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Effect of *Curvularia andropogonis* infection on secretory tissues and phenylalanine ammonia lyase enzyme of *Cymbopogon nardus*

ROFIATUN SOLEKHA[1,2,♥], NI N. T. PUSPANINGRUM[4], AISYAH H. RAMADANI[2,3], FEBIANTI D. HAPSARI[2], DINI ERMAVITALINI[1], HERY PURNOBASUKI[5,♥♥]

[1]Doctoral Program of Mathematics and Natural Science, Faculty of Science and Technology, Universitas Airlangga. Jl. Dr. Ir. H. Soekarno, Mulyorejo, Surabaya 60115, East Java, Indonesia. Tel.: +62-31-5936501, Fax.: +62-31-5936502, ♥email: rofiatunsolekha2@gmail.com

[2]Department of Biology, Faculty of Science, Technology and Education, Universitas Muhammadiyah Lamongan. Jl. Raya Plalangan KM 02, Lamongan 62218, East Java, Indonesia

[3]Doctoral of Philosophy, Faculty of Environment and Resources Studies, Mahidol University. Phutthamonthon Sai 4 Rd, Salaya, Phutthamonthon District, Nakhon Pathom 73170, Thailand

[4]Department of Chemistry, Faculty of Science and Technology, Universitas Airlangga. Jl. Dr. Ir. H. Soekarno, Mulyorejo, Surabaya 60115, East Java, Indonesia

[5]Department of Biology, Faculty of Science and Technology, Universitas Airlangga. Jl. Dr. Ir. H. Soekarno, Mulyorejo, Surabaya 60115, East Java, Indonesia. Tel.: +62-31-5936501, Fax.: +62-31-5936502, ♥♥email: hery-p@fst.unair.ac.id

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Abstract. *Solekha R, Puspaningrum NNT, Ramadani AH, Hapsari FD, Ermavitalini D, Purnobasuki H. 2024. Effect of* Curvularia andropogonis *infection on secretory tissues and phenylalanine ammonia lyase enzyme of* Cymbopogon nardus*. Biodiversitas 25: 598-604. Cymbopogon nardus* L. oil production decreased due to red spot disease caused by *Curvularia andropogonis* fungal infection. The objective of this study was to investigate the infection effect of *C. andropogonis* on secretory tissue formation in citronella leaf epidermis and the enzymatic defense expression response in *C. nardus*. Anatomical responses were observed from leaf secretory structure slide while PAL reactions were measured from enzyme activity in leaf samples of three treatment groups with healthy, wounded, and infected conditions. The morphological results of infected leaves changed from green to brownish triggered by pathogenic infection conditions that activated the PAL enzyme pathway and phenylpropanoid production as a defense. The results showed that the density of cells storing secondary metabolites flavonoids, tannins, and saponins in infected leaves increased compared to wounded and healthy ones. Secondary metabolites flavonoids, tannins, and saponins in wounded leaves increased compared to healthy leaves. It was observed that enzymatic response escalated when infection occurred. PAL enzyme activity test of *C. nardus* leaves showed that infected leaves had the highest value (0.0051 U/mL), followed by wounded leaves (0.0033 U/mL) and healthy leaves (0.0027 U/mL). Likewise, the specific activity of PAL enzyme in infected leaves was highest (0.121 U/mg), followed by injured leaves (0.040 U/mg) and healthy leaves (0.022 U/mg). The overall results exhibited that *C. andropogonis* infection affects the quality of secretory tissue and increases PAL enzyme activity as a form of defense. By detecting secretory tissue and defense enzymes after infection, further research can be carried out regarding controlling *Curvularia andropogonis* infections by engineering enzymes and secondary metabolites.

