Increase of coffee tree susceptibility to stem canker disease mediated by glyphosate application

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Abstract. Stem canker is a major disease of coffee in Indonesia, caused by Fusarium solani. Previous studies showed a correlation between stem canker incidence and glyphosate application. The study aimed to determine the effect of glyphosate application on coffee stem canker and the underlying physiological mechanisms. The research used glyphosate treatments with the concentrations of 0, 4, 8, and 16 mL⁻¹. In vitro experiments were carried out by measuring the growth of F. solani colony after glyphosate treatment. In planta, tests were conducted by artificial inoculation on glyphosate-treated coffee seedlings. The severity of stem canker was assessed. In a separate test, coffee seedlings were treated with glyphosate without pathogen inoculation. In the second experiment, physiological change was determined by measuring photosynthetic pigments, gas-exchange properties, lipid peroxidation, and activity of ascorbate peroxidase (APX) and catalase (CAT) enzymes. In vitro tests showed that glyphosate had antifungal activity against F. solani. In planta, the test showed that glyphosate treatment caused higher susceptibility to stem canker in coffee seedlings, indicated by the increase of canker wound depth. Glyphosate treatment on coffee decreased the chlorophyll, carotene contents, and reduced photosynthesis rate, caused oxidative stress depicted by higher lipid peroxidation and increased enzyme APX and CAT activity.

Keywords: Fusarium solani, herbicides, oxidative stress, physiological changes

1. Introduction

Coffee tree (Coffea sp.) is one of the industrial commodities in Indonesia with high economic value. Indonesia is the third-largest coffee producer and exporter globally, with 6.8% of total world coffee production after Brazil and Vietnam [1]. Recent coffee plantations in Indonesia face coffee stem canker, a new and destructive disease caused by Fusarium solani. It was reported that severe disease infections could cause the death of coffee trees [2].

Previous exploratory field survey on stem canker showed that herbicide use is associated with the high severity of coffee stem canker. The majority of herbicides used by coffee farmers are glyphosate [3]. Several studies have shown increased disease severity after glyphosate application. For instance, the increase of sudden death syndrome soybean caused by Fusarium solani [4], Charcoal rot in soybean caused Macrophomina phaseolina [5], and wheat head scab caused Fusarium graminearum [6]. However, the effect of glyphosate on the coffee stem cancer disease system is not yet known. This research aims to determine the effect of the herbicide glyphosate on coffee stem cancer and the underlying physiological mechanisms.
2. Materials and methods

2.1 Experiment on the effect of glyphosate on Fusarium solani in vitro

Fusarium solani SYW-5 isolate from Lampung were obtained from Klinik Tanaman, Department of Plant Protection, IPB University. Isolates were seeded on Potato Dextrose Agar (PDA) medium and incubated for seven days at room temperature. The concentration of glyphosate treatment was studied following the methods described by [7] with slight modification. The glyphosate herbicide used was a commercial formulation (Roundup 486 SL) and divided into four concentrations (0, 4, 8, 16 mL⁻¹). The F. solani isolate was grown on PDA medium + glyphosate mixture media. Observations were made by measuring the diameter of F. solani colonies. Measurement was taken every day until colonies in control Petri dishes grew maximum. The experiment was done with a randomized complete design (RCD) with four herbicide treatments, with four replications. Data were tabulated then analyzed statistically using analysis of variance according to Statistical Analysis System 9.4 (SAS 9.4 M4). The differences of means were identified by SNK (Student Newman Keuls) Test at 0.05 level.

