

INFECTION PROCESS OF *Fusarium oxysporum* FUNGUS: A CAUSE OF DAMPING-OFF ON *Acacia mangium*'s SEEDLINGS

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ABSTRACT

Fusarium oxysporum is the causal agent of damping-off disease. The fungus attacks seedlings of many plant species, including *Acacia mangium*. In order to effectively control the disease, detailed information on how the fungus infects seedlings of *A. mangium* and how the plant responds to the fungal infection is essentially needed. The objectives of this research were to investigate: (1) the infection process of *F. oxysporum* on seedlings of *A. mangium*, (2) the defence response of *A. mangium* seedling to infection by *F. oxysporum*. The fungal pathogen was identified, followed by performance of pathogenicity test. The infection process was followed by macroscopic observation as well as microscopic observation. The result indicated that fungal spore germination was observed at two-day post inoculation *in planta*. At four-day post inoculation, hyphae of *F. oxysporum* had penetrated the collar root of *A. mangium* seedling via stomata aperture. In addition, fungal hyphae had grown intercellularly in to the vascular tissue. Correspondingly, hypersensitive response was also detected at the stomata aperture. However, this defence mechanism is not effective in stopping the fungus since *F. oxysporum* is a necrotropic pathogen. Moreover, accumulation of lignin, but not callose, was observed.

Keywords: *Fusarium oxysporum*, damping-off, *Acacia mangium*.

INTRODUCTION

Demand for wood material from industrial plantation forest has increased after its introduction in 1984. Hardiyanto and Arisman (2004) reported that as an important tree species in industrial plantation forest, *Acacia mangium* Will has some advantages, such as fast growth, suitable characteristic of its timber for pulp and paper raw material, construction material, light construction and coal. To support raw material availability, many healthy *A. mangium* seeds are required to achieve the expected maximal production target.

Acacia mangium seedling is susceptible to disease in nursery such as damping off. *Fusarium oxysporum* is a soil borne pathogen fungus, causing damping-off disease in *A. mangium*. The fungus is also reported to cause damping-off disease in forestry plant nurseries particularly in *A. mangium* seedling (Wardani, 2007), *Pinus merkusii* (Achmad *et al.*, 2012), *P. Pinea* (Machon *et al.*, 2009), *Eucalyptus viminalis* (Salerno *et al.*, 2004). The objectives of this research were to investigate the infection process of *F. oxysporum* on seedlings of *A. Mangium* and the defence response of *A. mangium* seedling to infection by *F. oxysporum*

MATERIALS AND METHODS

Fusarium oxysporum Isolations and *Acacia mangium* Seedling

Isolated *F. oxysporum* was obtained from two-week old, infected *A. mangium* seedling indicating damping-off symptom. The *F. oxysporum* from infected *A. mangium* seedling was isolated and grown in *Potato Dextrose Agar*

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(PDA) media. *Fusarium oxysporum* was identified when colony was 4 days old in PDA media. The identification process included size and shape of the observed spore as well as its color and the change of colony color (Leslie and Summerell, 2006). *Acacia mangium* seedling was obtained by planting *A. mangium* seed in sterile soil media.

Defense Response of *Acacia mangium* Seedling Against *F. oxysporum*

Infected *A. mangium* seedling was observed microscopically. The infected seedling was treated with chlorophyll removal by soaking the seedling using 96% ethanol solution, heated at 100°C for 10 minutes, and left for cooling for 1 hour at room temperature. Then, the seedling was treated using transparent tissue and soaked into 20 mL (2.5 g/mL) chloral hydrate solution (Merck) for 12 hours. Observation of the infection process of *F. oxysporum* in *A. mangium* seedling was conducted consecutively at 2, 4, 6, and 8 days after the suspended spore inoculation in the 1-week *A. mangium*. Infected *A. mangium* seedling was then stained with lactophenoltrypan blue (Merck). The modified Ruzin's (1999) method was used for lignin accumulation. Tissue that was treated with transparent tissue was soaked in 20 mL phloroglucinol (Merck) solution for 30 minutes. Callose accumulation assay was done using the modified Ruzin's (1999) method as well. Tissue treated using transparent tissue agent was soaked into 20 mL aniline blue (Merck) solution for 10 minutes. Then, microscopic observation was done using Axioskop 40 fluorescent microscope and was recorded with Canon Power Shot A 620 camera digital.

