aceh, Tapanuli and Kerinci pines provided a type II categorization, both dolichol and polyprenol were traced in pine tissues. Total lipids in stem tissue ranged from 564.4-58.9 mg/g dw, polyisoprenoid values ranged from 1.34-2.44 mg/g dw. In the leaves, total lipids ranged from 590 to 669.43 mg/g dw while polyisoprenoid values ranged from 1.29 to 5.70 mg/g dw. The dolichol carbon chain length in the Pine stem of strain composed C_{70}-C_{90}, C_{65}-C_{90}, and C_{65}-C_{95}. Meanwhile, carbon chain-length of C_{85}-C_{105}, C_{70}-C_{95}, and C_{50}-C_{65} were found in leaf tissues. The length of the polyprenol carbon chain in the stem was C_{60}-C_{90}, C_{50}-C_{90}, and C_{70}-C_{90} respectively, while the chain lengths on the leaves were C_{60}-C_{115}, C_{50}-C_{95}, and C_{30}-C_{90}. The present study suggested the presence of both dolichol and polyprenols in *Pinus merkusii* without predomination either dolichol or polyprenol.

Keywords
Pinus Merkusii, Polyisoprenoid, Two-Dimensional Thin-Layer Chromatography, Leaf, Stem

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1. INTRODUCTION

Plants in the territory of Indonesia are part of the Malesiana plant, which is estimated to have about 25% of flowering plant species, which ranks seventh in the world with several species reaching 30,000 species (Paramita and Rahmadi, 2020; Kusmana and Hikmat, 2015). Living things produce secondary metabolites or natural materials through secondary reactions of primary organic materials (carbohydrates, fats, proteins). Secondary metabolite compounds that are widely found in plants are: alkaloids, flavonoids, steroids, saponins, terpenoids and tannins (Ergina and Pursitasari, 2014).

The biological activity of isoprenoids themselves, such as triterpenoids and phytosterols, is considered important as a potential natural resource for medicinal compounds. One of the secondary metabolites that are abundant in plants is polyisoprenoid alcohol compounds (Swiezewska and Danikiewicz, 2005; Sparg et al., 2004). Alcoholic polyisoprenoids are secondary metabolites along with plant sterols, ubiquinone, and specific isoprenoids, which are the largest class of naturally occurring compounds. Alcoholic polyisoprenoids are linear five-unit carbon polymers found in almost all living cells (Basyuni et al., 2016).

This research focused on long-chain polyisoprenoid in *Pinus merkusii*. *P. merkusii* is the only pine species, native to Indonesia (Harahap and Aswandi, 2006), grows naturally in three strains in Sumatra, namely Aceh, Tapanuli and Kerinci (Suhaendi, 2005). *P. merkusii* is often used by the Government of Indonesia in the program to save forests, land and water, especially reforestation and afforestation activities through the Ministry of Forestry which has been implemented since the 1960s (Harahap et al., 1995). *P. merkusii* is a pioneer species that can survive in critical land, fire-resistant, infertile land, and fast-growing species. Besides, there are sufficient seeds to make it easier every time reforestation (Sallata, 2013; Cahyono, 2011). Given the importance of this species in the rehabilitation and conservation program, no composition and occurrence of polyisoprenoids in *P. merkusii* have been previously available. The present study, therefore, aimed to analyze the distribution of polyisoprenoids in *P. merkusii* strains Aceh, Tapanuli, and Kerici for the first time.

2. EXPERIMENTAL SECTION

2.1 Chemical Material

The standard compound mixture of dolichol (C_{90}-C_{105}) was purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA) and polyprenol (C_{90}-C_{100}) was from *Malus* sp. Swiezewska and Danikiewicz, 2005 were used to identify polyisoprenoids in this study, as previously described (Basyuni et al., 2016). The dolichol and polyprenol standards were used to confirm the polyisoprenoid composition in this study. High-performance silica gel 60 thin layer chromatography (HPTLC) glass plate and high-performance RP-18 reverse phase silica thin layer chromatography (RP-TLC). HPTLC and RP-TLC glass plates were obtained from Merck (Darmstadt, Germany). All other chemicals and solvents are from the reagent class and were purchased from Merck (Darmstadt, Germany). Verification of polyprenol or dolichol family was performed at three replications.

2.2 Plant Material

The stems and leaves of mature Pinus (*Pinus merkusii* Jungh. et De Vriese) strains of Aceh pine, Tapanuli pine and Kerinci pine were collected from the Research and Development Center for Environment and Forestry (BP2LHK) Aek Nauli, North Sumatra with three replications in February-August 2018. The age of plants was about 15 years. All samples collected were cut into small pieces and stored in the freezer to keep it fresh before utilization.