Keywords: *Curvularia andropogonis*, *Cymbopogon nardus*, phenylalanine ammonia lyase, secretory tissues

INTRODUCTION

Cymbopogon nardus L. oil also known as citronella oil, is one of the agro-industrial export commodities that is very prospective in producing high-value industrial products. This opportunity can be used to optimize the quality of essential oils by increasing the productivity of *Cymbopogon nardus* leaves as raw material for making essential oils for Indonesia. However, there are several obstacles due to the reduction in the quality of essential oils, one of which is the attack of pathogens (Braga et al. 2019). Pathogen infection causes a decrease in the quality and oil production of *Cymbopogon nardus*. The most common pathogens that infect *Cymbopogon nardus* are *Fusarium* sp., *Pestalotia* sp., and *Curvularia* sp. Of these three pathogens, *Curvularia* sp. is the most serious pathogen as it causes huge loss by affecting leaf growth (Pandey et al. 2019). The infection of this pathogen causes long blotchy symptoms along the tips and edges of the leaves resulting in drying of the entire leaf and a significant reduction in leaf numbers and oil production in plant (Mapuranga et al. 2022).

One of the responses of plants to pathogen infection is changes in leaf morphology. Morphological changes may indicate alterations in its anatomical structure and in the development of specific tissues/organs (Kong and Yang 2023). The anatomy of the leaf blade indicates that the plant is suffering from pathogen infection. Anatomy in leaf tissue includes three tissue systems, epidermis, mesophyll, and vascular tissue (Oguchi et al. 2018). Derivatives of the epidermis consist of cuticle, fan cells, stomata and trichomata (Albornoz and Cantwell 2016). This tissue is one the structural protection found in plants due to the process of fungi infection (Doehlemann et al. 2017). The derivates of trichome cell are divided into glandular trichomes and non-glandular trichomes. Glandular trichomes or secretory cells are glandular hairs whose cells have a secretory function and are able to secrete certain compounds (Uzelac et al. 2021). The results of secretion through secretory structures,

included essential oils, lipid, resins, latex, mineral salts, derivatives of hydroxybenzoquinone, and a wide variety of secondary metabolite compounds (Costa et al. 2020), such as flavonoids, tannins, alkaloids, terpenoids, and saponins. These secondary metabolites can inhibit the growth of pathogens (Solekha et al. 2022).

Biotic stress affects plant physiology and growth, causing increased production of secondary metabolites as an initial response to plant resistance affected by the enzyme Phenylalanine Ammonia Lyase (PAL) (Anzano et al. 2022). PAL is an enzyme involved in the formation of phenol compounds, such as lignin, salicylic acid, flavonoids, and their derivatives. This PAL enzyme reacts with its substrate (L-phenylalanine) in metabolism phenylpropanoid pathway, produces intermediate compounds in the form of trans acids cinnamate is converted into benzoic acid and coumarin. The enzyme reacts with phenylalanine, which was previously generated from the shikimic acid pathway (Rigsby and Parker 2016). The PAL enzymatic reaction then produces an intermediate compound in the form of trans cinnamic acid which is converted into phenol compounds and their derivatives as defense through the phenylpropanoid pathway metabolism. If more phenylalanine is absorbed, the PAL enzyme increases and affects the formation of higher phenol compounds to suppress pathogen development (Song et al. 2016). The objective of this study was to investigate the effect of *C. andropogonis* on secretory tissue formation in citronella leaf epidermis and the enzymatic defense expression response in *C. nardus*.

MATERIALS AND METHODS

Procedures

Wound treatment and Curvularia andropogonis *infection*

Cymbopogon nardus plants grown for 2 months were wounded and infected. Infected leaves were cut at the ends along 2 cm using sterile scissors by leaf cutting method (Solekha et al. 2019). Leaves were inoculated with fungal suspension of spore density of 105 CFU/mL. Inoculation was carried out for 8 hours by dipping the leaves into a prepared glass bottle (Saia et al. 2019).

Preparation of secretory structure slide of Cymbopogon nardus

Secretory observation slides were made using transverse slices of *Cymbopogon nardus* leaves by incubating the leaves using schiff reagent for 30 minutes, followed by washing the leaves for 10 minutes with a solution of 0.5% sodium metabisulfite in 0.1% HCL. Then, slices were placed on a glass slide and the amount of secretion was observed under a microscope (de Andrade Santiago et al. 2016).