RESULTS AND DISCUSSION

Fusarium oxysporum Pathogenicity in *Acacia mangium* Seedling

Acacia mangium seedling in nurseries showed symptom of *damping-off* disease at two weeks after germination (Figure 1). Early symptom was indicated with wilted seedling, and the rot began from base up to whole seedling stem when seedling was 6 days old that caused damping off and death.

The fungus was isolated from *Acacia mangium* seedling showing the symptom and sign. The fungus was then sub cultured in PDA media at 27 °C and pure culture was obtained

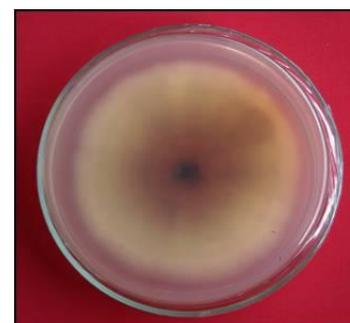
(Figure 2). Observation of the fungal colony morphology showed that the pathogenic fungi grown in PDA media were thin and rare, white as cotton to pale violet. Another side of the colony was yellowish to purplish.



Figure 1. Eight-day infected *Acacia mangium* seedling by *Fusarium oxysporum*



(A1)



(A2)

Remarks: (A₁) = colony upper surface (A₂)= colony lower surface

Figure 2. Eleven day old *Fusarium oxysporum*



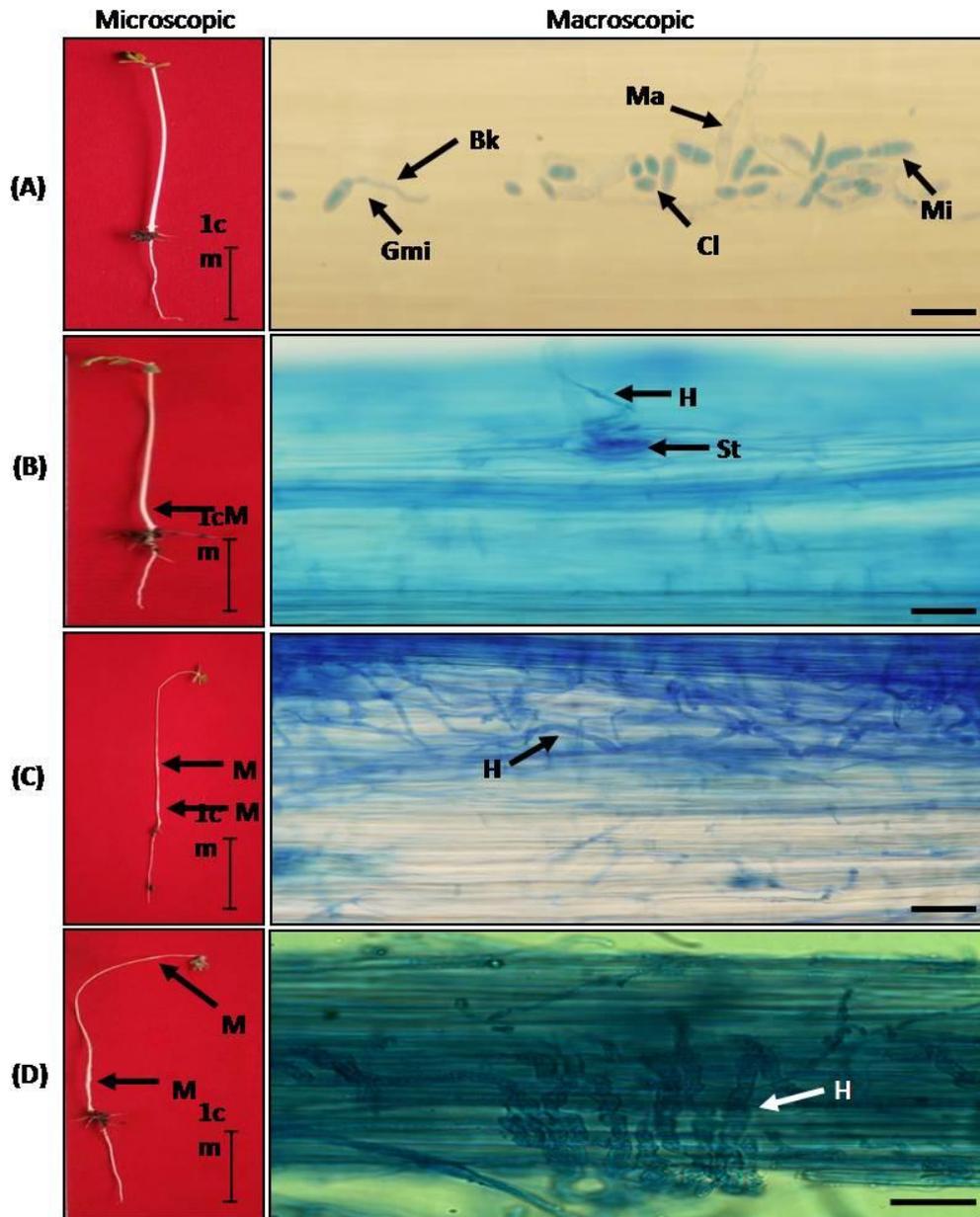
Remarks: (A) Macro conidia, (B) Micro conidia, (C) and (D) Chlamyospore (arrow sign)
(H) *F. oxysporum* hypha (Bar = 10 μ m)

