Resistant mechanisms of red jabon seedlings (*Anthocephalus macrophyllus* (Roxb.) Havil.) against *Botryodiplodia theobromae* Pat. causing dieback diseases

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Abstract. *Anthocephalus macrophyllus* (Roxb.) Havil. seedlings are the most preferred plant for nursery because they have a lot of benefits. Further, its benefits can increase economic prosperity of this plant. The main problem in nursery of forestry plants is pest and disease attacks. The disease that is a focus on this research is dieback disease. The dieback disease is caused by *Botryodiplodia theobromae* Pat. that may lead the host plant to death. Every plant has its resistant mechanisms to pathogen attacks both before and after pathogen attacks. This research aims: (1) to study *B. theobromae* attack with wounded and non-wounded stem infection methods on red jabon seedlings; (2) to study resistant mechanism of red jabon seedlings both in structural and biochemical resistance against *B. theobromae* attacks. The structural resistance was done by studying the microscopic stem of the red jabon seedlings, which was conducted through scanning electron microscope. While, the biochemical resistance was done by studying the chemical compounds of red jabon seedlings’ stem using phytochemistry analysis. Red jabon seedlings had the structure of trychomaes on the epidermis and necrotic resistance as structural resistance before and after pathogen attacked, respectively. Red jabon seedlings also had secondary metabolites such as flavonoid, phenol hydroquinone, tannin, saponin, triterpenoids and steroids. Biochemical resistance of red jabon seedling after pathogen attacks showed the increase of accumulation of phenolic compounds as phenol hydroquinone and tannin and the increase of accumulation of triterpenoids and steroids compounds.

1. Introduction

Red jabon (*Anthocephalus macrophyllus* (Roxb.) Havil.) is a fast-growing plant, which is the most preferred forestry plant nowadays [18]. This jabon is mostly used as light construction, furniture, plywood, ceiling, box, traditional medicine, etc [11]. Because of its benefits and excellence, red jabon is widely cultivated at the level of nursery.

Red jabon (*Anthocephaluscadamba* (Roxb.) Miq.) is a fast-growing plant, which is the most preferred forestry plant nowadays [18]. This jabon is mostly used as light construction, furniture, plywood, ceiling, box, and traditional medicine [11]. Because of its benefits and excellence, red jabon is widely cultivated at the level of nursery.
The red jabon timber is categorized into the strength classes of II-III and durability class of IV [11]. According to Oey (1990), the durability classes of timber are grouped into 5 categories, i.e., highly resistant (I), resistant (II), moderate (III), not durable (IV), and highly not durable (V). Red jabon is more preferred than white jabon because it has harder wood and more resistant to pest and disease [11]. The main problem that often occurs in forestry nursery is pest and disease attacks. The diseases that most frequently occur in the forestry nursery are dieback, patches and blight of leaves. This research focused on dieback disease caused by \textit{Botryodiplodia} sp. Molecular identification by [25] showed that the pathogenic species causing dieback disease is \textit{Botryodiplodia theobromae}. According to [6], \textit{Botryodiplodia} sp. was reported as pathogen of some forestry plants in Indonesia, causing leaf spots on \textit{Alstonia} sp., \textit{Intsiabijuga} Kuntze., \textit{Rhizophora mucronata} Lamk., \textit{Macaranga gigantean} Muell., root rot on \textit{Shorea} sp., \textit{blendok} on \textit{Calophyllum phylum} Linn., and stem disease on \textit{Aquilaria malaccensis} Lamk. According to [4], \textit{B. theobromae} could attack white jabon seedlings through wounded and non-wounded stem infection methods. [7] showed that white jabon seedlings aged 3, 4, and 5 months suffered disease incidence of 100% with disease severity of 61, 42, and 54%, respectively. Dieback disease that occurs on red jabon seedling can cause destructions and the death of the seedling, which may lead to the reduction of economic benefits. Healthy trees are derived from quality seedlings which are not infected by pests and diseases. According to [1], the increase of pine seedling age could cause the rise in resistance to seedling rot disease. This research, therefore, employed five months red jabon seedlings for evaluation of their resistance mechanisms against \textit{B. theobromae}.