Secretory structure slide of Cymbopogon nardus *leaf*

Microscopic slides of flavonoid, alkaloid and tannin observations were made by cross-sectioning leaf samples, with the leaf placed in the middle of a carrot and then thinly sliced with a sharp blade (Hunter et al. 2017). The slices were placed on a Petri dish containing distilled water, and then transferred to a glass object containing 10% NaOH reagent for flavonoids observation. Flavonoid content was indicated by a bright yellow color. Potassium dichromate dye reagent was used for the investigation of tannins. Tannins were marked with orange color and Wagner's reagent was used for alkaloid observation and was marked with yellowish brown color.

The slides were observed using a binocular microscope with a magnification of 400x with observation of three fields of view and documented using a digital camera. Observations were made by examining the changes occurring after dropping the reagent. The secretory cell density containing secondary metabolites (flavonoids, tannins, and alkaloids) was calculated by using the following equation (dos Santos Tozin et al. 2017):

$$\text{Secretory Density} = \frac{\text{Total of secretory structures}}{\text{FOV (mm}^2\text{)}}$$

Field of View (FOV) in 400x magnification = ¼ π d^2

Diameter of FOV (10x40) = 5 x 10^{-1} mm= 0.5 mm

Determination of PAL enzyme activity

Determination of PAL enzyme activity was analyzed by mixing 2 mL of 100 mM TrisHCl (pH 8.5), 1 mL of 10 mM L-phenylalanine, 0.8 mL of sterile distilled water, and 0.2 mL of protein crude extract. The reaction mixture was incubated at 30°C for 30 minutes using a waterbath, followed by stopping the reaction by adding 1 mL of 6 M HCl. The result was measured using UV-Vis spectrophotometry at a wavelength of 280 nm with cinnamic acid as a standard at concentrations of 2, 4, 6, 8, and 10 µg/mL, then as a blank, the same mixture of solutions was made but without phenylalanine (Medda et al. 2020). The PAL enzyme activity was calculated using the following formula:

$$\text{AE} = \left(\frac{\text{C}}{\text{BM} \times \text{t}}\right) \times \left(\frac{\text{H}}{\text{E}}\right)$$

Where:

AE : Activity of enzyme (U/mL)

C : Concentration of cinnamic acid (µg/mL)

BM : Cinnamic acid molecular weight (148 g/mol or 148 µg/µmol)

t : Incubation time (minute)

H : Total volume of enzyme-substrate (mL)

E : Volume of enzyme (mL)

PAL enzyme specific activity calculation formula is as follows:

$$\text{AS} = \frac{\text{AE}}{\text{Protein levels}}$$

Where:

AS : Enzyme specific activity (U/mg)

AE : Activity of enzyme (U/mL)

Protein levels : mg/mL protein

Data analysis

Data from the secretory structure were analyzed qualitatively and quantitatively. The qualitative analysis was in the form of visual images and the quantitative analysis was done using paired t-test. The calculation of PAL enzyme analysis used a Completely Randomized Design (CRD) with three treatments, i.e., healthy, wounded, and infected. The data were analyzed using SPSS with Analysis of Variance (ANOVA) test. If significant differences were found with a confidence level of 95% (alpha=5%), then the Least Significant Difference (LSD) test was conducted to determine which treatment was significantly different.

RESULTS AND DISCUSSION

Leaf morphology of *Cymbopogon nardus* L.

Based on morphological observations, healthy, wounded, and infected *Cymbopogon nardus* leaves had different leaf morphologies. Healthy leaves were evenly colored green without spots (Figure 1A), while wounded leaves were brown at the tips (Figure 1B), and leaves infected with *Curvularia andropogonis* had small red spots that later merged and extended from the ends of the leaf (Figure 1C). Leaves infected with *Curvularia* sp. showed yellow discoloration, this may be fungus producing toxins.

The macroscopic characteristics of *Curvularia andropogonis* were round-shaped colonies, greyish-brown to dark grey color with flat and thick growth, but with uneven edges and grayish white color (Figure 2A). Microscopic characteristics of *Curvularia andropogonis* a single or more brown conidia were present (Figure 2B). The conidia were 3-4 µm wide, variable in length, unbranched, curved, and the third cell of the conidia was larger than the other cells.