Figure 3. *F. oxysporum* spore

Next identification indicated that pathogen fungi infecting *A. mangium* seedling had three spore types i.e. macroconidia, microconidia and chlamyospore (Figure 3). The spore size was various. Macroconidia had size ranging from (12-18) x (3-5) μ m, with 3 septa and no septa, the fusiform was slightly bent and pointed at two ends. Microconidia had 2-4 septa and no septa. Its shape was ovoid ellipse, and its size was between (4-8) x (1.5-2.5) μ m. Microconidia was smaller than macroconidia and chlamyospore. Chlamyospore presented at hypha end/ terminal and within hypha or intercalary, had smooth wall, round shape, in single and pairing configurations with diameter of 2.25-3.75 μ m. Based on macroscopic and microscopic observations, isolation of pathogenic fungi infecting *A. mangium* seedling was *F. oxysporum* Schlechtendahl emend. Snyder and Hansen (Leslie and Summerell, 2006).

***Fusarium oxysporum* Infection on *Acacia mangium* Seedling**

Growth stages of *F. oxysporum* infection process at *A. mangium* seedling stem is presented in Figure 4. Macroscopic examination indicated that at 2 and 4 days after inoculation, there were no white mycelia appeared at the stem of *A. mangium* seedling. Microscopic examination of two-day-old seedling showed microconidia, macroconidia, hypha and chlamyospore on the surface of *A. mangium* seedling. At 4 days old seedling, macroscopically thin white mycelia appeared in base stem of *A. mangium* seedling. Microscopic examination indicated hypha growth that infecting seedling through stomata. Six days after *F. oxysporum* inoculation, macroscopically white mycelia was wider not only in stem base, but also up to central part. At 8 days after inoculation thin white mycelia even covered all stem surfaces until the seedlings died. Then, microscopically, there appeared addition of hypha on seedling stem surface.



Remakrs: (A)Two-day-old *A. mangium* seedling after inoculation, (B)*A. mangium* seedling, 4 days after inoculation, (C)*A. mangium* seedling, 6 days after inoculation, (D)*A. mangium* seedling, 8 days after inoculation, (H)*F. oxysporum* hypha, (Mi) Microconidia, (Gmi) Germinated microconidia, (Ma) Macroconidia, (Ch) Chlamydospore, (St) Stomata, (M) Mycelium *F. oxysporum*, (Bk) Shoot reed. (Bar = 50µm)

Figure 4. *Fusarium oxysporum* infection process at *Acacia mangium* seedling stem

Microscopic observation indicated that *F. oxysporum* hypha penetrated through stomata causing *damping-off* disease when *A. mangium* seedling occurred at 2 and 4 days after inoculation (Figure 4). Penetration continued into plant tissue in intercellular way at phloem and xylem when seedling was at 6 and 8 days after inoculation resulting in seedling death. Vidhyasekaran (2008) stated that *F. oxysporum* hypha performed direct penetration and colonized cortex. The colonization process caused fungi to be able to reach xylem vessel tissue and spread fast to stimulate wilt sign of the plant.