2.2 In planta test on the effect of glyphosate on stem canker disease

One-year-old robusta coffee seedlings were planted in a polybag 30 cm x 30 cm, polybag containing 2.5 kg each of sterilized soil and manure. Glyphosate was applied 30 days after transferring the coffee seeds to sterile soil in a greenhouse by spraying glyphosate on the soil surface at the rate of 17 ml/polybag. The pathogen was inoculated 14 days after glyphosate application. Pathogens inoculation began with surface sterilization of coffee stem, then seven-day-old F. solani cultures on PDA medium with a diameter of 60 mm were inoculated into the coffee stem. The inoculation area was about 10 cm from the base of the stem. Inoculation was performed by making wound the stem using a sterilized needle 1-3 mm deep, with 15 punctures. Seven-day-old F. solani cultures on PDA medium with a diameter of 60 mm were attached to the stem, then covered it with sterile moist cotton. Observation of coffee stem canker was done one month after inoculation. The measured disease variables were measured by length, width, and depth of stem canker wound. The experiment was done with RCD with four herbicide treatments (0, 4, 8, 16 mL⁻¹), repeated four times. Data obtained were analyzed statistically using analysis of variance using the Statistical Analysis System 9.4 (SAS 9.4 M4). The differences of means were identified by SNK (Student Newman Keuls) Test at 0.05 level.

2.3 In planta test of the effect of glyphosate on coffee tree seedlings physiology

The separate experiment was conducted without pathogen inoculation. The herbicide application was sprayed at a volume of 17 ml/polybag. Measurement of physiological characters was done at 2 and 12 days after treatment, and leaf samples were the second leaf from the shoots. Measurement of photosynthetic pigments in coffee leaves was done using the [8] method. Gas-exchange properties measurements were done using LI-COR 6400 (LI-COR Inc, USA). Observations were also made on Gas-exchange properties i.e., photosynthesis rate, CO₂ intracellular concentration, transpiration rate, and stomatal conductance. The experiment was set with RCD with four herbicide treatments (0, 4, 8, 16 mL⁻¹). Each treatment was repeated four times. Data were tabulated using the Microsoft Excel program 2010 then analyzed statistically using analysis of variance according to Statistical Analysis System 9.4 (SAS 9.4 M4). The differences of means were identified by SNK (Student Newman Keuls) Test at 0.05 level.

Lipid peroxidation was analyzed by the method described by [9]; 0.2 g leaves were extracted with 5 ml 0.1% TCA at 4°C, then centrifuged at 12000 rpm in 5 mins. 1 ml supernatant of the enzyme extract was mixed with 5 ml of a solution containing 0.5% TBA and 20% TCA and then incubated for 30 mins at 90°C (using a water bath) then cooled to ice bath. Absorbances were read at wavelengths of 532 and 600 nm using spectrophotometer Jenway 7315 (Bibby Scientific Ltd UK). The experiment was set with RCD with four herbicide treatments (0, 4, 8, 16 mL⁻¹). Each treatment was repeated three times.

Enzyme extraction for measured CAT (catalase) and APX (ascorbate peroxidase) was conducted by [10] method at two days after treatment. 0.2 g of leaf samples were crushed with 4 ml solution containing 50 mM phosphate buffer (PH 7.0), 1% polyvinyl polypyrrolidone and 0.2 mM ascorbic acid. The crushed results was centrifuged at 15,000 g for 30 mins to obtain the supernatant. CAT enzyme activity
was measured according to the [11] method with slight modifications. 20 µl of the supernatant was mixed with a 1.5 ml solution containing 100 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 20 mM H$_2$O$_2$. Measurement of H$_2$O$_2$ reduction was performed at a wavelength of 240 nm and calculated with a molar coefficient (36 mM$^{-1}$ cm$^{-1}$). APX enzyme activity was calculated based on the method used by [12]. A solution of 3 ml containing 50 mM potassium phosphate (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 2% H$_2$O$_2$, and supernatant 0.1 ml. The absorbance decline was measured at a wavelength of 290 nm for 1 minute, and the amount of oxidized ascorbate was calculated using a coefficient (3 =2.8 mM$^{-1}$ APX expressed in units of 1 mM per min at 25°C cm$^{-1}$). The experiment was set with RCD with four herbicide treatments (0, 4, 8, 16 ml.L$^{-1}$). Each treatment was repeated three times.

