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Pathogenesis-related proteins response in citronella grass (*Cymbopogon nardus*) infected by *Curvularia andropogonis*

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Abstract. *Solekha R, Mahbubillah MA, Tamam MB, Sofialana F, As-Syifa AH, Djazuli A, Purnobasuki H. 2025. Pathogenesis-related proteins response in citronella grass* (Cymbopogon nardus) *infected by* Curvularia andropogonis. *Biodiversitas 26: 5485-5494.* The development and growth of citronella grass (*Cymbopogon nardus*) are often hindered by *Curvularia andropogonis* fungal infection, which causes leaf disease. One key approach is to identify the expression of Pathogenesis-Related (PR)-17 and PKS type III defense proteins in citronella after *C. andropogonis* infection, serving as a model for counterattacking fungal infection. This study aimed to (i) determine chalcone synthase enzyme activity in *C. nardus* against *C. andropogonis* infection, (ii) identify the PR-17 response of C. *nardus* leaves, and (iii) identify the metabolic variations of flavonoid derivatives of *C. nardus* leaves. High-Performance Liquid Chromatography was carried out to analyze the enzyme activity. Protein identification was performed using sodium dodecyl sulfate. The variation of secondary metabolites was measured using Gas Chromatography-Mass Spectrometry. The results indicated that the enzyme CHS activity in infected *C. nardus* leaves had increased significantly compared to healthy leaves. PR-17, PR-2, and PR-3 were only expressed in *C. nardus* infected with *C. andropogonis*. Profile of flavonoid-derived metabolites in *C. nardus* infected with *C. andropogonis* changed significantly, involving seven flavonoid-derived metabolites. The activation of CHS, accompanied by elevated expression of defense-related proteins and increased levels of flavonoid derivatives, creates a synergistic defense strategy in infected *C. nardus*. These responses strengthen the plant immunity, restrict fungal colonization, and enhance overall resistance, making them important indicators of plant-pathogen interactions.

Keywords: Chalcone synthase, citronella, *Curvularia andropogonis*, flavonoid derivatives, PR-17

INTRODUCTION

Citronella (*Cymbopogon nardus* L.) is an aromatic plant that produces Java citronella oil, a significant export commodity (Bayala et al. 2020). Its essential oil contains citronellal, geraniol, and citronellol, which are extensively used in perfume, cosmetics, aromatherapy, and as natural antimicrobial and insecticidal agents (Handa et al. 2020). Despite its economic and pharmaceutical value, *C. nardus* is susceptible to pathogenic fungi, particularly *Curvularia* spp., which cause leaf spot disease, reducing photosynthesis and essential oil yield. Such infections can spread rapidly under humid conditions, leading to significant economic losses (Pang et al. 2017).

Research on the cellular responses of *C. nardus* to fungal infection remains limited, particularly regarding defense-related genes such as Pathogenesis-Related (PR) proteins and enzymes like Chalcone Synthase (CHS). Understanding these molecular responses is crucial for developing disease-tolerant varieties. Previous studies have highlighted the biochemical diversity of citronella, showing its adaptability under different environmental conditions. For instance, shade levels influence growth, physiology, and oil quality, with the Serai Wangi 1 variety demonstrating superior performance (Danata et al. 2023). Pathogen infection also alters metabolite profiles, reducing citronellol and geraniol but increasing carboxylic acids and other stress-related compounds (Solekha et al. 2024b).

Beyond its industrial applications, *C. nardus* has ecological and pharmaceutical significance. Its essential oil has demonstrated antimicrobial effects against oral pathogens and *Candida* species (Kamal et al. 2020). Conservation initiatives, including organogenesis, somatic embryogenesis, and in vitro preservation, have been successfully applied to *C. nardus* and related species, supporting biodiversity conservation and sustainable utilization (Gantait et al. 2020). These biotechnological approaches underscore the multifaceted importance of plants in both agricultural and ecological contexts.

The interaction between plants and pathogens represents a dynamic coevolutionary process that shapes biodiversity and ecosystem stability. Biochemical traits such as metabolite composition, enzyme activity, and signaling compounds differ among accessions of *C. nardus*, reflecting significant intraspecific variation. Some genotypes can sustain essential oil production under stress, while others show strong suppression, highlighting trade-offs between growth and defense (Rai et al. 2021). Plants must allocate resources strategically, and this balance influences ecological interactions and community structure (Hong 2023). In addition, interactions with herbivores and nematodes increase complexity, as both mobilize the same and unique biochemical defenses (Meresa 2024).