2.3 Methods

2.3.1 Isolation of Polyisoprenoid Compounds

The stems and leaves of Aceh pines, Tapanuli pines, and Kerinci pines that were collected were cut into small pieces to facilitate purification, then dried at 60°C-75°C for 48 hours (2 days). The crushed dry sample was then weighed (3 grams each) dw and put into a shaker bottle then extracted with chloroform and methanol in a ratio of 2:1 then incubated at 40°C for 48 hours (Sagami et al., 1992). After 48 hours the extracted liquid was filtered with a funnel and filter paper into the extract bottle. The results from the liquid filter were drained in the oven again before the next stage of saponification (Basyuni et al., 2018).

2.3.2 Lipid Extraction

Fat extracts from the stems and leaves of the three pine (*Pinus merkusii*) strains that had been dried were saponified at 65°C for 24 hours with a concentration of 0.45 g KOH (2 grains), 2 mL of ethanol, and 2 mL of water solution. The distilled water was then covered with parafilm and ape to prevent water from entering the extract bottle. After 24 hours, the product was dried again until the sample was completely dry. After the stems and leaves of the three pine are completely dry they were dissolved with n-hexane for sample analysis (Sagami et al., 1992).

2.3.3 Two-Dimensional Thin-Layer Chromatography Analysis (2D-TLC)

The first dimensional TLC was performed for 60 minutes on silica gel (20 x 3 cm) with a toluene-ethyl acetate (9:1) solvent system. In TLC analysis, polyprenol compounds moved slightly faster than dolichol compounds. The longitudinal edge of the first dimensional TLC with a width of 1 cm and the concentration zone of reverse phase TLC C-18 were clamped using two magnetic bars (4.0 x 1.1 x 0.8 cm) facing each gel phase. The bound TLC plate was then expanded perpendicular to the first dimension to transfer the polyprenol and dolichol to the TLC reserve phase concentration zone (Sagami et al., 1992; Basyuni and Wati, 2017).

The second dimension of the RP-18 TLC silica gel reserve phase was done with acetone solvent for approximately 30 minutes. The position of the polyisoprenoid alcohol was separated and developed with a 2D-TLC, then identified and visualized with iodine vapor. The chromatographic images obtained were directly scanned using a Canon E400 printer. The contents of polyprenol and dolichol found on HPTLC and RP-18 TLC were calculated using ImageJ 1.46r with dolichol and polyprenol standards as references (Schneider et al., 2012).

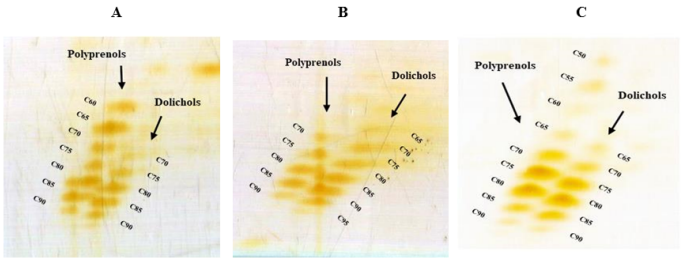

Figure 1. Two-Dimensional Thin Layer Chromatography (2D-TLC) of A Polyisoprenoid Alcohol Aceh Pine (A), Tapanuli Pine (B), Kerinci Pine (C). The Carbon Number Refers to the Length of the Alcohol Polyisoprenoid Carbon Chain

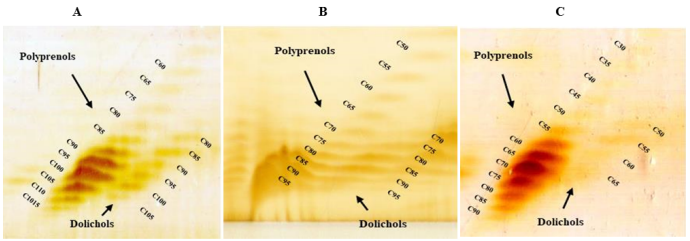

Figure 2. Two-Dimensional Thin Layer Chromatography (2D-TLC) Polyisoprenoid Alcohol from Leaves of Aceh Pine Strain (A), Tapanuli Pine Strain (B), Kerinci Pine Strain (C). The Carbon Number Refers to the Length of the Alcohol Polyisoprenoid Carbon Chain

Table 1. Total Lipid Value and Distribution of Polyprenol and Dolichol on the Stems of Aceh, Tapanuli, and Kerinci Strains