3. Result and discussion
Glyphosate application affected the depth of the canker wound but did not affect on length and width of the wound. The significant effect was indicated by the significantly higher wound size of tree seedlings treated by 16 ml.L$^{-1}$ glyphosate than untreated tree seedlings (Table 1). However, at glyphosate treatments of 8 ml L$^{-1}$, which is the recommended concentration, there was no significant effect. Stem canker symptoms started from a necrotic appearance inoculation, then the bark peeled off and later on developed into canker. Necrotic symptoms were seen when the point of inoculation is cut transversely (Fig. 1).

| Glyphosate treatment (ml.L$^{-1}$) | Length (cm)  | Width (cm)  | Depth (cm)  |
|-----------------------------------|--------------|-------------|-------------|
| 0                                 | 1.74 ± 0.42a | 1.37 ± 0.28a| 0.17 ± 0.04a|
| 4                                 | 1.84 ± 0.58a | 1.00 ± 0.10a| 0.18 ± 0.05a|
| 8                                 | 2.12 ± 0.79a | 1.13 ± 0.38a| 0.18 ± 0.04a|
| 16                                | 2.50 ± 0.75a | 1.36 ± 0.29a| 0.26 ± 0.02b|

Glyphosate is the most widely used herbicide in agricultural practices, including coffee tree cultivation. Weed control using herbicides is considered more effective, saves time, and more quickly suppresses weed growth. However, herbicides have undesirable effects on non-target plants, such as causing nutritional deficiencies and increasing disease severity. Glyphosate application can increase the severity of various plant diseases because it affects three components in the "disease triangle," consisting of plants, environment, and pathogens. Each of these components can have a significant influence on the severity of the disease, and when one component changes, it will affect the severity of plant diseases [13].

![Figure 1. Symptoms of a coffee stem canker with glyphosate treatment; a, 0 ml.L$^{-1}$; b, 16 ml.L$^{-1}$](image)

In vitro experiments were conducted to determine the glyphosate effect on the growth rate of
F. solani. Growth of fungal colony diameter was inhibited along with the increase in glyphosate concentration (Table 2). At the rate of 8 ml.L⁻¹, glyphosate inhibited the fungal growth completely.

Table 2. Effect of glyphosate on the growth of F. solani in vitro.

| Glyphosate treatment (ml.L⁻¹) | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 |
|------------------------------|----|----|----|----|----|----|----|----|----|----|
| 0                            | 0a | 1.6a | 2.48a | 3.4a | 4.35a | 5.20a | 6.13a | 7.28a | 7.78a | 8.5a |
| 4                            | 0a | 0b | 0b | 0.98b | 1.18b | 1.28b | 1.50b | 1.55b | 1.70b | 1.83b |
| 8                            | 0a | 0b | 0b | 0c | 0c | 0c | 0c | 0c | 0c | 0c |
| 16                           | 0a | 0b | 0b | 0c | 0c | 0c | 0c | 0c | 0c | 0c |

This study has proven that glyphosate directly inhibits pathogens, but glyphosate has also been shown to increase the severity of stem canker. Glyphosate plays a role in reducing plant resistance which results in increasing the severity of stem canker. Glyphosate affects the severity of plant disease due to interactions between its direct effects on pathogens and indirect effects through plant response [4].

Table 3. The value of photosynthesis rate, stomatal conductance, respiration, and transpiration in glyphosate treatment.

| Glyphosate treatment (ml.L⁻¹) | Photosynthesis (µmol CO₂ m⁻² s⁻¹) | Stomatal Conductance (mol m⁻² s⁻¹) | Intracellular CO₂ Concentration (µmol mol⁻¹) | Transpiration (mmol H₂O m⁻² s⁻¹) |
|------------------------------|-----------------------------------|----------------------------------|---------------------------------------------|---------------------------------|
|                              | 2 DAT | 12 DAT | 2 DAT | 12 DAT | 2 DAT | 12 DAT | 2 DAT | 12 DAT |
| 0                            |       |       |       |       |       |       |       |       |
| 4                            |       |       |       |       |       |       |       |       |
| 8                            |       |       |       |       |       |       |       |       |
| 16                           |       |       |       |       |       |       |       |       |

Glyphosate treatment effect of the coffee plant are shown in Table 3. Glyphosate treatment on coffee plants significantly affected photosynthesis rate at 12 days after treatment (DAT). This study also shows that glyphosate treatment significantly affected chlorophyll content, and carotene at 12 DAT (Table 4).