One major pathogen of *C. nardus* is *Curvularia andropogonis* (Zhang et al. 2020). This fungus, characterized by multicellular conidia, penetrates host tissues and forms haustoria that extract nutrients, thereby facilitating disease progression (Ullah et al. 2017; Ruimassa et al. 2023; Wang et al. 2023). Plants respond through immune signaling pathways that activate PR proteins and enzymes such as CHS, which restrict the spread of pathogens (Vandana et al. 2020). PR proteins, including PR-1, PR-2, and PR-17, have been associated with antifungal, antiviral, and glucanase activities, respectively (Yang et al. 2016; Jiang et al. 2020; Venkateswarlu et al. 2020). Likewise, CHS catalyzes the initial step of flavonoid biosynthesis, contributing to the production of antimicrobial and antioxidant compounds that are essential for stress tolerance (Yeshi et al. 2022).

Metabolomic studies using HPLC and GC-MS have shown that *C. andropogonis* infection suppresses the production of essential oil components while stimulating the production of phenolic and flavonoid derivatives (Solekha et al. 2024a). These shifts suggest a metabolic reprogramming strategy, in which the plant mobilizes secondary metabolite pathways to defend against pathogens. PR-17, in particular, has emerged as a defense-related protein with potential antifungal functions, previously linked to systemic acquired resistance and cell wall reinforcement in other species (Goyal et al. 2023; Jiang et al. 2023). Meanwhile, CHS upregulation under biotic stress enhances flavonoid and phytoalexin production, which is part of the salicylic acid- and jasmonate-mediated defense pathways (Mierziak et al. 2017; Panche et al. 2017; Ferreyra et al. 2021).

Therefore, investigating the expression of PR-17 and CHS in *C. nardus* under fungal stress is essential for understanding its defense strategies. Such insights can inform breeding and biotechnological approaches aimed at enhancing disease resistance. To date, no international peer-reviewed study has comprehensively examined the molecular defense mechanisms of *C. nardus* against *C. andropogonis*. While essential oil composition has been studied, gene-level defense responses remain poorly characterized. This study was designed to fill that gap by analyzing the expression of PR proteins, particularly PR-17, and the activity of CHS, alongside changes in flavonoid metabolites. By integrating proteomic, enzymatic, and metabolomic analyses, the research aims to uncover molecular markers of resistance and provide a foundation for developing *C. nardus* varieties with enhanced tolerance to fungal pathogens.

MATERIALS AND METHODS

Procedures

Sampling of Cymbopogon nardus

Inoculated leaf samples were collected 3 days after infection by collecting 10 cm cuttings from the infected plant, placing them in plastic bags, and storing them in a freezer at -20°C.

Enzyme extraction

One gram of *C. nardus* leaf (10 cm cuttings from the leaf tip of infected plants) was crushed in liquid nitrogen and homogenized with 1 mL of 0.2 M borate buffer, pH 8.8. The extract was centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was taken as the crude extract (Solekha et al. 2024).

Measurement of protein concentration

The total protein content was analyzed using the Bradford method. Bovine Serum Albumin (BSA) was used as the protein standard. Protein content was measured by absorbance using a spectrophotometer at a wavelength of 595 nm. Based on the absorbance value, the protein content of each sample was calculated using a linear equation ($Y = aX + b$) obtained from the BSA standard curve. The total protein content of the sample was expressed as g/ml protein.

Determination of CHS activity

CHS activity was determined by analyzing the naringenin chalcone content using High Performance Liquid Chromatography (HPLC). A total of 100 μL of extract was incubated in a water bath at 40°C for 5 minutes, after which 50 μL of 30 mM comaril CoA and 50 μL of 30 mM malonyl CoA were added as substrates (Tong et al. 2021) and incubated for 30 minutes at 30°C. And then, 5 μL of 5 N HCl was added. The extract was stored in the dark at 4°C until analysis by HPLC, under the following conditions: YMC-Triat C18 Reserved-Phase column (250 mm × 4.6 mm, 5 μm), with a mobile phase of 1% glacial acetic acid: methanol (70:30), and UV-Vis detector at 278 nm.