Strain	Tissue	TL (mg/g dw)	PI (mg/g dw)	Pol (mg/g)	Dol (mg/g)	% in Total Lipid			% in Polyisoprenoid		Group
						PI	Pol	Dol	Pol	Dol	
Aceh	Stems	564.4 ± 18.6	2.4 ± 2.5	1.7 ± 2.1	0.6 ± 0.4	0.42	0.3	0.12	72.44	27.56	II
Tapanuli	Stems	573.3 ± 15.3	1.3 ± 1.2	0.8 ± 0.6	0.6 ± 0.5	0.23	0.13	0.1	55.75	44.25	II
Kerinci	Stems	589 ± 14.9	2.4 ± 0.2	1.3 ± 0.3	1.2 ± 0.1	0.42	0.21	0.21	51.39	48.61	II

* dw = Dry weight, PI = Polyisoprenoid, Pol = Polyprenol, and Dol = Dolichol, Sd = Standard deviation. Total lipid, polyisoprenoid, polyprenol and dolichol from average data ± Sd (n=3).

Table 2. Total Lipid Value and Distribution of Polyprenol and Dolichol on the Leaves of Aceh, Tapanuli, and Kerinci Strains

Strain	Tissue	TL (mg/g dw)	PI (mg/g dw)	Pol (mg/g)	Dol (mg/g)	% in Total Lipid			% on Polyisoprenoid		Group
						PI	Pol	Dol	Pol	Dol	
Aceh	Leaves	590.0 ± 112.7	5.70 ± 4.9	4.3 ± 3.7	1.4 ± 1.0	0.97	0.72	0.24	74.85	25.15	II
Tapanuli	Leaves	610.0 ± 20.0	3.8 ± 3.6	1.1 ± 0.8	2.7 ± 2.3	0.63	0.18	0.44	29.45	70.55	II
Kerinci	Leaves	669.4 ± 11.3	1.3 ± 1.1	0.7 ± 0.6	0.6 ± 0.5	0.19	0.1	0.09	53.62	46.38	II

* dw = Dry weight, PI = Polyisoprenoid, Pol = Polyprenol, dan Dol = Dolichol, Sd = Standard deviation. Total lipid, Polyisoprenoid, Polyprenol, and Dolichol from average data ± Sd (n=3).

3. RESULTS AND DISCUSSION

3.1 Polyisoprenoid Compounds using 2D-TLC Analysis

Profiles and distribution of polyisoprenoids in polyisoprenoids found in the stem and leaf tissue of Aceh, Tapanuli, and Kerinci pines using 2D-TLC (Basyuni et al., 2018; Basyuni et al., 2016; Sagami et al., 1992) led to a clear separation of polyprenol from dolichol concerning carbon chain length. Table 1 and 2 outlines the total lipid value and distribution of polyprenol and dolichol contained in the stem and leaf organ of Aceh, Tapanuli, and Kerinci pines. The total lipids in the pine trunk tissue of Aceh, Tapanuli, and Kerinci ranged from 564.4-58.9 mg/g dw. The lowest total lipid was detected in Aceh pine strain, while the highest total lipid was found in Kerinci pine strain. Polyisoprenoid values ranged from 1.34 to 2.44 mg/g dw. Where the lowest polyisoprenoid value was found in the Tapanuli pine strain with a value of 1.34 mg/g dw, while the highest polyisoprenoid value was found in the same strain with the highest total lipid value, namely Kerinci pine with a value of 2.44 mg/g dw.

In Table 2, the total lipids in the pine leaf tissue of Aceh, Tapanuli, and Kerinci ranged from 590-669.43 mg/g dw while the polyisoprenoid values ranged from 1.29-5.70 mg/g dw. With the lowest total lipid found in Aceh pine strain and the highest total lipid found in Kerinci pine strain. The lowest polyisoprenoid value was found in the kerinci pine strain with a value of 1.29 mg/g dw. Meanwhile, the highest polyisoprenoid value was found in Aceh pine strains of 5.70 mg/g dw.

Table 1 and 2 showed that the analysis of polyisoprenoid in the stem and leaf tissue of Aceh, Tapanuli and Kerinci pines, only one type of polyprenol and dolichol profile group in leaves was detected, namely type-II for the polyisoprenoid type: the presence of polyprenols and dolichol (Basyuni et al., 2018; Basyuni et al., 2016). Whereas the type I and type III classifications were not detected. Type-I depicting the predominance of dolichol over polyprenol and type-III exhibiting polyprenol dominated over dolichol (Basyuni et al., 2016). The occurrence of polyprenols and dolichols in this study is by previous findings in tropical plants and world plant leaves (Basyuni et al., 2018; Arifiyanto et al., 2017; Swiezewska and Danikiewicz, 2005; Tateyama et al., 1999).