Table 4. The value of photosynthetic pigments in glyphosate treatment.

| Glyphosate treatment (ml.L⁻¹) | Chlorophyll a (mg.g⁻¹) | Chlorophyll b (mg.g⁻¹) | Carotene (mg.g⁻¹) | Total chlorophyll (mg.g⁻¹) |
|------------------------------|-----------------------|------------------------|------------------|---------------------------|
|                              | 2 DAT | 12 DAT | 2 DAT | 12 DAT | 2 DAT | 12 DAT | 2 DAT | 12 DAT |
| 0                            |       |       |       |       |       |       |       |       |
| 4                            |       |       |       |       |       |       |       |       |
| 8                            |       |       |       |       |       |       |       |       |
| 16                           |       |       |       |       |       |       |       |       |

The results of the physiological study show that the decrease of coffee tree resistance against stem canker pathogen is related to changes in physiological processes such as the decrease in the rate of photosynthesis which is in line with the decrease in chlorophyll content, carotene content, and the occurrence of oxidative stress (increased MDA, APX, and CAT enzyme activity). Similar research conducted by [14] showed a change in physiological processes in willow plants (Salix miyabeana) after glyphosate application.
Table 5. Lipid peroxidation in glyphosate treatment.

| Glyphosate treatment (ml.L⁻¹) | 2 DAT (MDA value (mM/ml)) | 12 DAT (MDA value (mM/ml)) |
|------------------------------|---------------------------|-----------------------------|
| 0                            | 0.40 ± 0.13               | 0.24 ± 0.03                 |
| 4                            | 0.56 ± 0.27               | 0.27 ± 0.10                 |
| 8                            | 0.56 ± 0.4                | 0.31 ± 0.01                 |
| 16                           | 0.59 ± 0.21               | 0.34 ± 0.13                 |

Measurements of lipid peroxidation were done at 2 and 12 days after glyphosate treatment. The results of this study show that the MDA content of coffee plants increased along with increasing glyphosate concentration (Table 5). The increase in the value of the MDA content indicates that the coffee plant suffers oxidative stress. The rescue mechanism is to increase the activity of antioxidant enzymes, including APX and CAT enzymes. Measurements of antioxidant enzymes were done two days after glyphosate treatment. This study showed that APX and CAT enzyme activity increased after glyphosate application (Table 6). This indicates that coffee tree seedlings treated with glyphosate suffers oxidative stress.

Table 6. APX enzyme and catalase in glyphosate treatment at 2 DAT.

| Glyphosate treatment (ml.L⁻¹) | APX (U.g⁻¹) | CAT (mU.g⁻¹) |
|------------------------------|-------------|--------------|
| 0 (Control)                  | 1.52 ± 1.66 | 61.47 ± 43.53 |
| 8                            | 5.94 ± 0.76 | 242.48 ± 30.04 |

Other research shows that abiotic stresses can cause physiological and metabolic changes that affect plant susceptibility to disease. Abiotic stresses such as high temperature, salinity, drought, and chemical toxicity can directly affect the interaction of pathogens with plants by changing plant physiological processes by reducing plant defense responses [15]. Like the previous research [16], drought stress in chickpeas results in a decrease in plant resistance due to increased transpiration, decreased water potential, and decreased stomatal conductance. The drought stress can make plants susceptible to *Macrophomina phaseolina* due to the weakening plant defenses or other metabolic changes. These events are called predisposition, where previous stresses (abiotic / non-genetic) can affect plants against further stress (biotic) by making the plant defenses weak and susceptible to pathogen infection [15, 17]. Thus it can be concluded that glyphosate treatment weakens coffee tree to stem canker infection.

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