Saprophytic microorganism can turn pathogenic in plant through parasitic adaptation and had various levels from obligate parasite to facultative parasite (Widyastuti *et al.*, 2005). *F. oxysporum* is one of saprophytic microorganism that can change into pathogenic microorganism that can cause *damping-off* on *A. mangium* seedling. Moreover, Widyastuti *et al.* (2005) stated that capacity of a microorganism to be a pathogen in plant was determined by its capacity to (1) penetrate into plant tissue, (2) break host defense, and (3) cause disease. Based on the current research result, soil-borne pathogen *F. oxysporum* was proved to have the capacity to penetrate phloem and xylem vessel tissue of *A. mangium* seedling, which finally caused *damping-off* disease over *A. mangium* seedling.

Defense Response of *Acacia mangium* Seedling due to *Fusarium oxysporum* Infection

Defense response of *A. mangium* seedling due to *F. oxysporum* infection was marked with lignin accumulation (Figure 5). Lignin accumulation in *A. mangium* seedling stem was detected when the seedling was at 4 and 6 days after inoculation. It was marked with light red to violet colour in area around phloem and xylem vessel tissue of the infected *A. mangium* seedling after stained with Phloro-glucinol (Ruzin, 1999; Vidhyasekaran, 2008). Observation showed that lignin was also accumulated in infected phloem

and xylem tissue, but not found as many as it was in area around infected phloem and xylem vessel tissue (Figure 4). Lignin was also not detected in uninfected xylem vessel tissue. It is expected that lignin in *A. mangium* seedling not inoculated by *F. oxysporum* was still produced but it was not as much as an infected *A. mangium* seedling. Lignin as chemical component in cell wall of xylem vessel tissue functioning to strengthen mechanical defense of plants was also produced (Vanholme *et al.*, 2008). Accumulated lignin was detected around stomata and xylem tissue in the inoculated *A. mangium* seedling stem, indicating response from the plants to strengthen mechanic system defense due to penetration of *F. oxysporum* hypha. Lignin was also one of polymers which is difficult to be degraded by pathogen (Nicholson and Hammerschmidt, 1992).

Widyastuti *et al.* (2005) indicated that wound or even death of host cells due to pathogen could often stimulate polyphenol oxidation or lignin accumulation in the healthy cells around the area of damaged cells. Accumulated lignin was detected to increase on cell wall of wheat (*Triticum sativum*) tissue, which is vulnerable to infection by *F. culmorum*. In contrary, lignin accumulation increased intensively on cell wall of wheat resistant to infection of *F. culmorum* (Kang and Buchenaur, 2000). Hadi (2001) reported that increased lignin in *P. merkusii* seedling due to *F. oxysporum damping-off* pathogen and *R. solani* occurred due to activities of peroxidase and polyphenoloxidase. Peroxidase and polyphenoloxidase are enzymes that will respond to oxidation of metabolites in plant related to phenolic compounds. The phenolic compound oxidation will affect production of lignin precursor that leads to lignin accumulation on infected tissue. Peroxidase activity was also detected to be related to the increase in lignin synthesis in pepper (*Piper nigrum*) in responding *Glomus intraradices* and *P. capsici* (Zheng *et al.*, 2005).

