



ANATOMICAL CHARACTERIZATION OF SELECTED PLANLET *Vanilla planifolia* Andrews. ON FUSARIC ACID, RESISTANT TO *Fusarium oxysporum* f. sp. *vanillae*

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ABSTRACT

Vanilla is one of the important agricultural export commodity of Indonesia. The vanilla from Indonesia is well known for its high levels of vanillin content, which is about 2,75%. Until recently, the most hazardous disease of vanilla is foot rot infection caused by *Fusarium oxysporum* f. sp. *vanillae* (Fov). One alternative way to control the foot rot disease is by using cultivars which are resistant to its pathogen. Selected plantlets have been induced by using fusaric acid on the *in vitro* medium. The goal of this research is to characterize the anatomical point of few especially on leaves, pseudo bulb and root with respect to resistance to Fov. Moreover, it is also addressed to determine the different characters of the phenotype vanilla plantlet which are total phenol, peroxidase enzyme activity, the thickness of lignin, and the chlorophyll content. The data on this research were recorded, compiled and categorized as descriptive qualitative and quantitative information. The result showed significant increase in chlorophyll a, chlorophyll b, and also the total of chlorophyll. The overall in line correlated with the increasing FA concentration. The total chlorophyll at 90 ppm FA could increase about 1,2 times, while at 100 ppm and 110 ppm FA could be enhanced about 1,4 to 1,5 times compared to control. Anatomical characters between vanilla control, infected Fov, and resistant to Fov, were shown clearly at the stele part especially at xylem as the main function for transporting water from root to the leaf, which formed thyllose. Phenol total at about 4,39 % in control, increased to 5,34% in 90 ppm, and followed by 5,52 % in 100 ppm and 5,87 % at 110 ppm. Peroxidase enzyme activity at FA 90 ppm produced about 0,25 U/mg/min, and at 100 ppm FA on 0,34 U/mg/min, while at FA 110 ppm generated 0.36 U/mg/min compared to control, at 0,12 U/mg/min. The thickness of lignin tracheid in the treatment of 90 ppm was at about 14,59 μ m; 100 ppm 18,16 μ m; and 110 ppm 19,39 μ m compared to controls 10,28 μ m. In conclusion overall showed that FA induced plantlet vanilla *in vitro* medium resulted in change of phenotype compared to control, which also affects its anatomy structure, especially on lignification and thyllose formation at surrounding tracheids, which could be used as a marker for the defense mechanism of plant to Fov.

Key words: *Fusarium oxysporum* f. sp. *Vanillae*, peroxidase enzyme, anatomical characters.

INTRODUCTION

Sub-sector plantation from agriculture has played an important role in order to elevate national economic growth. This sector contributes to foreign exchange value and also works plan (Anonymous, 2010). Vanilla (*Vanilla planifolia* Andrews) is an industrial plantation, which is planted in tropical countries including Indonesia, as an export commodity (Hadipoentiyanti *et al.*, 2007). Indonesian vanilla, *Java Vanilla Beans* is widely known for its high production and best quality. It contains about 2,75% vanillin, followed by Madagascar vanillin (1,91 - 1,98%), Sri Lanka (1,48%), and Mexico (1,89-1,98%) (Hadisutrisno, 2004). *United Nations Development Programme* (UNDP), ranked Indonesian vanilla in the same level as *Bourbon vanill*, which has already been known in the international vanilla market for its quality (Umamaheswari & Mohanan, 2011). This is an advantage for the enhancement of Indonesian vanilla export and increasing farmer income (Barani, 2008). Vanillin is used in food or beverages, medicinal beverage, and in aromatic perfume industries as raw materials (as *tincture*

or absolute form) (Mengesha, 2012). Vanilla productivity level in Indonesia was not stable. Therefore, it is required huge quantity plantlet, which was resistant to *Fusarium oxysporum* f.sp. *vanillae* (*Fov.*). One alternative way is to grow a new cultivar, which is resistant to *Fov.*, and also high yield product (Tombe, 2010).

Foot root disease on pseudo bulb of vanilla (BBV) is the main constrain for production of vanilla fruit caused by *Fov*, which affected 50-100% death plant. Although some of them still survives, but causes lower quality of product (Hadisutrisno, 2004). This disease, usually spread via storage plantlet from vegetative breeding, which has already 7-32% infected *Fov*.microspores during propagation (Tombe, 2010).