Secretory structure of infected *Cymbopogon nardus* leaves

Secretory structures have a function as site for the secretion of compounds from plants such as mucus, gum and oil. *Cymbopogon nardus* plants contained oil which in microscopic observations was marked with a purple color using Schiff reagent staining. The infection also affected the secretory tissue, anatomically. Pathogen infection also affects the secretory tissue, on the appearance of *Cymbopogon nardus* leaf preparations before infection, it can be seen that there is essential oil content in each gland, while after infection the oil glands are found empty (Figure 3). The empty glands indicate that no essential oil was formed. This fact could be an indicator of the disruption of essential oil biosynthesis mechanism due to tissue damage caused by fungal infection.

From these observations, the secretory cell density of leaves before infection had high-density value of 21.57/mm^2 than the infected leaves 10.42/mm^2 (Figure 4). The average secretory cell density in healthy leaves was 29.26/ mm2. The decrease in secretory cell density may be affected by pathogen stress which can reduce the secretion mechanism of the amount and quality of bioactive components secreted by *Cymbopogon nardus*.

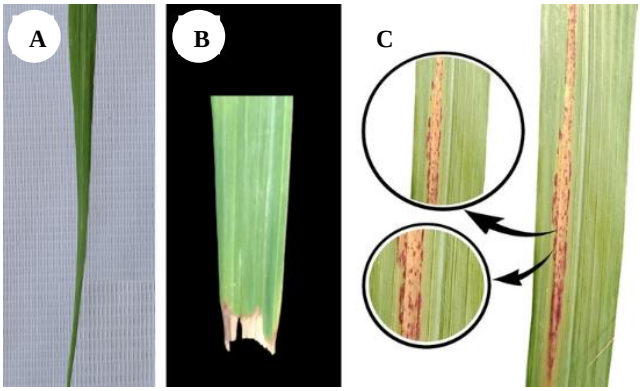


Figure 1. Morphology of *Cymbopogon nardus* leaves. A. Healthy, B. Wounded, C. Infected by *Curvularia andropogonis*

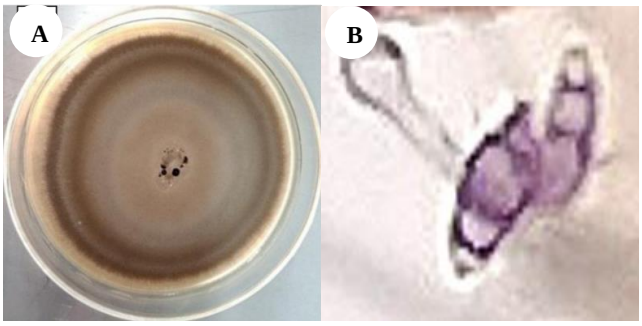


Figure 2. A. Culture of *Curvularia andropogonis*, B. Morphology of *C. andropogonis*

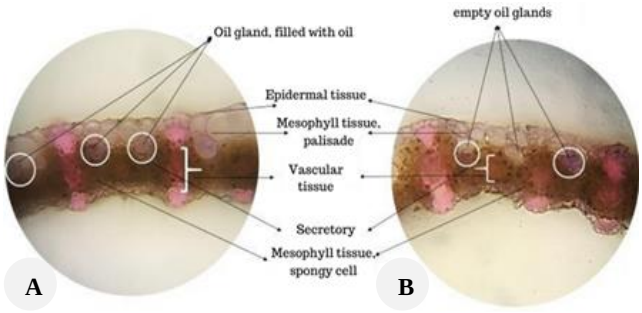


Figure 3. Slide view of secretory-indicated *Cymbopogon nardus*. A. Secretory tissue in healthy leaves accumulated oil in each oil gland, B. Infected leaf secretory contained empty oil glands