1 unit of CHS activity is the amount of enzyme that produces 1 μmol in a unit of time (minutes) and enzyme volume (mL). Under experimental conditions (Agrawal and Verma 2019), the formula is as follows:

$$AE = \frac{P \times fp}{t \times v}$$

Where:
AE : Specific enzyme activities CHS (U/mL)
P : (μmol/mL)
t : Incubation time (minutes)
v : Enzyme volume
fp : Dilution factor

SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis)

SDS is an anionic detergent that denatures proteins by disrupting non-covalent bonds (hydrogen, ionic, hydrophobic) and wraps them into linear chains with uniform negative charge—approximately one SDS molecule binds per two amino acids (~1.4 g SDS per 1 g protein). PAGE is a technique using a cross-linked polyacrylamide gel as a molecular sieve. The gel consists of two layers: a stacking gel (low % acrylamide, pH ~6.8) that concentrates proteins into tight bands, and a resolving (separating) gel (higher % acrylamide, pH ~8.8) that resolves proteins by size. A 12% gel was used in this analysis. Samples were analysed at constant current (100 V, 25 mA) for 100 minutes. The gel was stained with silver nitrate (Ag). The relative molecular mass of the proteins (kDa) was calculated using the GelAnalyzer 23.1 application for 1D electrophoresis (Solekha et al. 2019).

Analysis of flavonoid derivatives

Ten cm of three-day-old disease-infected leaves were cut and dried at 40°C. After drying, they were pulverized using a blender and macerated using 70% methanol in a ratio of 1:6 for 72 hours. Maceration was carried out three times to maximize the filtered compounds. After maceration, the extract was evaporated at 60°C until it thickened. The extract was analyzed using Gas Chromatography-Mass Spectroscopy (GC-MS), and the results are presented based on the molecular weight of the compound, which was identified using an available library (Bansal et al. 2022).

Experimental design

The experiment on the CHS enzyme activity was conducted using a completely randomized design with five replications and three treatments: healthy, wounded, and *C. andropogonis*-infected plants.

Data analysis

The data were analyzed qualitatively and quantitatively. The appearance of spots characterized protein profiles, while their relative molecular mass was calculated using the GelAnalyzer 23.1 application. The results of the metabolomic profile were presented in the form of molecular weight and library-based compound analysis. GC-MS analysis data were processed using Mass LynX v4.1 software. The CHS enzyme activity was analyzed using Analysis of Variance (ANOVA) to determine differences. Further testing was performed using Duncan's Multiple Range Test (DMRT) to determine the significant differences at 5% level between treatments. The analysis was performed using the Statistical Analysis System (SAS) 9.1.3.

RESULTS AND DISCUSSION

(PR-proteins) of *Cymbopogon nardus* infected with *Curvularia andropogonis*

PR proteins play a crucial role in the plant defense against both biotic and abiotic stressors. The comparative samples were healthy, wounded, and infected *Cymbopogon citratus*. Protein patterns were obtained by performing 12% SDS-PAGE (Figure 1), which revealed differences in the number of protein bands in each treatment and cultivar. Significant differences of PR-proteins were observed in sample no. 7, i.e., the infected citronella sample.

Based on the results of proteomic analysis (Table 1), healthy *C. nardus* samples had two protein bands with relative molecular masses of 63 kDa and 27 kDa, wounded *C. nardus* had two protein bands with relative molecular masses of 63 kDa and 27 kDa, and infected *C. nardus* had six protein bands with relative molecular masses of 110 kDa, 83 kDa, 63 kDa, 31 kDa, 27 kDa, and 25 kDa. These relative molecular masses indicated that PR-17 was expressed in all *C. nardus* samples. There is a lack of research comparing PR-17 expression in healthy plants and those exposed to biotic and abiotic stresses.

Four proteins were not present in either the healthy or wounded *C. nardus*, namely those with relative molecular masses of 110 kDa, 83 kDa, 31 kDa, and 25 kDa. An increase in protein expression was observed in the infected sample, as indicated by a more intense band at ~27 kDa, whereas corresponding bands in other samples were less intense. The infected sample exhibited a more intense protein band at approximately 27 kDa, indicating higher expression levels compared to other samples. An unidentified protein with a relative molecular mass of 110 kDa may be a novel protein; therefore, it needs further research.