An alternative method to encompass that constrain is using fusaric acid (*5-n-butylpicolinic acid*) as a metabolite resulted from *Fusarium* as a selective agent to the plantlets explants. At concentration above 10^{-5} M, the fusaric acid could hinder respiration at mitochondria and also sitokinin oxidation process. In addition, it is also decrease *Adenosin Tri Phosphat* (ATP) on plasma membrane as well as reduction on polifenol activity so hinder plant growth and regeneration. Contrastly, concentration below of 10^{-6} M, could induce fitoaleksin sintesis, as plant response for pathogenic activity inhibition (Bouizgarne *et al.*, 2006). This kind of plant defence mechanism would be expressed after patogen attachement (Huang, 2001). Such parameters could be measured including: increasing fenolic coumpound as well as increasing peroxidase activity (PR-protein), also lignifications especially at xylem wall (Lea & Leegod, 1999). There is positive correlation between resistance plantlet to FA with resistance plantlet to *Fusarion* respectively (Arai & Takeuchi, 1993). Such researchs identification *in vitro* insensitif variety plantlet to FA was done on tomato (Toyoda *et al.*, 1984), banana (Matsumoto *et al.*, 1995), gladiolus (Remotti *et al.*, 1997), and ananas (Borras *et al.*, 2001), which showed that these clones were also resistant to pathogen. Similar experiments on vanilla, were also reported by Kosmiatin *et al.* (2000), and Inayati (2003), but their result still un-stable yet.

In this context FA possibly would affect the changing of base promoter and trigger the expression of peroxidase gene (Saravanan *et al.*, 2004). Then this protein could be identified by *Sodium Dodecyl Sulphate-Polycrylamide Gel Electrophoresis* (SDS-PAGE) according to Maniatis *et al.* (1982). Beside that, genetic variety on vanilla plantlet could be detected by molecular marker, *Randomly Amplified Polymorphic DNA* (RAPD), which base on *Polymerase Chain Reaction* (PCR) with 10 bases random primer (Williams *et al.*, 1990). This research was aims to get a selected plantlet AF and characterized base on morphological, physiological and anatomical perspective.

MATERIALS AND METHODS

Preliminary study was done on *in vitro* micropopagation of vanilla nodus using MS medium plus *2,4-Dichlorophenoxy Acetic Acid* (2,4-D), *Naphthalene Acetic Acid* (NAA), and *Benzyl aminopurine* (BAP) (Rout *et al.*, 2006). Phenolic compound has estimated for resistant vanilla and the control by using the method of Aberouman & Deokule, 2008. Chlorophyll content was measured using plantlet leaves between controls in the company of resistant vanilla plantlet according Harbourne (1987). This analysis was including the total chlorophyll, chlorophyll a and Chlorophyll b respectively. Protein and DNA profiling also have

been detected. Character anatomical as the main concerned was showed in the pictures. It also another character which supported this research has been added as supplementary results.

RESULTS AND DISCUSSION

Characterisation of resistant plantlet vanili

The results showed that a combination of 2,4-D 2,0 mg/L plus NAA 10 mg/L was the most effective for callus initiation and shoot development from shoot tip explants and the BA 1,0 mg/L was the fastest shoot initiation from a nodes explants. Nodal indeed was the best explant for vanilla plantlet regeneration. In one node explants could produce 3 to 6 shoots within 12 weeks. The increased of capability in nodal explants to form shoot is probably caused by the existense of axillaries meristem, so the explants easily regenerated to form shoots. The optimum concentration of FA on plantlet selection with vanilla steady growth was between 90 ppm-110 ppm. They showed yield in plantlet vanilla at about 17,14 % at 90 ppm, 12,00% at 100 ppm and 10,59 % at 110 ppm, all of them insensitive to FA. FA insensitive plant may occurred as result of higher expression peroxidase enzymes on variety of resistant plantlet for detoxification. By using FA concentration of 110 ppm was already effective for suppression of *Fov* growing up to 25%, compared to the concentration of 90 ppm and 100 ppm respectively. In other words by using 110 ppm fusaric acid, it could increased the category criteria from sensitive to resistant. Molecular analysis throughout a protein (SDS PAGE) and DNA (RAPD) pattern proved that Plantlet characters of vanilla indeed resistant to *Fov*. The protein profile of vanilla plantlet showed a thicker band of about 18 kD compared to the control. We predicted this band like patogen related (PR)-protein may be as the peroxides protein (enzyme), which was responsible for vanilla resistant to *Fov*. A new band of Random Amplified Polymorphic DNA in a resistant plantlet were found in size of 930 bp by using (OPB_14) primer, 430 bp (OPB_20), 230 bp and 270 bp (OPD_19) respectively. These bands predicted as a candidate marker RAPD which responsible for vanilla resistant to *Fov*. Actually by increasing FA concentration would enhance also the total phenol, peroxides enzyme activity, and thickness of lignin in vanilla resistant to *Fov*. Elevated levels of total phenol of about 4,39 % in control, increased to 5,34% in 90 ppm, followed by 5,52 % in 100 ppm and 5,87 % at 110 ppm Harniet *al.*(2012). The peroxides enzymes activity enhanced at about 2 to 3 times at 90-110 ppm FA concentrations compared to the control.