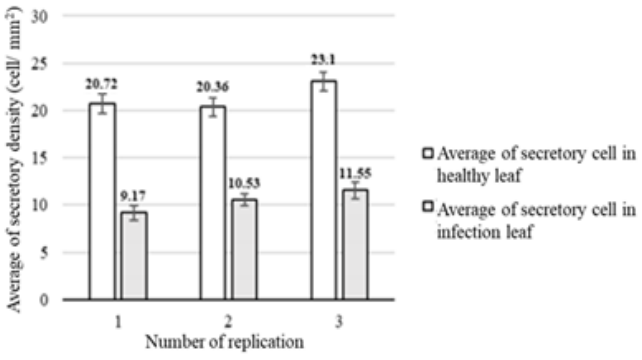


Figure 4. Average secretory density before and after infection

Anatomy and density of secondary metabolites (Flavonoids, alkaloids and tannins)

Secondary metabolite increase in plants can be triggered by pathogen stress experienced. The correlation between anatomical structure and the secondary metabolite content was observed because fungal infection damaged stomatal cells causing the rate of photosynthesis to decrease, thus affecting the biosynthesis of secondary metabolites from the primary metabolite pathway.

The presence of flavonoids in healthy and infected leaves is presented in Figure (5A-B) Flavonoids were characterized by a bright yellow color in the mesophyll tissue. A significant color difference was observed in the cross section. Figure 5A (healthy) showed the presence of flavonoids marked with yellow color, but the yellow color did not fill every cell.

In Figure 6B (*Curvularia andropogonis* infection) solid yellow color filled the cells. The significance of flavonoid content between the two can be observed in (Figure 6) regarding flavonoid density. Secondary metabolite compounds stored in vacuoles in secretory cells were secreted when the plant experienced stress, the secretion of secondary metabolites passes through the plasma membrane and tonoplasm. The number of infected flavonoid density was higher than the healthy flavonoid density value (Figure 6). This can also be inferred from the *p*-value <0.05 indicating a significant effect on the treatment before infection and after infection.

The observation of tannin in the leaves of *Cymbopogon nardus* is presented in Figure (7A-B). The detected tannins were characterized by orange color in the mesophyll tissue. Result revealed that both leaf sections showed a significant color difference. healthy leaf showed the tannins marked with orange color but the orange color did not fill every cell. In infected leaves (*Curvularia andropogonis* infection) orange appeared and filled in every cell.

The significance of tannin content between the two can be seen in (Figure 8) regarding tannin density. The amount of tannin density of infected leaves was higher than the tannin density of healthy/uninfected *Cymbopogon nardus* leaves, as seen from the *p*-value <0.05.

The observation of alkaloids in healthy and infected leaves is presented in Figure (9A-B). The detected alkaloids were characterized by the presence of brown color in the mesophyll tissue. Both figures A and B produced a significant color difference. Figure 9A (healthy) showed the alkaloid marked with a brown color but did not fill every cell. In Figure 9B (*Curvularia andropogonis* infection) brown appeared and filled in every cell.

The significance of alkaloid content between the two figures is presented in (Figure 10) regarding alkaloid density. The density of alkaloids in infected leaves was higher as compared to the healthy/uninfected *Cymbopogon nardus* leaves, as can be observed from the *p*-value <0.05.

Phenylalanine Ammonia Lyase (PAL) enzyme activity

The results of enzyme activity test and PAL enzyme specific activity of *Cymbopogon nardus* leaves indicated that leaves infected with *Curvularia andropogonis* had the highest value, followed by wounded leaves and healthy leaves (Figure 11). Healthy leaves had the lowest enzyme activity value with an average of 0.0027 U/mL, and then increased after being wounded to 0.0033 U/mL and continued to increase after being infected with *Curvularia andropogonis* to 0.0051 U/mL.

Enzyme activity and specific enzyme activity showed a significant and synchronous increase. In infected *C. nardus* leaves, the activity was higher (0.121 U/mg) compared to wound (0.40 U/mg) and normal (0.022 U/mg) treatments (Figure 12).