Table 1. Protein characterization analysis of *Cymbopogon nardus* leaves

Sample name	Code	Band	Rf	Mr (kDa)	kDa GelAnalyzer
Healthy *Cymbopogon citratus*	2	1	0.227	39.0109	61
		2	0.759	13.819	26
Wounded *Cymbopogon citratus*	3	1	0.268	36.0123	63
		2	0.75	14.0637	26
Infected *Cymbopogon citratus*	4	1	0.179	42.8402	83
		2	0.268	36.0123	63
		3	0.714	15.0869	27
Healthy *Cymbopogon nardus*	5	1	0.268	36.0123	63
		2	0.705	15.3541	27
Wounded *Cymbopogon nardus*	6	1	0.268	36.0123	63
		2	0.705	15.3541	27
Infected *Cymbopogon nardus*	7	1	0.098	50.1735	110
		2	0.179	42.8402	83
		3	0.268	36.0123	63
		4	0.589	19.253	31
		5	0.732	14.5653	27
		6	0.821	12.2447	25

Note: Rf: Retention factor, Mr: Relative molecular mass

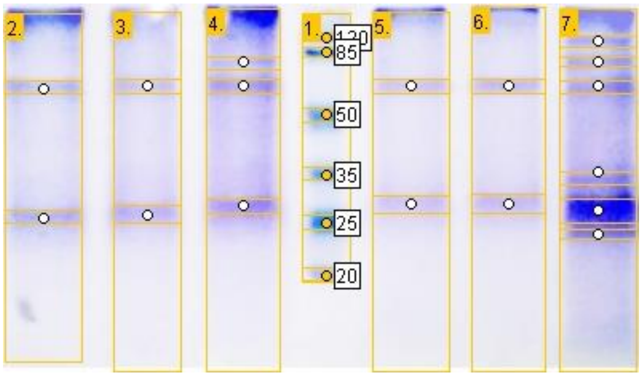

Figure 1. Protein expression using a 12% SDS gel, and were calculated using a gel analyzer application: 1. Marker, 2. *Cymbopogon citratus* healthy, 3. *Cymbopogon citratus* wound, 4. *Cymbopogon citratus* infection, 5. *Cymbopogon nardus* healthy, 6. *Cymbopogon nardus* wound, and 7. *Cymbopogon nardus* infection. The arrow points to the band with a molecular weight of 27 kDa corresponding to PR-17

CHS enzyme activity of *Cymbopogon nardus* leaves infected with *Curvularia andropogonis*

CHS enzyme activity was assessed using HPLC to measure naringenin chalcone as its product. The chromatogram revealed that the highest levels of naringenin chalcone were in infected *C. nardus* (Figure 2).

Naringenin chalcone levels were calculated to estimate CHS enzyme activity in the three *C. nardus* samples. Leaves infected with *C. andropogonis* had the highest value of naringenin chalcone, followed by wounded leaves and healthy leaves (Figure 3). The enzyme activity of infected *C. nardus* leaves differed significantly from that of healthy *C. nardus* leaves.

Leaves infected with *C. andropogonis* had the highest enzyme activity compared to wounded and healthy leaves (Figure 3). Healthy *C. nardus* leaves had the lowest enzyme activity value, averaging 0.134 U/mL, which then increased to 0.336 U/mL in wounded leaves and 0.436 U/mL in leaves infected with *C. andropogoni*. Presumably, wounded and infected leaves of *C. nardus* infected with *C. andropogonis* induced biochemical responses in plants via the activation of defense pathways involving CHS enzymes to produce flavonoid derivatives. It results in higher enzyme activity than in healthy plants.

Table 2 indicates a significant increase in CHS enzyme activity between the healthy, wounded, and infected leaves. It reflects an induced defense mechanism in citronella in response to *C. andropogonis* infection.

Flavonoid derivatives of *Cymbopogon nardus* leaves infected with *Curvularia andropogonis*

The chromatogram of flavonoid derivatives of *C. nardus* leaves by GC-MS is presented in Figure 3. Peak fluctuations at retention times indicate the presence of accumulated compounds.

Flavonoid derivatives in *C. nardus* differed among the three treatments. Healthy leaves had lower flavonoid derivatives, followed by wounded plants and leaves infected with *C. andropogonis*. It might be caused by stress conditions in wounded and infected *C. nardus*, resulting in the accumulation of flavonoid derivatives.