Studies about \textit{Botryodiplodia} spp. that attacks white jabon seedlings had been previously carried out by many workers. These workers included [7] on the pathogenicity test of \textit{Botryodiplodia} sp. on white jabon seedling, [4] on the virulence test of \textit{Botryodiplodia} sp. on white jabon seedling (\textit{A. cadamba}) and [11] on the bioactivity test of mahogany extract and molecular identification of \textit{Botryodiplodia} sp. on white jabon seedlings. However, studies on red jabon and its resistance mechanisms have never been done.

Every plant has its own resistance mechanism against pathogens. According to [2], the disease resistance mechanisms can be distinguished into two categories, namely structural and biochemical resistance, which occur both before and after the pathogen attack. Structural resistance includes the surface structures of plants and tissues, cells, cytoplasm, and necrotic resistance mechanism. Biochemical resistance includes the existence of inhibitor substances in plant cells such as secondary metabolites and the increase in accumulation of phenolic compounds. Some secondary metabolites are the phenolic compound such as flavanoid, quinone, and tannin.

Based on the above description, research on the resistant mechanism of red jabon seedling against \textit{B. theobromae} attack is essential. Therefore, this study aims to: (1) to study \textit{B. theobromae} attack with wounded and non-wounded stem infection methods on red jabon seedlings; (2) to study resistant mechanism of red jabon seedlings both in structural and biochemical resistance against \textit{B. theobromae} attacks.

2. Materials and Methods
This research was conducted from April to December 2014 in the Laboratory of Forest Pathology, Faculty of Forestry, and Laboratory of Analytical Chemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University (IPB), Bogor, Indonesia, plants nursery of BPDAS Citarum-Ciliwung, Dramaga, Bogor, Indonesia and Division of Zoology, Research Center for Biology, Indonesian Institute of Sciences, Cibinong, Bogor, Indonesia.

2.1. Rejuvenation and multiplication of isolated \textit{B. theobromae}
The isolates of \textit{B. theobromae} is a collection of Forest Pathology Laboratory, Bogor Agricultural University, Bogor, Indonesia [4]. Isolate multiplication was done by purifying the existing isolates. Rejuvenation was done based on [17] modification. The pathogen was planted on a PDA using a 5 mm diameter corebore, and then was incubated at 25°C in Laminar Air Flow until the pathogen growth filled the petridish. The pure culture of \textit{B. theobromae} isolates were then used as the inoculum source.
2.2. Research design
This research used a factorial treatment design assigned in a complete randomized design that combined pathogen inoculations (control and inoculated with pathogen isolate) and stem infection methods (wounded and non-wounded) in seedlings with 10 replications. Samples were placed in paranets and arranged accordingly to suit the treatment design.

2.3. Research evaluation
Redjabon seedlings aged five months were used in this research. Evaluation of resistance was carried out using a jelly block pasting method based on [13] with modification. In the wounded stem infection method, the inoculation was carried out using syringes. The control treatment was inoculated with jellyblock without the fungal pathogen isolate. A seven-day-old fungal isolate was used in the inoculation treatment. The observations were done for 14 days. In the non-wounded infection method, inoculation was done on the side of the stem with no any lenticels (examined using a loop). The observed parameters were the disease incidence [1] and disease severity (Townsend and Heurberger (1943) in Stevic et al. (2010), the incubation period, temperature and air humidity in the nursery (in the morning, at noon and night).

2.4. Structural resistance analysis of red jabon seedling by using Scanning Electron Microscopy (SEM)
Stems of healthy redjabon seedling were used as samples of control treatment and infected stems of redjabon seedlings through wounded and non-wounded stem infection methods. The analysis was done based on the Guide Book of Zoology Research Center, Indonesia Science Institution, Cibinong. The samples were observed using a scanning electron microscopy (model JSM-5310LV).

