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THE GENOTYPE OF *AQUILARIA MICROCARPA* INTERACTING WITH *FUSARIUM* SP BASED ON MICROSATELLITE MARKER

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Abstract– *Aquilaria microcarpa* is one of the potential trees species for producing agarwood. The formation of aloes only occurs when the plant interacts with pathogenic substance. The process of a secondary metabolite is strongly influenced by gene activities and environmental factors as supportive component. However, the genotype information of *A. microcarpa* is still lacking. The genotype of *A. microcarpa* interacting with *Fusarium* sp is expected to be produced by using microsatellite markers in order to obtain the molecular characters of the plant. This result indicates that the process of morphological change occurs one week after plants were inoculated by *Fusarium* sp which were characterized by discoloration of leaves. With amplification testing of DNA fragments by four pairs of microsatellite primers in the population of *A. microcarpa* population, it was found that only primers: 10 PA 17 and 16 PA 17 were capable to amplify 5 to 6 polymorphic alleles. The molecular information is pivotal and would be used to consider the development of its culture and conservation strategic in nature.

INTRODUCTION

Thymeleaeceae is one of the family of tropical plant forests that can produce aloes. The family has less than 50 genera and only seven genera may be able to produce aloes, including; *Aetoxylon, Enkleia, Gyrinops, Gonystilus, Phaleria, Wikstroemia* and *Aquilaria* (Sumarna, 2005). He further stated that of the seven genus, *Aquilaria* is the most commonly found in Indonesia. Aloes is one of non-wood forest products having high economic value, because the price which could reach 30 million rupiah per kg for a super quality in global market (Siburian *et al*., 2013). Aloes have been sold for industry needs to produce perfume, cosmetics, incense, various types of preservatives and medicines (Sumarna, 2005) and also for the religious purposes (Barden *et al*., 2000).

Research on the phenotype characters of aloes plant (Siburian *et al*., 2015) and the anatomical characters (Siburian *et al*., 2013) has been indicated by quantitative measures. These features were generally controlled by many genes and also were affected by environmental factors in which the differences are often times hard to distinguish.

The phenotype marker has limitation because it requires the information on vegetative and generative characteristics and which generally can only be observed after plants reach adult stage. Hence to get the distingushed information criterion of plants with and without aloes, there is a need to do genotype characterization (Finkeldey, 2005). The genotype characterization would be undertaken with the facilitation of molecular marker, due to having a level of accuracy and with higher efficiency. Molecular marker often known as fingerprints DNA is in line with ribbon polymorphism of fragments of DNA. The advantage of a marker is the accuracy of molecular data which were not affected by the environment and would be tested at all the level of plant development. In pest and disease tests, the marker is independent and is not affected by a disturbance organism. As such, the use of marker in cultivation for the selection of plants facilitates the speed up of the selection and more efficient in the field testing (Kasim and Azrai 2004).

The technology of molecular marker in the genus of Aquilaria advanced in line with many available

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options of DNA marker, including the use of marker based on the Polymerase Chain Reaction (PCR) by using nucleotide sequences as a primer such as Randomly Amplified Polymorphic DNA (RAPD) which was conducted by Lee *et al*. (2011), Sequence Characterized Amplified Regions (SCARS) by Lee *et al*. (2011). Furthermore, Meiling *et al*. (2011) found 567 polymorphic ribbon from 112 accessions of *A. sinensis* by using marker of Inter-Simple Sequence Repeats (ISSR) and Sequence-Related Amplified Polymorphism (SRAP) from 11 locations.

Microsatellite markers are genetic markers with codomain feature and having a high level of polymorphism and even may be inherited following Mendel's law (Powell *et al*., 1996; Hancock 1999). Eurlings and Gravendeel (2009) designed four pairs of microsatellite primaries for plant of *A. crasna* which own a high polymorphism. In addition, Eurlings *et al*., (2010) redesigned a new primary (71pa17) to test some samples *A. crassna* from several areas in Asia. A number of considerations on the use of microsatellite markers such as markers plentiful distribute and equitable in a genome. The variability is significantly high and the location of the genome can be identified. In addition, microsatellite markers assist to distinguish genotype clearly to evaluate the seed purity, mapping and genotype selection for intended characteristics in the genetic population studies and the analysis genetics diversity (Powell *et al*., 1996).

Based on the consideration, the genotype of *A. microcarpa* plant interacting with *Fusarium sp* is expected to be conducted by using microsatellite markers in order to obtain molecular characteristics to assist the cultivation plan of *A. microcarpa* plant in the future.