According Fahn, (1991) in monocot, the bundle sheath there was no cambium except in the some family of Liliales and this bundle sheath spread in the stele. That's why the stem of monocot relatively constant in the size one to another and could not develop anymore (Dickison, 2000). Lignin was formed as a plant response to the fusarium in order to prevent further damage at epidermal and tracheal parts (Raven *et al.*, 1999). The root of vanilla showed that xylem is consisted of trachea, tracheids, fibers and parenchyma and located in the stele like some finger print so called diarch, triarch and *polyarch*. Xylem consist of protoxylem located at the perifer and metaxylem located to the central. The thickness of lignin in the resistant plantlets was increased about 1.4 times at 90 ppm, 1.8 times at 100 ppm; and 1.9 times at 110 ppm compared to the control. At non-toxic concentrations (10^{-7}) FA would induced the H_2O_2 from peroxisome organel inside of the cells (Agrawal *et al.* 1999).

This peroxide was a compound which could trigger the increase of peroxides enzymes activity and these enzymes possibly would oxidized phenol compound and catalyzed this compound reaction to quinone compounds, which produced H_2O_2 that naturally toxic to pathogen (Bouizgarne *et al.*, 2006). Peroxides enzyme plays an important role as regulator catalyst for biosynthesis inducing lignin.

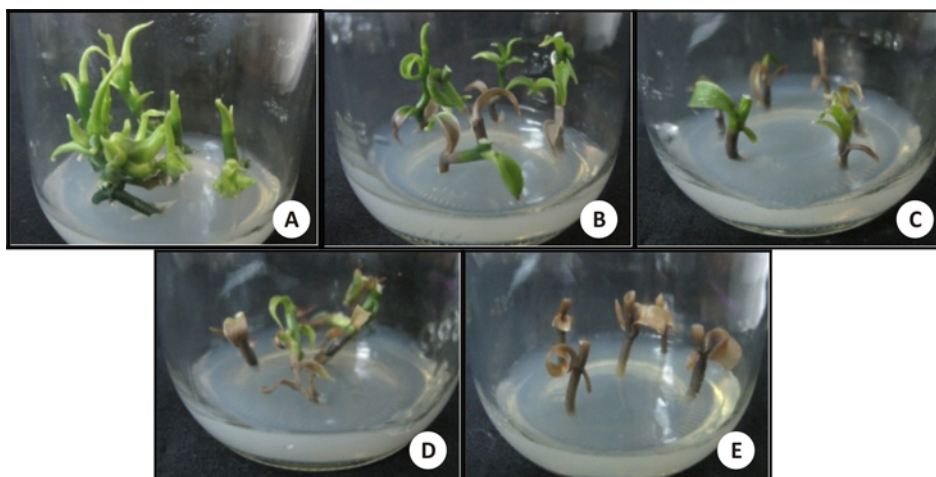


Figure 1. Vanilla plantlet 12 weeks on different fusaric acid concentration A= 0 ppm (control), green all, B= 90 ppm, green to few brown, C= 100 ppm green and a little brown, D= 110 ppm green to brown, E= 120 ppm, brownness