2.5. Biochemical resistance of secondary metabolite analysis of red jabon seedling by using phytochemical analysis
The analysis was done following the method of [12] on the stem of healthy red jabon seedlings as control treatment and infected red jabon seedlings through wounded stem infection methods. Samples from red jabon seedling’s stem were used in a powder of 500 mg in each testing. The secondary metabolite analysis included alkaloids, flavonoid, phenyl hydroquinone, tannin, saponin, triterpenoid, and steroid assays.

3. Results and Discussion
Disease incidence and severity data were subjected to analysis of variance, which then followed by the Tukey test to separate the treatment means when the treatment effect was significant. Structural and biochemical resistances data were descriptively analyzed and presented in forms tables and figures.

3.1. Disease incidence and severity on red jabon seedling
The disease incidence on redjabon seedling in control treatment with wounded and non-wounded stem infection methods were all 0% (Figure 1.A and 1.E). The disease severity of redjabon seedling in control treatment with wounded and non-wounded stem infection methods were also 0%. Control treatment (inoculated without pathogen isolate) of redjabon seedlings showed no dieback symptoms. According to [7], white jabon seedling aged 3, 4, and 5 months, showed symptoms of dieback disease; nevertheless, white jabon seedling inoculated without pathogen (control) showed no dieback disease symptoms.

The disease incidence on redjabon seedling in inoculated with pathogen isolate treatment through wounded (Figure 1.B, 1.C, and 1.D) and non-wounded (Figure 1.F, 1.G, and 1.H) stem infection methods were 100% and 80%, respectively. The disease severity of redjabon seedling in plants inoculated with pathogen isolate treatment through wounded and non-wounded stem infection methods were, respectively, 38% and 16%. The conditions of red jabon seedling with wounded and non-wounded stem infection methods are presented in Figure 1.
The response of pathogen inoculations and stem infection methods in red jabon seedlings showed that the disease incidence on the plants inoculated with pathogen treatment through wounded stem infection was not significantly different from those inoculated with the pathogen through non-wounded stem infection but significantly different from the control treatment (both wounded and non-wounded stem infection methods) (Table 1). The disease incidence on red jabon seedlings inoculated with the pathogen through wounded stem infection (100%) was more extensive than on red jabon seedlings inoculated with the pathogen through non-wounded stem infection (80%). According to [4] Botryodiplodia spp. showed symptoms of dieback disease on wounded and non-wounded stem infection methods. This fungi could attacks red jabon seedling, but not worse than white jabon seedling.

| Wounded | Control | 2 DAI | 7 DAI | 14 DAI |
|---------|---------|-------|-------|-------|
|         |         | A     | B     | C     | D     |

| Non-wounded |         | E     | F     | G     | H     |

**Figure 1.** The condition of red jabon seedling during 14 days observation. A. Control treatment with wounded stem infection method; B. 2nd day after inoculation (DAI) through wounded stem infection method; C. 7th DAI through wounded stem infection method; D. 14th DAI through wounded stem infection method; E. Control treatment with non-wounded stem infection method; F. 2nd DAI through non-wounded stem infection method; G. 7th DAI through non-wounded stem infection method; H. 14th DAI through non-wounded stem infection method.

The response of pathogen inoculations and stem infection methods in red jabon seedlings showed that the disease severity on the plants inoculated with pathogen treatment through wounded stem infection was significantly different from those inoculated with the pathogen through non-wounded stem infection and the control treatment (both wounded and non-wounded stem infection methods) (Table 1). The disease severity on red jabon seedlings inoculated with the pathogen through wounded stem infection (38%) was more extensive than on red jabon seedlings inoculated with the pathogen through non-wounded stem infection (16%).