MATERIALS AND METHODS

To determine response of plant on *Fusarium* sp, the test was conducted to 40 young plants (seedlings) with four strains isolates of *Fusarium sp*. The types and strains of *Fusarium sp* were still in the research phase at the Forestry Research and Development Bogor. Therefore, the code for *Fusarium sp* strains was based on researcher code namely; *Fusarium* 512, *Fusarium* 500, *Fusarium* 509 and *Fusarium* 2969. The observation was conducted on the plant response from the inoculation until early symptoms were visible, in days unit after inoculation (day). In addition, the severity of the impact of inoculation was calculated according to Mak *et al*., (2008); 0 = no symptoms, 1 = there is no infection, 2 = few leaves turn to a bit yellow colour, 3 few leaves become yellow, 4 = leaves turn to brown yellow and 5 = deciduous leaves. The scale was calculated by the severity disease formula (disease severity index/dsi) as follow;

$$Severity = \frac{\sum(n \times V) \times 100\%}{Z \times N}$$

where; n = Number of leaves in each category
v = Score of value in each category
Z = Score of value of the highest severity
N = Number of observed leaves

The level of virulence of each isolate was determined by the average score of all sampled plants on the last observation by using the criteria in Table 1.

Table 1. The virulence criteria of *Fusarium* sp isolates on *A. microcarpa*

Score (v)	Virulence level
0	No Virulence
$0 < v \leq 5$	Low Virulence
$5 < v \leq 10$	MiddleVirulence
$v > 10$	High Virulence

DNA Extraction

The method used to extract DNA was the CTAB (Cetyl Trimethyl Ammonium Bromide) method which was modified from Murray and Thompson (1980). Most of DNA extraction methods from plant tissue in literature, require a longer time period and expensive chemical compounds such as chesium khlorida so it is inefficient (Brown *et al*., 1993). The leaves were cut with size 2x2 cm, then were crushed by adding liquid nitrogen in pestel/cleaned mortar. The results of crushed leaves later were incorporated into micro tube 2 mL and were added by buffer solution as much as 500 - 700µL. To make leaves mixed with buffer solution PVP 2 % evenly so tube contains the extracted solutions were vortexed. Then the mix was incubated in water bath between 45 minutes and 1 hour and was shaken slowly every 15 minutes. The optimal temperature used in the process incubation ranged between 65-70 °C. When the temperature incubation exceeds the optimal temperature then DNA would be broken down.

After the incubation, the micro tubes were lifted up and cooled for 15 minutes then were added chloroform as much as 500 µL and phenol as much as 10 µL, and then were centrifuged at speed of 13.000rpm for 2 minutes. Supernatant separated

from precipitate reduce gel using micro pipet and moved into a tube micro then moved into the new micro .It is chloroform and phenol twice that aims to deprive of organic solvents left in solution.

The seperated supernatant from organic phase was added cool isopropanol as much as 500μL and NaCl 300μL. The mixture was then kept in the freezer for 45 minutes until 1 hour. The deposition was centrifuged at speed 13.000 rpm for 2 minutes later a liquid in a tube micro disposed. The deposition will be pellets of DNA format. The pellets of DNA were added with ethanol 300 μL and were centrifuged for 2 minutes at speed of 13.000rpm, then a liquid ethanol were dumped. Then left of DNA pellets in a tube micro were dried in desiccator with reversed position for 10 minutes then were added by TE solution as much as 20 μL vortex then stored in the freezer.

During the drying of DNA pellets, agarose 1% (0.33 g agarose in 33 mL TAE) was prepared. To the process of electrophoresis, it was taken 3 μL DNA and was added 2 μL blue juice 10X and then was running on voltage 100 volt for approximately 30 minutes. The DNA would move at positive (anode) side. The electrophoresis result was then soaked in solution etidium bromide (ETBR) 10 μL per 200 mL aquades for 3 - 5 minutes and then was visualized in UV transiluminator.

The Primer Selection

The primary used in research consist of four specific primers to suitable for Aquilaria (Eurlings *et al*., 2009). The primers were isolated and designed by Eurlings from *A. crassna*. The four primers were primers: 6pa18, 10pa17, 16pa17, and 71pa17 (Table 2).

PCR (Polymerase Chain Reaction)

The PCR process requires 4 main components such as H_2O, Hotstar mix, primary and DNA. DNA result from extraction process before the amplification process of PCR must be diluted by using aquabidest, depending on the tickness of extracted DNA. For PCR process, 1.5μL DNA got mixed with hotstar mix 7.5 μL, nuclease-free water 2.5 μL and 1.5 μL primary and then was centrifuged during 5-10 seconds and then was incorporated into PCR machine.