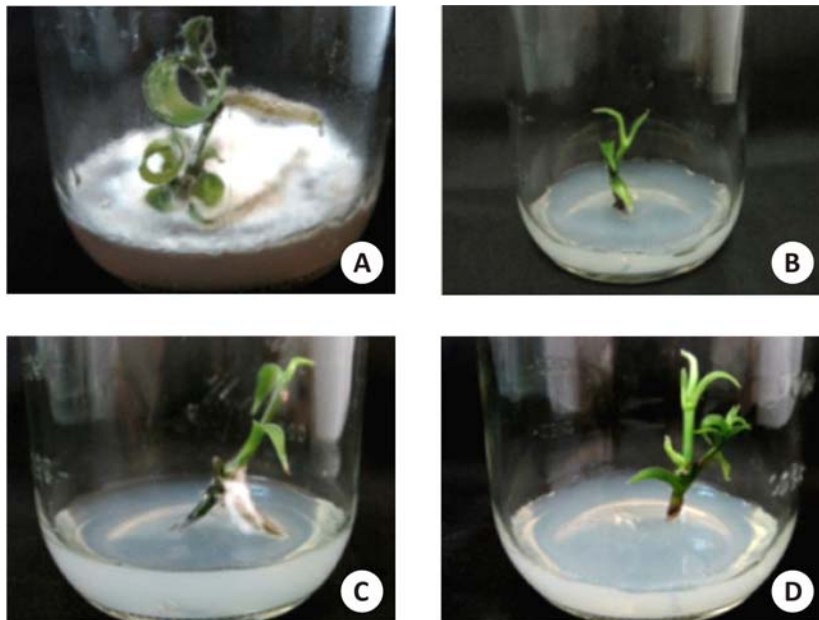


Figure 2. Infected of *F. oxysporum* sp. *vanillae* on vanilla plantlet at different FA concentration 29 days after selection. A= 0 ppm, totally die, B= 90 ppm, no infection, C= 100 ppm, no infection, D= 110 ppm, no infection