The disease severity on the seedlings inoculated with pathogen through wounded stem infection method was worse than the those inoculated with pathogen through non-wounded stem infection.
According to [21], *B. theobromae* is a weak pathogen that needs injuries/wounds to infect the host, but it can be a serious disease.

In this research, we employed five months old red jabon seedlings. Age of the plant could increase the resistance mechanism of the plant. According to [1], the increase of pine seedling age could cause the increase of resistance of seedling to root rot disease. [7] showed that white jabon seedlings aged 3, 4, and 5 months had disease incidence of 100%, and the disease severity was, respectively, 61%, 42%, and 54%.

3.2. Incubation period

The incubation period is time intervals from inoculation to the appearance of disease symptoms. The incubation period of red jabon seedling was one day after the inoculation. The number of infected red jabon seedlings during 14 observation days was none for control, ten for inoculation with the pathogen treatment through wounded stem infection and eight for inoculation with the pathogen treatment through non-wounded stem infection. On the 14th days after pathogen inoculation, the numeric value of disease category and disease severity of red jabon seedlings, both wounded and non-wounded stem infection were, respectively, 3 and 1. The incubation periods are presented in Table 2.

The incubation periods of red jabon seedlings inoculated with the pathogen through both wounded and non-wounded stem infection methods were similar, i.e., one day after inoculation. Research by [20] employing *Corchorus olitorius* seedlings inoculated with seven days old inoculum of *L. theobromae* showed the disease symptoms on 7-10th days after inoculation. According to [2], incubation period is time intervals from inoculation to the appearance of disease symptoms. If plant has more time to be infected, that plant is more resistant.

3.3. Temperature and air humidity

Temperature and air humidity are the most important environmental factors for the growth of both the pathogens and the host plant, that will affect the development of the disease. The mean temperature for 14 days of observation was 27.53 °C in the morning, 33.27 °C during daytime, and 26.07 °C at night.

Environmental conditions important factors for the development of a disease are the temperature and air humidity. Means of temperature recorded during 14 days of observation were 27.53 °C in the morning, 33.27 °C in the daytime, and 26.07 °C at night. Means of air humidity recorded during 14 days observation were 84.67% in the morning, 72.47% at daytime, and 92.60% at night. During 14 days of observation, the *B. theobromae* fungal pathogen were in the optimum temperature, so the symptom occurred was caused by the biotic factors. According to [20], *L. theobromae* optimally grows at a temperature of 30°C. [5] [14], *L. theobromae* and *F. solani* are optimally grows at a temperature of 25 ± 2 °C.

**Table 1.** Mean dieback disease incidence and severity on red jabon seedling

| Treatment                                      | Replication | Disease incidence (%) | Disease severity (%) |
|------------------------------------------------|-------------|-----------------------|----------------------|
| Redjabon (control/inoculated without pathogen) | 10          | 0^b                   | 0^b                  |
| through wounded stem infection method          |             |                       |                      |
| Redjabon (control/inoculated without pathogen) | 10          | 0^b                   | 0^b                  |
| through non-wounded stem infection method       |             |                       |                      |
| Redjabon (inoculated with the pathogen) through| 10          | 100^a                 | 38^a                 |
| wounded stem infection method                   |             |                       |                      |
| Redjabon (inoculated with the pathogen) through | 10          | 80^a                  | 16^b                 |
| non-wounded stem infection method               |             |                       |                      |

Note: *Means within the same column with the same letter are not significantly different at Tukey test (α = 95%).*
Table 2. The incubation period of redjabon seedling against *B. theobromae* attack

| Treatment                                                                 | Number of infected seedlings | Incubation period (day) | The highest numeric value* |
|--------------------------------------------------------------------------|------------------------------|-------------------------|---------------------------|
| Redjabon (control / inoculated without pathogen), through wounded stem infection method | 0                            | 0                       | 0                         |
| Redjabon (control / inoculated without pathogen), through non-wounded stem infection method | 0                            | 0                       | 0                         |
| Redjabon (inoculated with the pathogen), through wounded stem infection method | 10                           | 1                       | 3                         |
| Redjabon (inoculated with the pathogen), through non-wounded stem infection method | 8                            | 1                       | 1                         |

Note: * = the highest numeric value of 10 replications recorded on the 14th days after inoculation. This value refers to the numeric value of disease category and dieback disease severity of red jabon seedlings (Townsend and Heurberger (1943) in Stevic et al. (2010).