The test of polymorphism was conducted by observing the ribbon resulted from PCR which was visualized based on the electrophoresis results. The test results was determined polymorphism if the ribbon pattern produced have at least more than one variation, while the result was monomorphic if there is no variation in the ribbon of electrophoresis results.

The molecular data analysis was conducted based on the ribbon score in DNA on gel polyacrylamide with the silvery color, where every locus was assumed as microsatellite alleles. Bands of DNA were measured in length, then the result was incorporated into the equation long-fragments obtained by software Minitab version 15. This is meant to ensure that the scored microsatellite DNA was located in the right position. The microsatellite alleles are co-dominant, then the genotype was identified based on the variation available ribbon alleles.The number and alleles frequency were obtained from POPGEN 1.32 version.

RESULTS

Testing Fusarium sp on seedling level *A. Microcarpa* baill

The observation result on morphology characteristics of *A. microcarpa* induced by *Fusarium* sp in seedling stage showed the variations of plant resilience against the type of tested *Fusarium* sp since the first week of inoculation, especially on the

Table 2. The microsatellite primers of *Aquilaria crasna* (Eurlings *et al.,* 2009)

Locus	*Primer sequence* (5′–3′)	*Repeat*	*Size range* (bp)	Number of alleles	TA (°C)
6pa18	F: TGAGGCGTGAGTGAGATATTGATT R: CCTTCCTCTCTTCTTACCTCACCA	$(CA)_8$	180–210	7	50
10pa17	F: ACACACTGTTATGGTCTACAGCTT R: CGCCATCTCATAATATTCTAATGTA	$(CA)_{12}$	152–156	3	50
16pa17	F: AGTGAACAACTTGACTAGGCTTG R: GCTGAACACAACAAGATATCACC	$(CA)_{19}$	143–155	6	59
71pa17	F: AGCAAACAGTGGGATAAGGTC R: AGAAAGGAGGCGAAACGAAT	$(CA)_{15}$	152–224	15	54

type of *Fusarium* 512. It is characterized by the color changes of leaves from green to yellow even caused deciduous leaves (Figure 1 and Figure 2).

The genotype of every seedling (Table 3) was

Fig. 1. The leave morphology of *A. microcarpa* after four days inoculated by Fusarium #512

Fig. 2. The leave morphology of *A. microcarpa* after 8 days inoculated by Fusarium #512

based on the locus 6PA18 and 71Pa17. The measurement result of detected length fragments, where locus 6PA18 has 10 genotypic variation such as 184/184, 184/198, 198/198, 198/210, 210/210, 198/198, 198/210, 198/222, 210/210, 210/222, while locus 71PA17 has 8 genotypic variation including 158/158, 166/166, 166/178, 178/178, 178/198, 194/195, 194 /198 and 198/198 (Table 3).

On the third and fourth week observations following inoculation, the dissimilaity was not apparent markedly between the four tested *Fusarium sp* (Table 3). There are many factors affecting this aspect, a few of them are the level of the virulence of *Fusarium sp*. as well as the plant defense system and also the environment.

The observed plant response after the inoculation was conducted such as leaves chlorosis was a response to hypersensitive plants to limit infection of *Fusarium* sp which occurs because the disruption in the nutrition availability as a result of the wound due to inoculation. The lack of the nutrition caused the leaves are lacking of chlorophyll pigments so leaves turn to yellowish color and finally deciduous (Nieamann and Visintini 2005).

The pathogen fungi that is capable to infect various plants including the crop of aloes is in the group of *Acremonium* sp and *Fusarium* sp. Williams (1997) stated that the infection of pathogenic fungi involved several mechanisms such as the mechanical force, enzymes and toxin. All of these mechanisms coincide with passive and active immune system of the host plant. One of the active defense system in plants is by producing phytoaleksin compound (Goodman *et al* 1986).

In the inoculated *A. microcarpa* plant with Fusarium sp, a defense mechanism was recognized by the reaction of yellowing in leaves to decidious. This reaction is very specific against differential pathogens and also differential plants. In the type of *Fusarium* 512, it was visible at plant A, B and C, the triggered reaction was very fast compared with the kind of *Fusarium* and other plants. Even though it was observed that after the fourth week the whole plant looked back to normal.