The healthy leaf of *C. nardus* only detected limonene as a flavonoid derivative. Four flavonoid derivative compounds were found in the wounded group, including 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl) prop-2-en-1-one, 3-deoxy flavonoid, flavan-4-ol, and 6'-deoxychalcone. Seven flavonoid-derivatives were detected in infected *C. nardus* leaves, namely 1,2-dirilpropan, flavan-4-ol, chalcone 3-benzopyranes, linalool, suphatelanool, 1,2-dirilpropan, and seven flavonoid-derived compounds, namely 5,7-ol, flavan-4-ol, chalcone 3-benzopyranes, linalool, suphatelanool, 1,2-dirilpropan. 5,7,4'-trihydroxyflavonoids, 3-benzopyranes, flavan-3-ol, flavan-3,4-diol, 3,7-dimethyl-1,6-octadien-3-ol, dihydroflavonols, and 4,2',4',6'-trihydroxychalcone. Flavonoid derivative compounds expressed in diseased leaves are more abundant than in injured and healthy leaves.

Flavan-3,4-diol is a direct precursor for anthocyanin synthesis and catechin production, and it is involved in plant resistance, making it a potential health-protective compound in foods (Tong et al. 2021). The 5,7,4'-trihydroxy flavonoid plays an important role in antioxidant activity (Kaur et al. 2021). Lin et al. (2020) noted that linalool or 3,7-dimethyl-1,6-octadien-3-ol is a common monoterpene alcohol with two enantiomers and related metabolites involved in the full spectrum of plant interactions with pollinators. This compound is widely found in essential oils. Linalool is a key defense metabolite present in many natural essential oils and is a volatile compound. Linalool repels certain insects and herbivores and protects against pathogens, including fungi (Wang et al. 2023). Katsumata et al. (2018) reported that linalool can inhibit the growth of *Penicillium digitatum* and *Penicillium italicum* spores on *Citrus* sp. Long et al. (2019) also noted that although linalool is a minor component (only 0.15%) in *C. citratus* essential oil, it has good antifungal activity against *Aspergillus flavus* and *Aspergillus parasiticus*.

Table 2. The CHS enzyme in healthy, wounded, and infected *Cymbopogon nardus* leaves

Sample	Enzyme activities (U/mg protein)
Healthy *Cymbopogon nardus*	0.134^{a}±0.001
Wounded *Cymbopogon nardus*	0.336^{b}±0.004
Infected *Cymbopogon nardus*	0.435^{c}±0.001

Note: Numbers followed by the different superscript letters (a-c) indicate significant differences based on the Tukey HSD test, α: 0.05

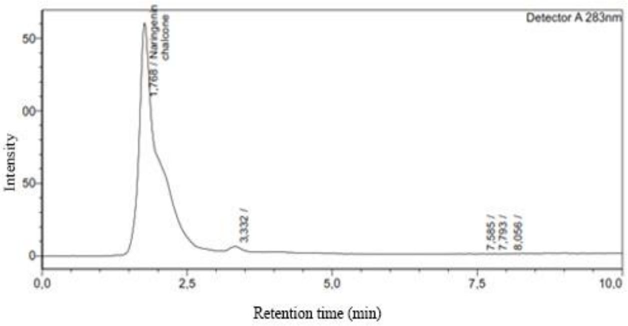

Figure 2. Chromatogram of naringenn chalcone resulting from CHS enzyme activity in infected *Cymbopogon nardus*