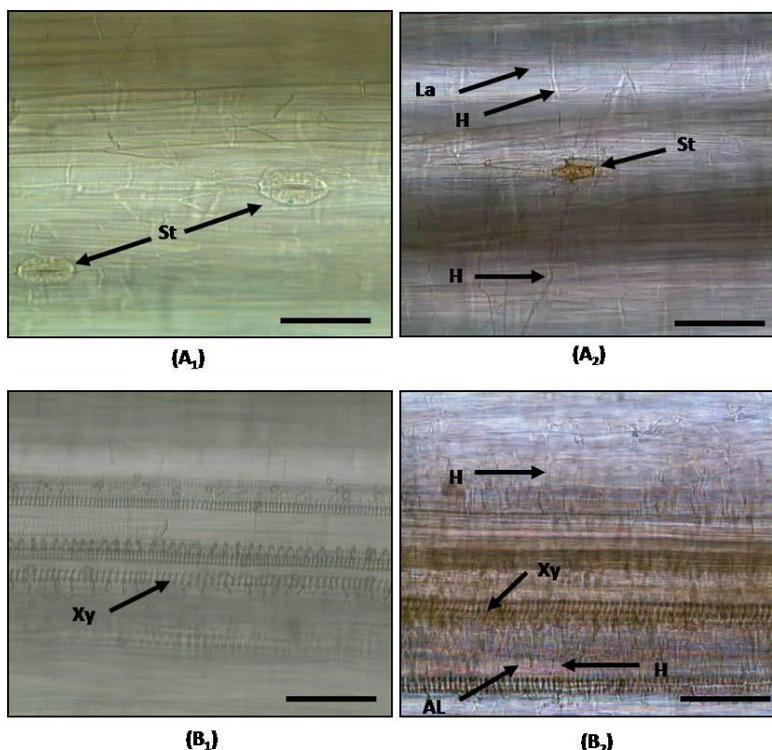


Figure 5. Lignin accumulation in *Acacia mangium* seedling due to *Fusarium oxysporum* infection. **(A₁)** Stomata of healthy *A. mangium*, **(A₂)** Stomata of *A. mangium* 4 days after inoculation, **(B₁)** Xylem of healthy *A. mangium*, **(B₂)** Xylem of *A. mangium* 6 days after infected, **(La)** Lignin accumulation, **(H)** Hypha *F. oxysporum*, **(St)** stomata, **(Xy)** Xylem, (Bar = 50 μ m).

Result of *A. mangium* seedling defense response with histological stain Aniline blue indicated that there was callose accumulation but only in infected and non infected xylem (Figure 6). Callose was expected to accumulate and increase in area around xylem vessel tissue of an infected *A. mangium*. Microscopic examination indicated that seedling at 4 and 6 days after inoculation had no callose accumulation at site around the infected xylem vessel tissue. Interaction occurring between *A. mangium* seedling as the host and *F. oxysporum* as pathogen was expected to form a compatible interaction. The interaction resulted in *A. mangium* seedling vulnerable to *F. oxysporum* infection. It was evidenced with wilted seedling at 4 and 6 days after inoculation so the seedling may be classified as vulnerable to pathogen (Figure 4).

Southerton and Deverall (1990) reported that great callose accumulation in cell of various wheat varieties in incompatible relation was observed and there was no callose accumulation in compatible relationship due to *Puccinia triticina* pathogen infection. It is also expected that undetected callose in infected site around xylem vessel tissue was due to *F. Oxysporum* which can produce enzyme β -1,3-glukanase that can degrade callose (β -1,3-glukan). Pathogen is known to produce β -1,3-glukanase that can degrade callose (β -1,3-glukan) (Vidyasekaran, 2008). Enzyme β -1,3-glukanase produced by pathogen in area between mycelium and tissue in plant will result in callose degradation (Brockmann *et al.*, 1992).