Dixon and Lamb (1990) stated that some cells in plants when attack occurred by pathogenic, the process is buffered by the mechanical defense (lignin, hydroksiproline, glycoprotein). However, if the mechanism fails, the plant will show necrosis symptoms. This is because the enzyme secreted by

Table 3. The severity score (mean+SD) of *A.microcarpa* after inoculated by *Fusarium sp*

Fusarium types	1 WAI	2 WAI	3 WAI	4 WAI
512	4.847±4.85	9.78±8.82	5.84±2.38	6.64±3.56
500	0.92±2.1	2.52±4.07	5.29±4.36	7.24±5.2
509	0	3.03±3.72	8.05±2.78	11.29±4.33
2969	0	6.68±5.1	8.12±4.48	11.63±6.67
Control	0	1.45±3.5	4.44±3.74	6.66±5.61

pathogenic would be confined by defense genes that will produce a variety of expression such as the formation of phytoaleksin, antagonism protein or the transfer of metabolism cycle, even sometimes these mechanisms would occur simultaneously.

The advance in the use of modern biological systems would not be separated from the principle of the chemical basis life. The DNA material genetic is the complex consisting of sugar, phosphates and bases which are composed accordingly to determine the development and growth of an organism and is inherited to the next generation. A certain sequence of a sugar-phosphate-basal chain is called gene. Genes control the chemical feature through the work of enzymes naturally. Through the technique, we should be able to do things like determine genes with specific function, isolate the specific genes, insert an intended specific gene into the DNA of other organisms and genes express to drive the host cells to make the material in accordance with its code (Crouch et al 1998).

Characteristic *A. microcarpa* with microsatellite marker

The results of amplification fragments testing of DNA by using four pairs of primary microsatellite in the population *A.microcarpa* showed that only two primaries are capable of amplification DNA with good condition. The profile of DNA electrophoresis of both locus was presented in Figure 3.

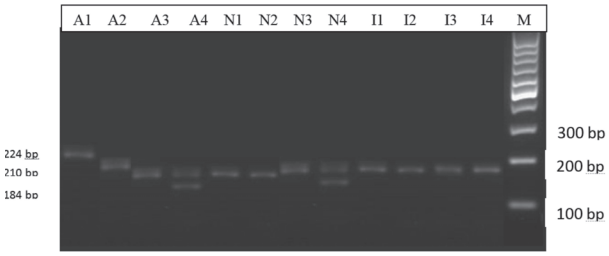

Fig. 3. The amplification results of PCR with primery 71pa17 in plant of *A. microcarpa*

Two other pairs of primers: 10PA17 and 16PA17 did not yield the amplification product. Eurlings *et al* (2009) organized a test at 12 microsatellite primers in *A. crassna* plant, 4 primers amplified the sample with a polymorphic pattern, while one primary was monomorphic and the rest 7 primers were having no amplification. The failure of amplification due to incorrect temperature had been minimalized because it dealt with in a touchdown decrease. The failure amplification in this research was possible because of the incompatible primers sequence with the DNA printed sequence, as reported by Eurlings *et al*. (2009), where of some samples of *A. crasna* were from different locations, the designed primer couples did not all amplify the tested sample plants. Primers sucessfully used in this study have been reported success in amplifying some DNA of the aloes crop from various areas (Siburian, 2017; Eurlings *et al*. 2009; Irmayanti, 2011). The couple of microsatellite primers: 6PA18 and 71PA17 tested in *A. microcarpa* were capable to amplify 5 to 6 alleles.

A gene loci is categorized polymorphic if at least there are two variations of different alleles and the frequency of its often found alleles was less than 95% (Finkeldey, 2005). The size and the sum of alleles according to (Leung *et al* 1993) were assumed that all the DNA ribbons with similar migration rate were classified as the homologous locus. A number of DNA microsatellite areas at an individual basis was expected to recognize the expression of a quantitative trait which was controlled by many genes (Dolf *et al* 1993). Microsatellite is a marker with a codomain trait so the genotype would directly be determined based on variations in alleles (Crouch *et al* 1998). The gained combination of specific alleles was constituted a specific phenotype because a molecular marker is generally found close to its gene (Muladno 2000). If this aspect is related to the resistance level of *Fusarium* inoculated plants so then specific alleles owned by the saplings population may be used as characterized plant interacting with to *Fusarium sp*.

CONCLUSION

The results testing of the DNA fragment amplification by using four pairs of microsatellite primers in the population of inoculated *A. microcarpa* by *Fusarium sp* showed that the two primers were able to amplify DNA, and were capable to demonstrate specific alleles traits as the nature characters of *A. microcarpa* plant that interacted with *Fusarium sp*.

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