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TOTAL LIGNIN CONTENT AND AGROMORPHOLOGICAL CHARACTER DIVERSITIES OF 30 INDONESIAN RICE (*Oryza sativa* **L.) ACCESSIONS**

Keragaman Kandungan Lignin Total dan Karakter Agromorfologi 30 Aksesi Padi (*Oryza sativa* **L.) Indonesia**

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ABSTRACT

Lignin is one of lignocellulosic components in vascular plants, essential in plant mechanical *properties, water transport, and defense against pathogens. Furthemore, lignin has been applied in various industry. This study aimed to explore variation of lignin content and itsrelated morphological traits of Indonesian rice accessions, providing beneficial information for breeding approaches to improve utilization characteristics of grass biomass. Therefore, the total lignin contents of thirty Indonesian rice accessions using Thioglycolic Acid Lignin (TGAL) method and their correlation to seven agromorphological characters using Pearson correlation analysis were investigated. Variation of lignin content ranged from 8.38 to 20.75% (of cell wal residue, CWR), and the average value was 13.55%. Correlation analysis showed that lignin total positively correlated with plant height, stem length, panicle length, stem diameter, total fresh weight, and panicle weight per tiller. On the other hand, the number of tillers had a significantly negative correlation to lignin contents.*

Keywords: agromorphological characters, biomass, lignin, rice, total lignin contents

ABSTRAK

Lignin merupakan komponen lignoselulosa yang berperan penting pada sifat mekanis tanaman, transportasi air dan pertahanan terhadap patogen pada tanaman berpembuluh. Selain itu, lignin telah banyak diaplikasikan di berbagai jenis industri. Tujuan penelitian ini untuk mengeksplorasi variasi kandungan lignin dan sifat-sifat agromorfologi aksesi padi Indonesia yang terkait, serta memberikan informasi yang bermanfaat untuk pendekatan pemuliaan sebagai pengembangan karakteristik pemanfaatan biomassa rumput-rumputan. Oleh karena itu, dilakukan pencarian informasi kandungan lignin pada tiga puluh aksesi padi Indonesia menggunakan metode *Thioglycolic Acid Lignin* (TGAL) dan korelasinya terhadap tujuh karakter agromorfologi menggunakan analisis korelasi Pearson. Keragaman kandungan lignin yang diperoleh adalah berkisar 8,38–20,75% (*Cell Wall Residue*, CWR), dengan nilai rata-rata 13,55%. Analisis korelasi menunjukkan bahwa akumulasi lignin pada batang padi berkorelasi positif dengan karakter tinggi tanaman, panjang batang, panjang malai, diameter batang, bobot total segar dan bobot malai per anakan. Sebaliknya, karakter jumlah anakan berkorelasi negatif signifikan terhadap kandungan lignin.

Kata Kunci: biomassa, kandungan lignin total, karakter agromorfologi, lignin, padi

INTRODUCTION

Agricultural residues such as those from oil palm (*Elaeis guineensis*), sugarcane (*Saccharum* spp.), sorghum (*Sorghum* spp.), maize (*Zea mays*), and rice (*Oryza sativa*) can be used as a lignocellulosic biomass, a potent source of bionenergy, woody materials, animal feed, organic fertilizer, medicinal raw materials, and paper feedstock. Rice, one of the Gramineae family, is a staple food source for Indonesian people and also provides lignocellulosic biomass with the highest potential energy value in the world. The potential energy value of Indonesia's agricultural land residues in 2019 was 244.15 PJ (Picojoule), with total rice productivity of 81.38 million tons (Mofijur et al. 2019). On the other hand, the utilization of this biomass residue has not been intensively done. Currently, 4.6 billion tons of lignocellulosic biomass residues are produced, but only 25% is used intensively in the world (Dahmen et al. 2019).

The composition and chemical structures of lignocelluloses from agricultural residues affect their potential energy values. Lignocellulose, which is derived from plant secondary cell walls, consists mainly of cellulose, hemicelluloses, and lignin (Silvy et al. 2018). Lignin, a phenylpropanoid polymer, fills the interspace of cellulose and hemicelluloses in the secondary cell walls (Loix et al. 2017). Lignin plays important roles in plant growth such as mechanical support, water transport, and defense against pathogens in vascular plants.