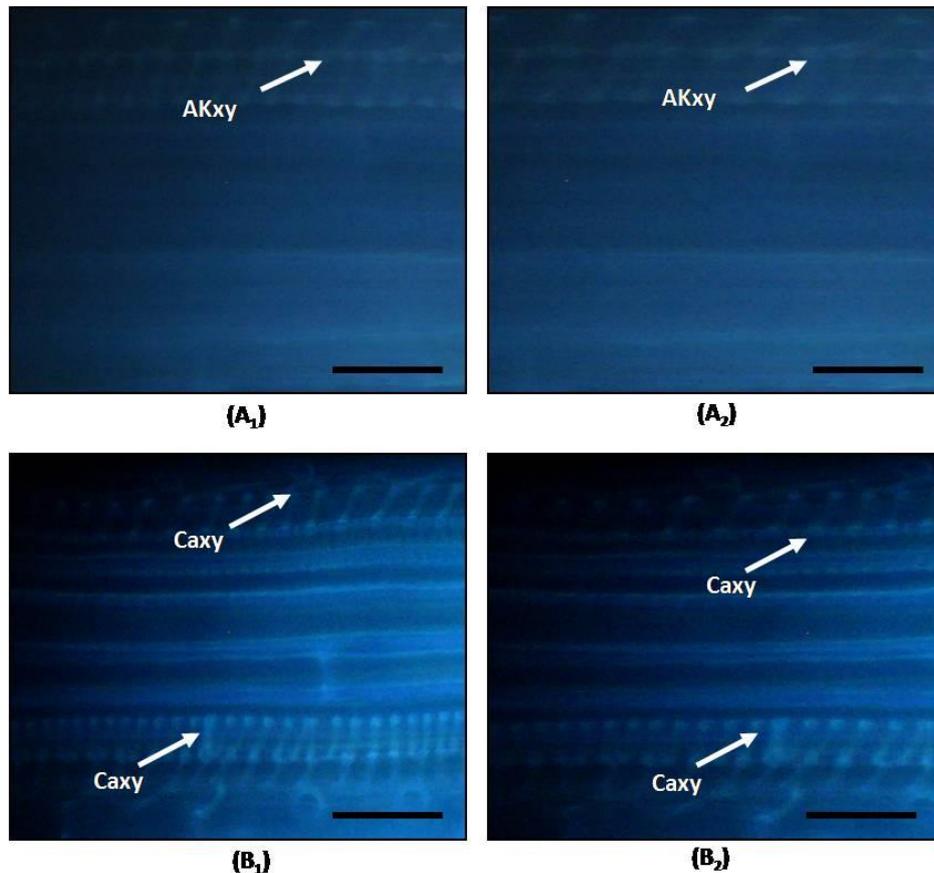


Figure 6. Callose accumulation in *A. mangium* seedling due to *Fusarium oxysporum* infection. (A₁) Xylem of healthy *A. mangium*, (A₂) Infected xylem of *A. mangium* 4 days after inoculation, (B₁) Xylem of healthy *A. mangium*, (B₂) Infected xylem of *A. mangium* 6 days after inoculation, (Caxy) Callose accumulation in xylem. (Bar = 50 μ m)

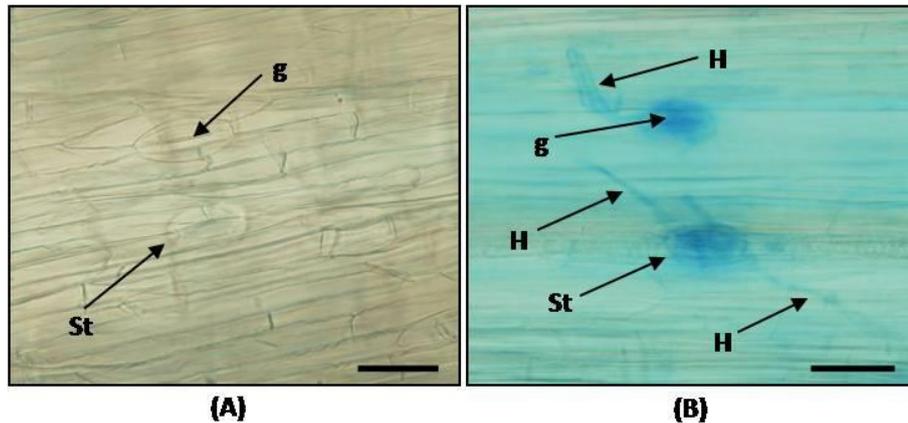
Hypersensitive reaction was found when there was *F. oxysporum* infection on seedling stem surface at 4 days after inoculation (Figure 7). Based on the figure, there appeared difference in stomata colour between healthy tissue and tissue infected with *F. oxysporum* at the same age. In infected tissue, stomata was darker blue than that around healthy tissue (Figure 7A), while stomata in healthy tissue indicated the same colour as other surrounding tissues (Figure 7B). Staining with lactophenol trypan blue can be used to detect the existence of fungi structure or dead cell in plant, marked blue which is darker than other tissue. Similar finding was also presented by Feys *et al.* (2001) that staining with lactophenol trypan blue on *Arabidopsis* leaf infected with *Pseudomonas parasitica* could detect mycelium and might also detect hypersensitive reaction. The detected hypersensitive reaction was marked with darker

colour in leaf tissue experiencing more hypersensitive reaction than other surrounding tissues.

Entirely, infection process and response of *A. mangium* seedling defense infected with *F. oxysporum* is presented in Fig. 8. The infection process occurred in 5 steps. The first step was the attachment of *F. oxysporum* spore at the 7-day *A. mangium* seedling stem or immediately after inoculated with *F. oxysporum* spore. The second step was marked with spore germination. In the first and second steps, *A. mangium* seedling tissue still appeared healthy. It was also proven with no symptom and signs appearing macroscopically. In the third step, *F. oxysporum* hypha had entered stem tissue of *A. mangium* seedling through stomata in stem surface. When *F. oxysporum* hypha entered the tissue through stomata, there was hypersensitive reaction in stomata. Then in the fourth step, *F. oxysporum* hypha entered the

phloem vessel tissue to xylem with intercellular way through intercellular space. In the fifth steps, *F. oxysporum* hypha developed in *A. mangium* seedling tissue and the seedling died. Lignin

accumulation occurred in the fourth and fifth steps. Lignin accumulation was not detected in the last fifth step because the seedling had died.