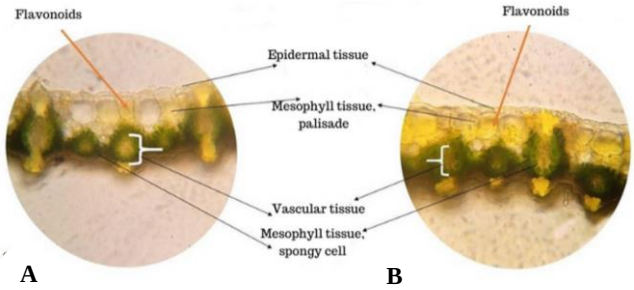


Figure 5. The yellow-colored mesophyll tissue indicated the presence of flavonoids. A. Uninfected leaf slide, B. Leaf slide after infection

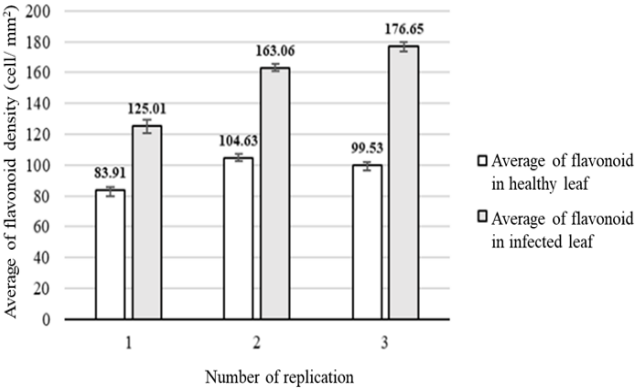


Figure 6. The average of leaf flavonoid density of *Cymbopogon nardus*

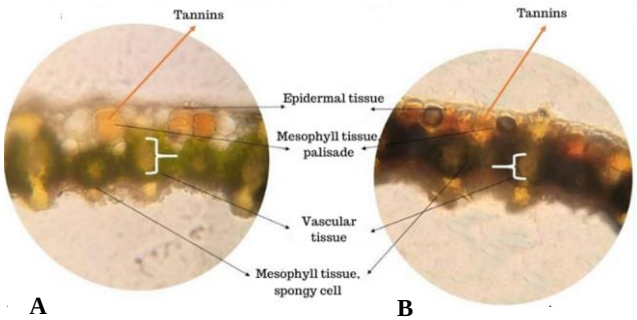


Figure 7. Mesophyll tissue producing orange color is indicated to contain tannin. A. Uninfected leaf slide, B. Leaf slide after infection

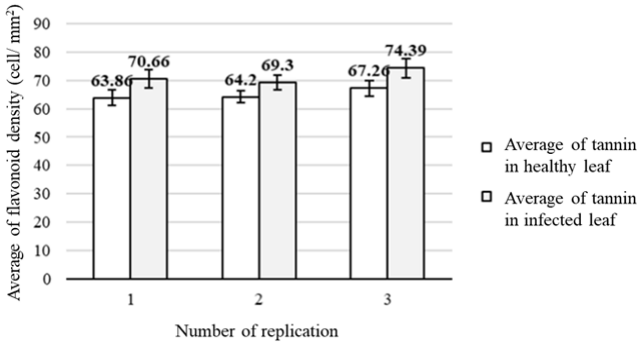


Figure 8. The average of tannin density of *Cymbopogon nardus* leaf

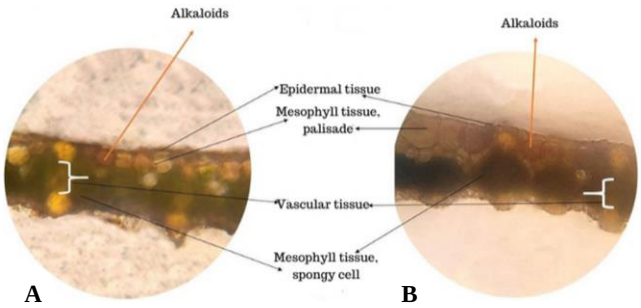


Figure 9. Mesophyll tissue producing brown color indicated to contain alkaloids. A. Uninfected leaf slide, B. Leaf slide after infection