Monolignols, lignin precursors, such as *p*-coumaryl, coniferyl, and sinapyl alcohols undergo dehydrogenation (radical formation) by peroxidase and laccase followed by polymerization (radical coupling) to form *p*hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units in lignin polymers, respectively (Tobimatsu and Schuetz 2019). Lignins from eudicots such as dicotyledonous plants and monocotyledonous grasses share H, G, and S units (Boerjan et al. 2003, Vanholme et al. 2010). The H unit generally exists at a much lower frequency in lignin polymers, although its level tends to be slightly higher in grasses compared with those in dicotyledonous plants (Boerjan et al. 2003, Vanholme et al. 2010). In addition, *p*-coumarate (Withers et

al. 2012, Marita et al. 2014, Petrik et al. 2014), ferulate (Karlen et al. 2016), and flavon tricin (del Río et al. 2012, Lan et al. 2015, Lam et al. 2021) were characteristic components of grass lignins. It is known that lignins affect the process of pulp and paper making from wood biomass (Santos et al. 2013) and the property of wood-based biopellet (Wistara et al. 2020, Umezawa et al. 2020). In addition, lignins have recently attracted attention as a source of materials and chemicals such as adhesive (Lempang 2016), adsorbent (Supanchaiyamat et al. 2019), bioplastic (Yang et al. 2019), and pharmaceutical and biomedical products (Figueiredo et al. 2018, Domínguez-Robles et al. 2020).

One of the characteristics of grasses is that delignification of lignicelluloses is easier than those from wood species (Umezawa et al. 2018). Delignification is critical step in the bioethanol (Wi et al. 2013) and animal feed industries (Yanuartono et al. 2017) because lignins have been regarded as a main obstacle for digestion of cell-wall polysaccharides. In addition, lignin can also inhibit ethanol formation fermentation (Zeng et al. 2014) and is cannot be digested easily by ruminants (Sheikh et al. 2018). On the other hand, in the biopellet industry, grass biomass with high lignin content may be preferred because lignin content positively correlated with the calorific value of combustion and the High Heating Value (HHV) of the biomass material (Vargas-Moreno et al. 2012, Carrillo et al. 2014, Menucelli et al. 2019).

Breeding to alter lignocellulose characteristics of grass species such as rice (*O. sativa*) (Takeda et al. 2019, Umezawa et al. 2020), sorghum (*S. bicolor*) (Wang et al. 2017), sugarcane (*Saccharum* sp.) (Bewg et al. 2016), and *E. arundinaceus* (Kasirajan et al. 2020) may be one of the strategies to promote applications of their lignocellulosic biomass. This strategy may also lead to reduced consumption of woods derived from natural forests and enhanced utilization of suboptimal or marginal lands. Many important studies using biotechnological approaches have been carried out to manipulate the content and/or structures of lignin toward lignin-oriented biorefineries

(Umezawa 2018, Pazhany and Henry 2019, Lebedev and Shestibratov 2021).

Indonesia has abundant rice germplasms, consisting of local varieties and wild species. Several rice varieties have been developed into superior varieties with improved quality. Rice can be classified into upland, lowland, and swamp rice based on the ecosystems. As a model of grass species, in addtion, rice has also been used as a reference for physiological information for grass crops and biomass plants such as *S. bicolor*, *Z. mays*, *S. officinarum*, *T. aestivum*, *P. purpureum*, and *M. sinensis* (Shimamoto and Kyozuka 2002, Bush and Leach 2007). Despite their significances as a model plant as well as potential source of lignocellulosic biomass, our knowledge about lignins of rice germplasms in Indonesia is still very limited. In the present study, therefore, we aimed to explore variation of lignin content and its-related morphological traits of established Indonesian rice accessions, providing beneficial information for breeding approaches to improve utilization characteristics of grass biomass.

MATERIALS AND METHODS

Place and time of the research

This research was carried out at the Laboratory of Genomics and Plant Genetics Engineering and Plant Testing Facility, Research Center for Genetic Engineering, National Innovation and Research Agency (BRIN), Cibinong, Indonesia. This research was conducted from May 2020 to February 2021.

Material

The genetic materials used were 30 Indonesian rice accessions belonging to the Research Center for Genetic Engineering, BRIN (Table 1). The standard material used was bamboo (*Phyllostachys heterocycla*) milled wood lignin.