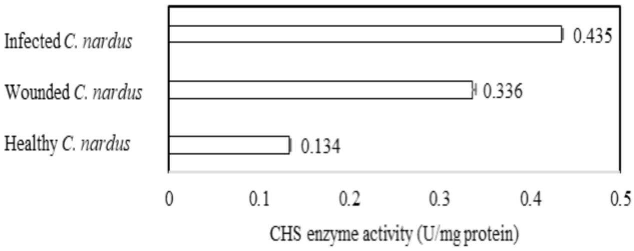

Figure 3. CHS enzyme activity in *Cymbopogon nardus*

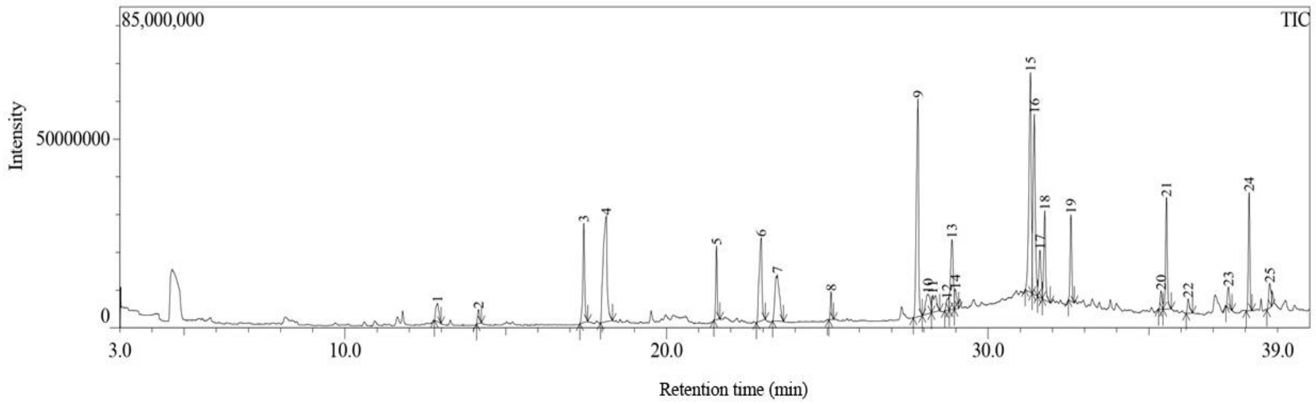

Figure 3. GC-MS chromatogram of infected *Cymbopogon nardus* leaf extracts

Table 3. Flavonoid-derivatives in *Cymbopogon nardus*

Species	Treatment	Flavonoid-derived metabolites	Contents (%)
Cymbopogon nardus	Healthy	Limonene	9.81
	Wounded	1-(2,4-Dihydroxyphenyl)-3-(4 hydroxyphenyl) prop-2-en-1-one)	3.45
		3-deoxy flavonoid	13.64
		Flavan-4-ol	0.62
		6'-deoxychalcone	1.04
	Infected	5,7,4'-Trihidroksi Flavonoid	13
		3-benzopyranes	2.25
		Flavan-3-ol	3.57
		Flavan 3,4-diol	3.27
		3,7-dimethyl-1,6-octadien-3-ol	1.92
		dihydroflavonols	12.37
		4,2',4',6'-Trihydroxy-chalcone	3.10

Discussion

PR-proteins of Cymbopogon nardus *infected with* Curvularia andropogonis

Biotic or abiotic stressors impact plant balance, resulting in a response involving the production of various defense compounds and proteins, such as PR proteins. PR proteins play a crucial role in increasing plant resistance to disease (Jiang et al. 2020). Ali et al. (2018) stated that the expression of PR proteins in plant defense against pathogens is an effective strategy producing broad-spectrum, disease-resistant, and long-lasting plant cultivars against fungal pathogens. Several in vitro studies have demonstrated that PR proteins target or hydrolyze fungal cell walls, leading to cell death. Proteomic analysis using a 12% SDS gel determines the complete profile of proteins regulated in the defense response to wounding and infection of *C. andropogonis* (Figure 1). SDS-PAGE profiling was used to assess the expression of PR-17 and type III polyketide synthase enzyme (PKS Type III). PR-17 expression illustrated the response to fungal attack. At the same time, PKS Type III is a protein indicating the presence of accumulated CHS enzymes, also known as a (PKS Type III), with a size of 40-45 kDa (Hu et al. 2017). A study by Venkateswarlu et al. (2020) demonstrated that the protein expression of PR-17 (27 kDa) functions as both an antiviral and an antifungal agent.

More protein was expressed in infected *C. nardus* compared to infected *C. citratus*. *Cymbopogon citratus* had three protein bands at 83 kDa, 63 kDa, and 27 kDa, which were also expressed in *C. nardus*. Protein expression indicates the interaction of proteins with pathogens in vitro, revealing the plant's response to pathogenic attack (Gupta et al. 2015). Proteins play a crucial role in various signaling pathways, including those that regulate metabolism, hormone signaling, cell division, and responses to biotic and abiotic stimuli. The more proteins that appear, the greater their role in regulating plant immunity to pathogens at different levels (Vo et al. 2021). The findings showed that *C. nardus* has stronger defenses than *C. citratus.* Plants exhibit an immune response when attacked by abiotic and biotic stressors. It includes the expression of PR-1, PR-17, and PR-5, which accumulate under stress conditions to enhance resistance against phytopathogens (An et al. 2019). PR proteins accumulate not only locally in infected and surrounding tissues, but also in uninfected tissues (Jain and Khurana 2018). The expression of PR-17 in *C. nardus* in all treatments requires further verification, particularly in uninfected leaves.