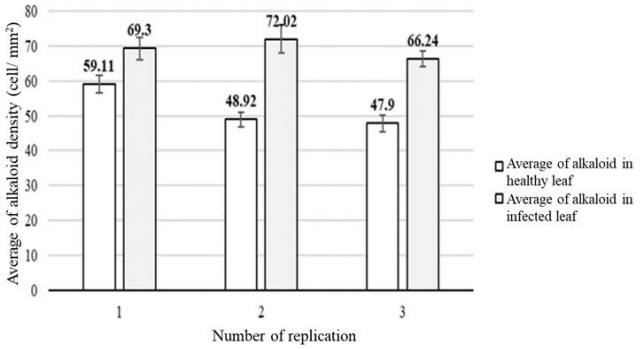
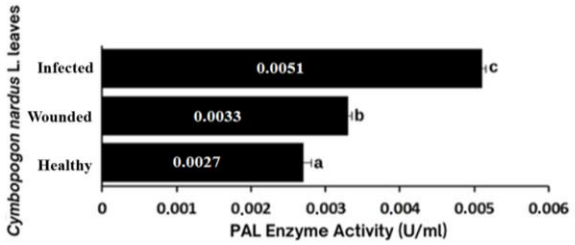
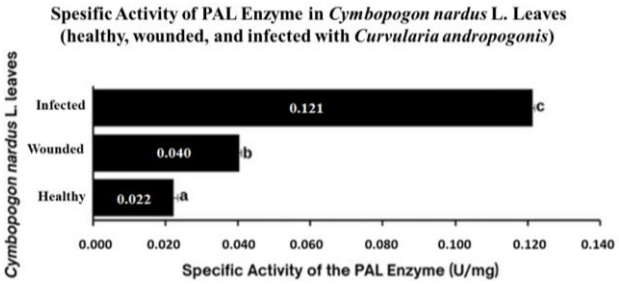


Figure 10. The average of alkaloid density of *Cymbopogon nardus* leaves



Note: Different letters indicate significantly different results based on the BNT test with a confidence level of 95%

Figure 11. The activity of PAL enzyme in healthy, wounded, and infected leaves of *Cymbopogon nardus*



Note: Different letters indicate significantly different results based on the BNT test with a confidence level of 95%

Figure 12. Specific activity of PAL enzyme in healthy, wounded, and infected leaves of *Cymbopogon nardus*

Discussion

Infection of *Curvularia andropogonis* causes changes in citronella plants both anatomically and physiologically, which is reflected in differences in defense enzymatic activity in the plant (Hu et al. 2022). Invasion of fungi on leaf tissue can be through direct penetration, but if there is a wound on the leaf plants the process gets easier (Zuo et al. 2019). The citronella leaves looked healthy, green and fresh when in normal condition. After infected by the fungi it can be seen that there are initial symptoms of red spots appeared on the leaves, later these spots merged and gradually formed long necrotic areas then covered the entire leaf area and eventually make the leaf dried up. This occurred because of the pathogenic toxin produced by fungus spread on the leaf cell. The toxin is assumed to have degrading activity leaf chlorophyll so it causes the leaves to turn yellow small black spots (Kaur et al. 2022; Wei et al. 2022). Color alteration in leaf is the manifestation of defense mechanisms in plants. Wounds in plants trigger stress and cause PAL enzyme activity and phenylpropanoid production increase which results in chlorophyll degradation resulting in brown discoloration or browning. The color change involves two main pathways, the phenylpropanoid and Polyphenol Oxidase (PPO) pathways (Hunter et al. 2017). The phenylpropanoid mechanism is significantly triggered by stress, injury and the presence of pathogens (Dixon et al. 2002). PPO responses associated with the activation of cell death and the lesion mimic phenotype as investigation in walnut leaf. Leaf color changes is resulted from polymerization of PPO which generate quinones and generate phytomelanins (Mesquita and Queiroz 2013).

Anatomical traits of citronella leaf before and after infection of *Curvularia* fungi show the distinction clearly under microscopic analysis. The secretory structure of healthy citronella leaves had higher density than infected leaves. The secretory structure functions as a place to release secretion compounds from plants such as mucus, sap and oil (Han et al. 2022). In healthy leaves, the oil was found in large quantities in the secretory tissues, but in lesser quantities in infected leaves. The observation results found a greater number of empty secretory cells that did not contain oil. The empty glands indicate that no essential oil was formed in infected leaves. This fact could be an indicator of the disruption of essential oil biosynthesis

mechanism due to tissue damage caused by fungal infection. Essential oil biosynthesis depends on the terpenoid pathway composed of sesquiterpenes and monoterpenoids. This pathway is originally derived from three compounds consisting of phenolic compounds, fatty acid derivatives, and isoprenoids (Rehman et al. 2016).