3.2 Carbon Chain Length Analysis

Table 3 and Table 4 showed dolichol and polyprenol found in the stem and leaf tissue of Aceh, Tapanuli, and Kerinci pines. From Table 3 it can be seen that the longest polyprenol carbon chain, namely C_{50}-C_{90}, is found in Tapanuli pine and the shortest dolichol carbon chain C_{70}-C_{90} is in the Aceh pine strain, while the shortest polyprenol carbon chain C_{70}-C_{90} and the longest dolichol C_{65}-C_{95} are in the strains that the same, namely Kerinci pine. Table 4 also shows that the longest polyprenol carbon chains and the shortest dolichols are in the same strain. Whereas the longest polyprenol carbon chain, C_{30}-C_{90}, and the shortest dolichol carbon chain, C_{50}-C_{65}, were found in Kerinci strain pine leaves. Meanwhile, the shortest polyprenol carbon chain, namely C_{50}-C_{95}, was found in the pine leaves of the Tapanuli strain and the longest dolichol carbon chains in the same two strains with different chain length values, where the values on the leaves were Pinus Aceh strains C_{80}-C_{105} and on the pine leaves of the Tapanuli strain namely the C_{70}-C_{95}.

The polyprenol and dolichol carbon chains (Figures 1 and 2) varied according to each tissue even within the same strains. In line with the statement of Tateyama et al. (1999) and Basyuni and Wati (2017) have reported in the same organ the distribu-

Table 3. Length of Carbon Chain (C) Polyprenol and Dolichol on the Stems of Aceh, Tapanuli, and Kerinci Pine Strains

Strain	Tissue	Polyprenol									Dolichol						
Aceh	Stems			60	65	70	75	80	85	90		70	75	80	85	90	
Tapanuli	Stems	50	55	60	65	70	75	80	85	90	65	70	75	80	85	90	
Kerinci	Stems					70	75	80	85	90	65	70	75	80	85	90	95

Table 4. Length of Carbon Chain (C) Polyprenol and Dolichol on the Leaves of Aceh, Tapanuli, and Kerinci Pine Strains

Strain	Polyprenol												Dolichol								
Aceh	90	95	100	105	110	115	60	65	70	75	80	85	95	100	105			80	85	90	
Tapanuli	90	95			50	55	60	65	70	75	80	85	95			70	75	80	85	90	
Kerinci	30 90	35	40	45	50	55	60	65	70	75	80	85	50	55	60	65					

tion of polyprenol chain lengths was not necessarily the same as the dolichol chain length. Figures 1 and 2 showed that the distribution of polyprenol is more dominant in leaf tissue than in the stem tissue studied. In the plant world, polyprenols are generally more dominant in leaf tissue with abundant concentrations compared to dolichols (Basyuni et al., 2016; Swiezewska and Danikiewicz, 2005; Tateyama et al., 1999). Recently, it has been demonstrated that short and long chain polyisoprenoid alcohols indicated different oxidation numbers (Molińska et al., 2015). These results indicated that the leaves are capable of synthesizing various secondary metabolite arrangements for self-defenses.

Polyisoprenoid chain lengths have occurred in various plant tissues (Swiezewska and Danikiewicz, 2005). Each stem and leaf of Aceh, Tapanuli, and Kerinci pines (Figures 1 and 2), depicted a different length of polyisoprenoid chain based on the occurrence and distribution of each strain. The length of the polyisoprenoid chain has occurred in various plant tissues, several factors could cause a difference in the length of the polyisoprenoid chain.namely tissue aging (increasing age) which can cause differences in the concentration of dolichol and polyprenol to increase due to differences in tissue, light, salinity (Basyuni et al., 2014; Basyuni et al., 2018; Swiezewska and Danikiewicz, 2005). In this circumstance, environmental factors can change the concentration of polyisoprenoid in plants, and physiological factors can determine the rate of polyprenol formation in leaves (Roslinska and Chojnacki, 2002).

4. CONCLUSIONS

The distribution of dolichol and polyprenol compounds on the stems and leaves of Aceh, Tapanuli and Kerinci pines grouped to a type II category, both dolichol and polyprenol occurred in plant tissue. These findings indicated no domination distribution of polyisoprenoids in stem and leaf tissue in the Aceh, Tapanuli and Kerinci strains.

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