Remarks: (A) Healthy stomata, (B) Infected stomata, (St) stomata, (g) Guard cell, (H) *F. oxysporum* hypha (Bar = 50µm)

Figure 7. Hypersensitive reaction on *Acacia mangium* stomata due to *Fusarium oxysporum* infection 4 days after inoculation

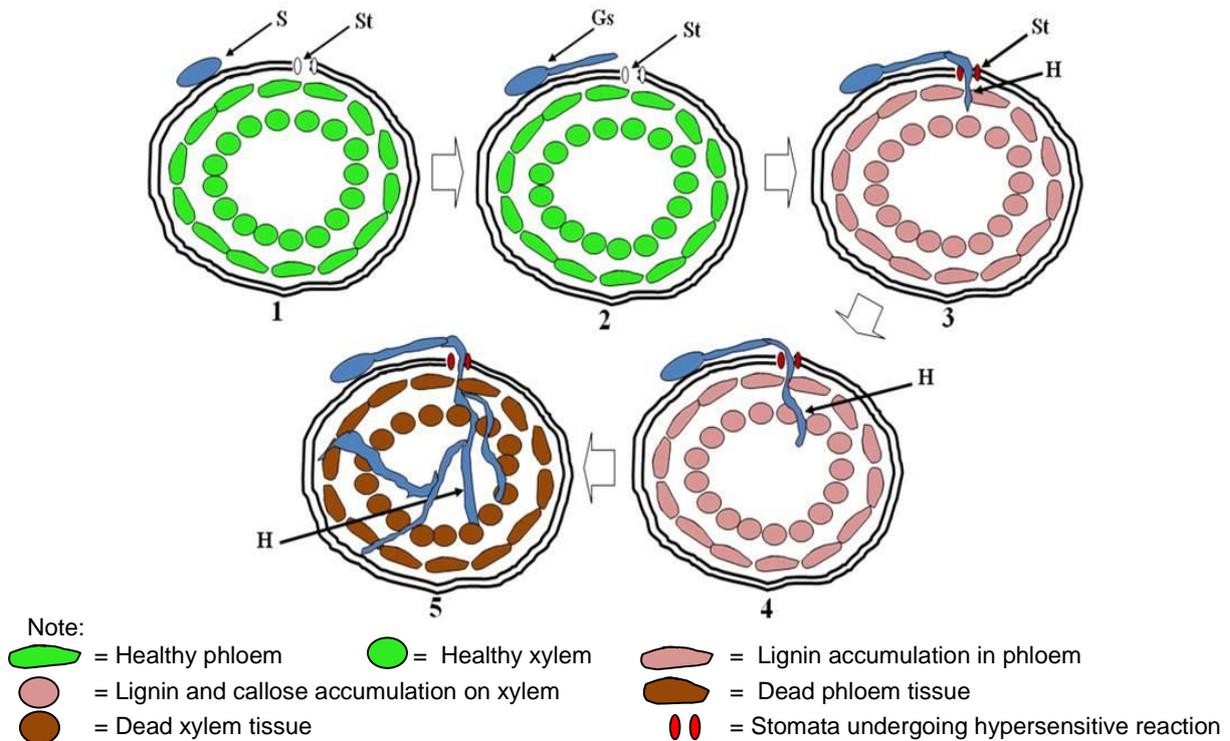


Figure 8. Infection process scheme and defense response on *Acacia mangium* seedling stem infected with *Fusarium oxysporum*. (Gs) Germinating spore, (St) stomata, (H) *F. oxysporum* hypha

CONCLUSIONS

In conclusion *F. oxysporum in vitro* penetrated through stomata into *A. mangium* seedling stem base until phloem vessel tissue and xylem intercellular through intercellular space. Hypersensitive reaction and lignin accumulation occurred due to *F. oxysporum* infection.

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