The production of secondary metabolites is regulated by nutrition, decreased growth rate, feedback control, enzyme inactivation, and induction of enzymes. Increasing secondary metabolite production has the relation to chemical defenses in plants such as the formation of enzymes involved in phytoalexin biosynthesis or as response to stress (Jeandet et al. 2017). The plant response against the pathogenic fungi infection is not only determined through plant morphology but also biochemical resistance characteristic. One of the biochemical resistances in plant can be identified from phenol compounds released by plants. High contains of phenol compounds in some parts of the plant are an indicator of the high stress inside the plant (Vandana and Lakpale 2020a). Abiotic and biotic stresses have significant effects on the production of secondary metabolites. These stresses can be used as a strategy to optimize the formation of secondary metabolites as a plant defense mechanism (Vagiri et al. 2017).

The detection of secondary metabolites in this research found that there are increasing number of the plant defense compound such as flavoniod, tannin, and alkaloid within the mesophyll tissue. Mentioned by Grote et al. (2013), the stress-induced secondary metabolite is mostly produced in mesophyll cells mainly for the volatile compound group. The non-volatile compound as flavonoid, tannin and alkaloid then be embedded in secretory gland such as idioblasts, secretory cavities, internal gland and ducts, however in some occasion typically also found in glandular trichomes (Marinho et al. 2011; Huchelmann et al. 2017; de Souza et al. 2018; Goodger et al. 2018). This study focused on screening the compound from the leaf mesophyll. The increased number found in secondary metabolite compounds of flavonoids and tannins began with the occurrence of stress from *Curvularia* infection which causes the activity of defense enzymes thereby producing cinnamic acid derivatives which are important for formation of defense compounds, such as flavonoids and tannins. Flavonoids which are one of the metabolites secondary produced by plants can be toxic to other organisms, the way flavonoids work disrupts the function of pathogenic cell proteins that infect plants (Katushova et al. 2021). Tannins function as anti-pests for plants so they prevent attacks from fungi (Al-Khayri et al. 2023). The alkaloids are an organic compound that functions as plant protector disease and pest attacks and as a regulator of development (Wang et al. 2018). Secondary metabolites alkaloids are responded to and are released from specific organelles where they are stored in the plant tissues that are attacked by pathogens (Dutton et al. 2019).

Wounds induced changes biochemistry in the plant *Cymbopogon nardus* related to signaling stress by activating the maintenance pathway involving the PAL enzyme in it stimulates browning. The average highest value of specific activity of PAL enzymes in infected leaves was 0.121 U/mg, followed by 0.040 U/mg in injured leaves; and healthy leaves was 0.022 U/mg. Infected leaves showed activity value. The specific PAL enzyme was highest because the protein content was small, while the PAL enzyme activity was high. Specific enzyme activity is influenced by two factors namely enzyme activity (U/mL) and protein content (U/mg) (Behera et al. 2017). PAL enzyme activity increased significantly in black rice resistance mechanism after infection with *Xanthomonas oryzae* pv. *oryzae* (Kaur et al. 2021). PAL enzyme activity in the leaves of resistant cultivars of barley genotypes increased against *Bipolaris sorokiniana* infection. The fungal infection *Fusarium oxysforum* f. sp *cube* (FOC) against male bananas causes increase in PAL enzyme activity (Kumar et al. 2022). Phenolic compounds associated with PAL enzymes increase as an initial response to plant resistance when plants are subjected to biotic and abiotic defense assays (Oliva et al. 2020).

In conclusion, the results revealed changes in secretory structure, density of flavonoids, alkaloids, tannins and PAL enzyme activity in the leaves of *Cymbopogon nardus* after infection with *Curvularia andropogonis* as a defense response.

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