3.4. Structural resistance in the red jabon seedlings’ stem

Red jabon seedlings had the structure of trychomaes on the epidermis, which is a form of structural resistance before pathogen attack and demonstrated necrotic resistance by activating hypersensitivity reaction, which is a form of structural resistance in response to the pathogen attack. The healthy red jabon seedlings (control) showed no hyphae of *B. theobromae* fungi (Figures 2.A and 2.B). Red jabon inoculated with pathogen treatment (infected) through wounded stem infection method showed the hyphae not on trychomaes and through into epidermal tissues in artificial wounded, cortex and through to stele tissues (Figure 2.C and 2.D). Red jabon seedlings inoculated with the pathogen (infected) through non-wounded stem infection method showed the hyphae through into the epidermal tissues until cortex and stopped in cortex (Figures 2.E and 2.F).

Figure 2 shows the microscopic of the transverse section of red jabon seedling’s stem using scanning electron microscope.

Red jabon seedling had the structure of trychomaes on epidermal tissues as structural resistance before pathogen attack and necrotic resistance through hypersensitivity reaction, which is a structural resistance mechanism that is activated after the pathogen attack. According to [19], the mechanism of structure of trychomaes as resistance is by capture microorganisms, include fungi spore and prevent the spore to reach the surface of epidermal. The trychomaes also have effect to humidity on epidermal. The spore carried by wind and sticks on trychomaes. The trychomaes have lowest humidity but spore need high humidity to grow. In addition, red jabon’s wood are harder than white jabon, both in seedling and plant (trees), so it difficult for pathogen to penetrate and infect the host. Faizah (2010), the high density of trychomaes was structural resistance mechanism of chili to against *Bemesiatabacito* transmit begomovirus, the cause of yellow curly leaves. According to [2], one of the active plant structural resistances is necrotic resistance activated through hypersensitivity reaction. The hypersensitive response causes damage to cellular membrane infected by the pathogen. It causes the plant tissues to respond to the pathogen by producing necrosis symptom.
Figure 2. Electronic micrographs of red jabon seedling’s stem: (A) epidermal tissues of healthy red jabon seedling; (B) stele tissues of healthy red jabon seedling; (C) cortex tissues, which were dried (red jabon seedling’s stem inoculated with pathogen through wounded stem infection method); (D) hyphae of \textit{B. theobroma} attacking the epidermal, cortex and through to stele tissues; (E) epidermal and cortex of destructed red jabon seedling inoculated with pathogen through non-wounded stem infection method; (F) hyphae of \textit{B. theobroma} on trichomes of red jabon seedling inoculated with the pathogen through non-wounded stem infection method.

Infection of \textit{B. theobroma} on red jabon seedlings occurred through the lenticel, artificial wounds, and direct infection through the epidermal surface using physical or biochemical weapons. On red jabon seedling with non-wounded stem infection methods, the mycelium on the structure of trichomes on epidermal tissues. Mycelium infected in epidermal, destroyed the cell, dried and died. Then the pathogen destroyed the cortex tissues and through to stele. According to [19], infection mechanism of pathogen \textit{M. grisea} is penetrating the host by apresorium. Some pathogens had cell wall destructed enzyme to infect host cells. These enzymes such as, xylanase, metilesterase pektin, endoglucanase, and polysaccharide deacetylase. \textit{Fusarium moniliforme} has polygalacturonase that infected tomato plant’s tissues.