Method

Rice planting and plants maintenance

Thirty rice accessions were germinated in soil and goat manure (1:1) media in the greenhouse. Fourteen days old rice seeds were transplanted to planting pots containing _ 10 kg of soil: goat manure (3:1) media. Five

biological replicate per accession were transplanted, and three biological replicates per accession were analyzed. The experimental design was randomized complete block design. Rice growing was carried out using a stagnant water system by maintaining as high as 5 cm water level from the soil surface. Plant maintenance such as fertilizing, watering, and controlling pests and diseases is carried out regularly using standard rice planting protocols until harvesting.

Agromorphological characters

The agromorphological characters were observed at the seed ripening or harvesting stage. Seven characters, based

Table 1. The thirty rice Indonesian accession by ecosystem type

N/A = Data Not Available

on the Standard Rice Plant Evaluation System (IRRI 2013), were observed, namely plant height (cm), stem length (cm), panicle length (cm), stem diameter (cm), number of tillers, total plant fresh weight (g), and panicle weight per tiller (g). Plant height was measured from the soil surface to the tip of the highest panicle. The number of tillers counted is the total number of tillers. The total fresh weight of the plant is the weight of all plant organs without panicles and roots. The length of the stem was measured from the base to the neck of the panicle, and the length of the panicle was measured from the neck to the tip of the panicle. The stem diameter was measured at 10 cm above the soil surface. Data collections were taken randomly from 3 plants per biological replication.

Total lignin analysis using the TGAL

The pretreatment was performed following the previous protocol by Suzuki et al. (2009). The rice biomass without panicles, and leaf blades was dried at 55ºC in a drying oven for five to seven days, cut into 5-10 mm, and then was pulverized using TissueLyser (Qiagen) for 2.5 minutes at 26 Hz as shown in Figure 1. Two hundred milligrams of crushed rice culm biomass were extracted using methanol and *n*hexane, and followed by washing with distilled water (Yamamura et al. 2012). The

lignin extract was dried using a freeze dryer for 16 hours and stored in a desiccator.

Lignin standard calibration curve

A standard solution was prepared using five weight variations of bamboo milled wood lignin (1–5 mg), with three technical replications. The total lignin was analyzed photometrically based on the Thioglycolic Acid Lignin (TGAL) method (Suzuki et al. 2009). The bamboo or samples powder was put into a 2 ml tube, and then 1 mL 3 N HCl and 0.1 mL thioglycolic acid (Nacalai Tesque, Kyoto, Japan) were added. The samples were heated at 80ºC for 3 hours. The supernatant was removed after centrifugation at 16100 \times g for 10 minutes at room temperature (RT). The pellet was vortexed for 30 seconds in 1 mL distilled water. The samples were centrifuged at 16100 \times g for 10 minutes at RT, the supernatant was discarded and the pellet was re-suspended in 1 ml 1N NaOH then shaken vertically at 80 rpm for 16 hours. The samples were centrifuged at 16100 g for 10 minutes at RT, and 1 mL the supernatant was transferred into 1.5 ml tubes and acidified with 0.2 ml HCl acid fuming 37%. The samples were incubated at 4ºC for 4 hours, and then centrifuged at 16100 \times g for 10 minutes at RT. The supernatant was removed and pellet was dissolved in 1 ml 1 N NaOH (lignin fractions). The lignin

Figure 1. (A) Rice stem parts for analysis of the total lignin contents, Scale bar = 3 Cm (B) Dried stem biomass, Scale bar = 5 Cm (C) Dried stem powder, Scale bar = 1 Cm

concentration was a dependent variable (Y), and the absorbance value at a wavelength of 280 nm was a independent variable (X).

Photometric quantification of total lignin

Fifteen milligrams of extracted lignin from rice stem were weighed on a semimicro analytical balance (Shimadzu Co, Kyoto, Japan), and the total lignin was prepared using the TGAL method as above (standard solution) (Suzuki et al. 2009). The lignin fraction was diluted with 25-fold 1 M NaOH, and then 200 µL of the diluted solution was transferred to each well on a 96-wells microplate (UV-Star, Greiner, Frickenhausen, Germany). The lignin concentration was measured using a UV spectroscopy microplate reader (Multiskan™ GO, Thermo Scientific, Waltham, MA, USA) at 280 nm.