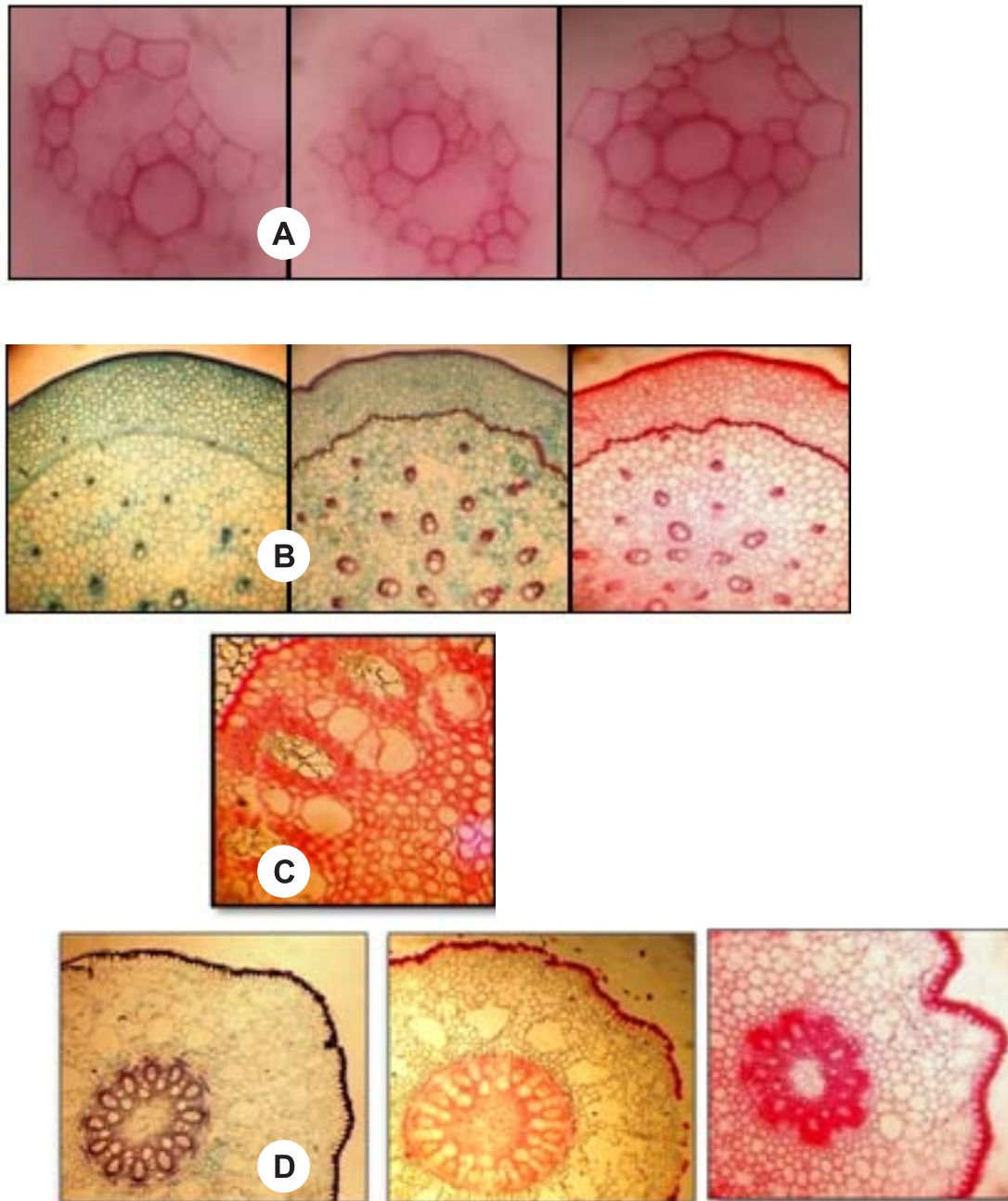


Figure 3. These picture A, B, and D from left to right was: uninfected fov (control), infected to fov. and resistant to fov. A. Lignification on different bundle sheath part for detection the lignin thickness B. Cross section of vanilla pseudobulb showed amiloplast located between cortex and stele part as an analogous of cambium at dicotyl stem C. Cross section of infected to fov. showed thyllose at xylem at tracheid lumen consisted with parenchymes tissue D. cross section of root uninfected, infected to fov. and resistant to fov. From outside to inside: epidermal root showed lignifications at U shape character, parenchyma cortex, endodermis, floem, xylem, pith.

There was significant increased in total chlorophyll at about 1.2 to 1.5 times compared to the control. This phenomenon in the plantlet proved that it was because of FA pressures, underwent adaptation and changed the basic structures of promoter for toxin (H_2O_2) detoxification, through activated peroxides gene, affected increasing of the chlorophyll in resistant plantlet.

This research was supported by Suharyanto *et al.* (2007) about *threonin deaminase defektif* (TD⁻) *Nicotiana plumbaginifolia* mutant complemented by cDNA TD tomato, resulted an increase twice more at the transgenic, even phenotypically better than those mutant. *Starvation* experiment also was reported from isolated *Nicotiana sylvestris* mutant resistant to β -Aminoethylcystein (AEC) when on medium supplemented by isoleusin, lysine, danvalin, and homozygot progeny showed better morphological and physiological growth than control (Suharyanto; personal communication, Frankard *et al.*, 1992).

The anatomical comparison between vanilla plantlet uninfected, infected, and resistant to *Fov*, showed on xylem as the main water transportation. The metaxylem root lumen formed new parenchymas and was called thylose. These outgrowth tilosis as parenchyma growth in the xylem may be due to of mechanical response to the presence of pathogen infection, and served to limit the pathogens. Daly (2006) reported how thylose was formed by *Fusarium oxysporum* f.sp. *cubense* (*Foc*) infected at banana stem. First at all, fungi infected in the form chlamidospores penetrated via root injury and then those spores went to xylem by water transport. Later spores would germinate to form micellium in which give a signal to the cell and peroxisomes produce peroxides. Xylem formed parenchymals in the lumen of tracheids was thylose to prevent water containing spores for further transport to the stem. Finally, plant would die because supply of water and mineral was blocking (Semangun, 2001).

CONCLUSION

The anatomy comparison between uninfected *Fov*, infected *Fov*, and resistant to *Fov*, vanilla viewed from part of the stele. They located on xylem as the main transportation. Metaxylem roots forms new parenchyma's in the xylem lumen named tilosis. These outgrowth tilosis in the xylem probably is a form of mechanical response to the presence of pathogen infection, and its function is to isolate the pathogens. In general, the increasing of fusaric acid concentration used for selection are followed by enhancing of phenolic compound, peroxide enzyme activity, chlorophyll content and thickness of lignin in epidermal as well as in surrounding tracheids.

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