3.5. Diseases The biochemical resistance of red jabon seedlings’ stem
A healthy red jabon seedling contained secondary metabolites such as flavonoid, phenyl hydroquinone, tannin, saponin, triterpenoid and steroid. In the seedlings inoculated with the pathogen (infected), there was an increase in some phenolic compounds such as phenol hydroquinone and tannin. This treatment also demonstrated an increase in triterpenoid and steroid contents. The secondary metabolites of red jabon seedling are presented in Table 3.
Table 3. Secondary metabolites of red jabon seedlings’ stem

| Active compounds | Treatment                  | Control (healthy, inoculated without pathogen) | Inoculated with pathogen (infected) |
|------------------|----------------------------|----------------------------------------------|------------------------------------|
| Alkaloids        |                            | -                                            | -                                  |
| Flavonoid        |                            | +                                            | +                                  |
| Phenol hydroquinone |                          | +                                            | ++                                 |
| Tannin           |                            | +                                            | ++                                 |
| Saponin          |                            | ++                                          | +                                  |
| Triterpenoid     |                            | +                                            | +++                                |
| Steroid          |                            | ++                                          | +++                                |

Note: (-) negative, (+) positive but weak, (++) positive and somewhat strong, (+++) strong positive, (+++++) very strong positive.

The biochemical resistance of red jabon seedlings did occur through the existence of inhibitory substances in the host cells such as secondary metabolite compounds. Red jabon seedlings contained flavonoid, phenol hydroquinone, tannin, saponin, triterpenoid and steroid compounds. According to [23], secondary metabolite is a compound of non-essential metabolites that serve as a resistance mechanism to environmental conditions, resistance to pest attacks, diseases, and attracts the pollinators.

Biochemical resistance after pathogen attack of red jabon seedling occurred through the increase in phenolic compounds accumulation. Several compounds of secondary metabolites included the phenolic compound such as the flavonoid, tannin, and phenol hydroquinone. The inoculated red jabon seedlings showed an increase of phenol hydroquinone and tannin compounds. According to [2], one of the plant biochemical resistances after pathogen attack is the increase in the accumulation of phenolic compound. The rise of accumulation of phenolic compound occurs soon after pathogen infection in resistant varieties. [24] found an increase of total phenol content in tomatoes infected by *Fusarium* sp. [3] the activity of polyphenoloxidase is higher on infected resistant variety than vulnerable plant.

Besides, we found in the present study that red jabon seedlings also showed an increase of triterpenoid and steroid compounds. [11] demonstrated that extract of leaves, bark, rind of the fruit, seeds, and roots of mahogany had antifungal compounds against *Botryodiplodia* sp. in in-vitro assay because these extracts contain limonoid derived from limonin and triterpenoid. Triterpenoid of annual plant roots, stems of two years old, leaves, flowers, and twigs of *Jatropha curcas* are potential antifungal compound against *M. albican* and *C. guiliermondii* [16]. According to [9], steroid serves as anti-inflammation, anticarcinogenic, and controller of diabetes. [15] also added that steroid could be used as a toxic compound.

4. Conclusions

The disease incidence of red jabon seedlings inoculated with the pathogen through wounded stem infection method was same than of red jabon seedlings inoculated with the pathogen through non-wounded stem infection method. However, the disease severity of red jabon seedlings inoculated with the pathogen through wounded stem infection method was worse than that of red jabon seedlings inoculated with the pathogen through non-wounded stem infection method. Red jabon seedlings had the structure of trichomes as structural resistance before pathogen attack and necrotic resistance through hypersensitivity reaction as the resistance after pathogen attack. Biochemical resistances of white jabon seedling both before and after pathogen attack were found as secondary metabolites such as flavonoids, phenol hydroquinone, tannin, saponin, triterpenoid and steroids, and the increase of phenolic compounds, such as phenol hydroquinone and tannin, and the increase of triterpenoids and steroids compounds.
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