Data analysis

The standard bamboo-milled wood lignin calibration curve was used to make a linear regression equation, which was then used to figure out the total lignin. Statistical analysis of total lignin was performed by comparing the mean value of each biological replicate $(n = 3)$ from each accession with One-way ANOVA and was continuing with Duncan's multiple range test $(P < 0.05)$. Total lignin content and agro-morphological character data were further analyzed using Pearson correlation analysis (Ratner 2009) and hierarchical cluster analysis using the average linkage (between groups) cluster method. Statistical analyses were performed using IBM SPSS Statistics 25.0 software.

RESULTS AND DISCUSSION

According to Suzuki et al. (2009), lignin quantification using the the TGAL method for lignin quantification is appropriate for herbaceous samples with a high level of polyphenols and proteins. Because grass tissues also contain much silica, a contaminant of the acid-insoluble fraction of Klason lignin (Suzuki et al. 2009), as well as proteins (Hatfield and Fukushima 2005), the TGAL method would be preferred for their samples with similar absorption coefficient. In addition, this method is also more efficient than other lignin quantification methods such as Klason (>200 mg) and acetyl bromide (5– 35 mg) methods, because requiring only 10– 20 mg of a sample (Suzuki et al. 2009). A standard calibration curve constructed using bamboo milled wood lignins provided the regression equation ($y = 200.42x + 10.28$) was obtained with a coefficient of determination (R^2) of 0.9909 was established on the standard calibration curve of bamboo milled wood lignin (Figure 2).

The established TGAL method to cellwall samples prepared from thirty Indonesian rice accessions was applied in this study. The variation of lignin content of the samples

Figure 2. The calibration curve to calculate the total lignin contents. The bamboo (*Phyllostachys heterocycle*) milled wood lignin as standard

ranged from 8.38 to 20.75% (of cell wall residue, CWR) (Figure 3); the mean value of total lignin content is 13.55% CWR. Gebang has the highest lignin content at 20.75%, while Tukad Petanu has the lowest (8.38%) (Figure 3). In the previous reports (Suzuki et al. 2009, Koshiba et al. 2017), the TGAL content of straw tissues without panicles $(12.7 \pm 0.74\%)$ and stem organs $(10-15\%)$ from a Japonica rice cultivar Nipponbare (wild-type plants) was in the range of the values in this study.

Lignin content in plants is affected by environments (Kuai et al. 2016, Hussain et al. 2019, Rivai et al. 2021, Rivai et al. 2022) and genetic variation (Koshiba et al. 2017, Lam et al. 2017, Wu et al. 2019), and it might interact with by several morphological characters such as leaf length and width, plant height, tiller length, tiller number, plant girth and biomass weight (Jahn et al. 2011). In supporting the notion, our study showed

that plant height, stem length, panicle length, stem diameter, number of tillers, total fresh weight, and panicle weight per tiller correlated with lignin contents (Figure 4). Pearson correlation test showed that the character of plant height $(R = 0.775)$ and stem length $(R = 0.774)$ had a strong and significant degree of correlation with lignin content at a 99% confidence level. The characters of total plant fresh weight $(R =$ 0.384), panicle length $(R = 0.467)$, stem diameter ($R = 0.524$), and panicle weight per tiller $(R = 0.646)$ had a moderate and significant degree of correlation with lignin accumulation at the 99% confidence level. The number of tillers had a moderate negative correlation $(R = -0.537)$ with lignin accumulation and was significantly affected at the 95% confidence level (Figure 4).

According to the correlation analysis, it can be concluded that the number of tillers was inversely proportional to the total lignin

significant difference in the DMRT (α = 0.05). Data is the mean value of n = 3

Figure 4. Correlation of lignin content to agromorphological characters. ** shows a significant correlation at P < 0.01 and * shows a significant correlation at P < 0.05

content. The analysis showed that rice accessions with relatively low lignin content, Ciherang and IR-64, have almost three times number of tillers compared to the high lignin content accessions, Lampung Kuning and Gebang (Table 2). Although there was only one accession in this study, Inpago 7, it had a relatively large number of tillers and a high lignin content. Based on the correlation data, lignin content may link to stem diameter and plant height in the rice accession tested. It is known that Inpago 7 has relatively larger stem diameter and higher plant height than the other accessions. Despite Lampung Kuning and Gebang had the few tillers, these accessions had relatively high plant height and stem diameter characteristics. It can be concluded that the character of the number of tillers cannot always be used independently reference in estimating the amount of lignin accumulated in a lignocellulosic biomass material. Other characteristics such as plant height, stem length, panicle length, stem diameter, total fresh weight, and panicle weight per tiller should be also considered.

In comparison to leaves and sheaths, rice stem contributes about 55% of the total lignocellulosic biomass, which is comparable to sorghum (Jahn et al. 2011). Because stem

PH = Plant height; SL = Stem length ; PL = Panicle length; SD = Stem diameter; NT = Number of tillers; TFW = Total fresh weight; PWT = Panicle weight per tiller

Figure 5. Dendrogram of hierarchical cluster analysis based on lignin content and seven agromorphological characters. The hierarchical cluster analysis was carried out using the average linkage (between groups) cluster method

tissues account for a large part of the total biomass from grasses, the correlation between stem diameter and lignin content in grasses described previously (Jahn et al. 2011) and observed also in this study (Figure 4) may be important for their biomass applications. Gramineae family members with larger height and stem diameter such as bamboo (*P. heterocycla* Mitf.), *Saccharum* spp., *S. bicolor* and *E. arundinaceus* generally have relatively higher lignin contents (Itoh 1990, Yamamura et al. 2013, Miyamoto et al. 2018, Wahyuni et al. 2019) compared with those of family members with smaller plant height and stem diameter such as rice (Jahn et al. 2011), which might reflect a positive correlation between stem diameter and lignin content. *Arabidopsis thaliana* mutants lack of genes associated with lignification showed decrease in stem diameter (Herrero et al. 2013, Li et al. 2015), further supporting an interaction between lignification and stem morphology.

Based on the characters of the lignin content and the seven agromorphological

characters, the dendrogram generated by Hierarchical Cluster Analysis obtained 4 clusters (Figure 5). Cluster 1 consists of Tukad Petanu, Cigenit, IR-64, Ciherang, and Merah had identical characteristics of plant height and relatively low stem length but had relatively large numbers of tillers. Based on the lignin content analysis, this cluster contained the three accessions (Tukad Petanu, Ciherang, and Cigenit) with the lowest lignin contents. These three low lignin (8–11% CWR) accessions can used as biomass crops for animal feed, bioethanol production, and biorefinery (Wang et al. 2015, Christensen and Rasmussen 2019).

Cluster 2 showed that Gebang, Lampung Kuning, and Fatmawati had similar characteristics of lignin content, plant height, stem length, and panicle weight per tiller which were relatively high. The correlation analysis in Figure 4 showed that plant height, stem length, and panicle weight per tiller were strongly correlated to lignin accumulation.

In cluster 4 with Ketan Kelapa, Inpago 7 had a relatively high lignin content (16.98%). Both accessions shared the characteristic of having a relatively high total fresh weight, especially Inpago 7. Inpago 7 had the highest total fresh weight among the 30 rice accessions. Accessions with relatively high lignin contents and biomass, such as Gebang, Lampung Kuning, Inpago 7, and Fatmawati, can be used as materials in high biomass rice variety development strategies. To develop rice varieties with desired lignin contents, this approach can be combined with lignin modification based on the lignin biosynthetic pathway (Key and Bozell 2016, Li et al. 2016, Umezawa 2018). However, the utilization of lignocellulosic biomass will be more comprehensive and optimal if the information on other important cell wall components such as cellulose, hemicellulose, silica, and others is also available (Guerriero et al. 2016, Reyes et al. 2021, Wang et al. 2021). In future studies, the obtained information can be used for breeding to modify lignin content and plant morphology for promoted applications of grass biomass.

CONCLUSION

Variation of lignin content determined

by the TGAL analysis of the 30 rice accessions ranged from 8.38 to 20.75% CWR, and the average value was 13.55% CWR. The accessions Gebang, Lampung Kuning, Inpago 7, and Fatmawati showed relatively high lignin contents (16.81-20.75% CWR) with high biomass productivity (133- 200 g per plant). On the other hand, relatively lowest lignin content were observed in Tukad Petanu (8.38% CWR), Ciherang (10.67% CWR), and Cigenit (11.04% CWR). The total lignin content in the rice accessions was correlated with their several agromorphological characteristics. Plant height, stem length, panicle length, stem diameter, total fresh weight, and panicle weight per tiller correlated significantly positive with lignin contents. On the other hand, the number of tillers had significantly negative correlation with lignin content in the accessions tested.

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