Four proteins that were absent in both healthy and wounded plants were present in infected *C. nardus* with relative molecular masses of 110 kDa, 83 kDa, 31 kDa, and 25 kDa. This study identified a novel protein with a relative molecular mass of 110 kDa. The protein with a relative molecular mass of 83 kDa was recognized as Heat Shock Protein 83 (HSP 83). HSP 83 is produced in response to various forms of stress to enhance tobacco chloroplast defense (Albarracín et al. 2015). It also serves as a carrier protein for expressing vaccine antigen in plants. HSP 83 is upregulated in response to aging, heat stress, hyperoxia, hydrogen peroxide, radiation, and stress from fruit flies, dust mites, and Leishmania parasites (Niu et al. 2020).

PR-2 (a 1,3-glucanase with a relative molecular mass of 31 kDa has been identified in *Polypodium polypodioides* under drought and fungal stress (Chakraborty 2018). The expression of a 31 kDa protein was also identified in maize under temperature and light stress (Covello et al. 1988a). The protein with a relative molecular mass of 25 kDa was identified as PR-3, also known as chitinase type V (Venkateswarlu et al. 2020). According to Dos Santos and Franco (2023), PR-3 hydrolyzes chitin, exhibits antifungal and antimycotic properties, and is salt-tolerant. A study by De Toledo et al. (2020). Studies state that proteins with a relative molecular mass of 15-30 kDa belong to the small HSP (sHSP) family, functioning as chaperone molecules that prevent the aggregation and misfolding of target proteins. Tong et al. (2021) noted that sHSP plays a role in resistance to pathogenic microorganisms. Wu et al. (2016) studied rice infected with *Magnaporthe oryzae* and identified a resistance response mediated by a protein with a relative molecular mass of 18-27 kDa. It is consistent with the hypothesis that PR-17 is expressed, but PKS Type III is not, in *C. nardus* attacked by *C. andropogonis*.

In *Hordeum vulgare* infected by *Blumeria graminis*, PR-17 genes are upregulated early in the apoplast and epidermal layers, key sites of pathogen entry (Zhou et al. 2010; Jiang et al. 2023). Although still undercharacterized, PR-17 proteins exhibit structural similarities to aminopeptidases and zinc metalloproteinases, and are predicted to function in proteolysis and antifungal defense (Goyal et al. 2023). A previous study by Jiang et al. (2023) suggests that PR-17 enhances both biotic and abiotic stress tolerance in cereal crops such as *Oryza sativa* and *Triticum aestivum*. Therefore, the expression of PR-17 and the observed flavonoid shift in *C. nardus* may represent synergistic defense layers, reflecting a similar resistance strategy to that of other members of the Poaceae under fungal stress.

CHS enzyme activity of Cymbopogon nardus *leaves infected with* Curvularia andropogonis

The CHS enzyme activity of infected *C. nardus* with *C. andropogonis* was the highest compared to wounded and healthy leaves (Table 2). Healthy *C. nardus* leaves had the lowest enzyme activity with an average of 0.134 U/mL, which increased to 0.336 U/mL in wounded leaves and to 0.436 U/mL in infected leaves. Presumably, *C. andropogonis* induced biochemical responses in plants by activating defense pathways involving CHS enzymes to produce flavonoid derivatives, so that enzyme activity was greater than in healthy plants. Plants experiencing stress can stimulate the metabolism of phenolic compounds. Phenolic compounds are formed in response to injury, as indicated by an increase in the synthesis of cinnamic acid derivatives (Parnidi et al. 2021). Wounded plants exhibited increased CHS enzyme activity, with the increase being higher in the infected plants. The increased stress triggers a higher production of flavonoid derivatives.

CHS regulation in plants under various stresses produces a range of secondary metabolites. Plants exposed to abiotic stresses such as UV light and drought exhibit increased CHS activity, producing metabolites like anthocyanins. Plants experiencing biotic stress due to pathogen infection resulted in the activation of CHS, leading to the formation of phenolic compounds, including 3-deoxyanthocyanidins, apigeninidin, luteolinidin, catesin, and isoflavonoids (Lin et al. 2020). The results of this study contradicted those of a previous study by Han et al. (2014), which stated that CHS enzyme activity did not occur under abiotic stress conditions but instead resulted in flavonoid accumulation. Furthermore, this study contradicts another study by Ashraf et al. (2018), which stated that CHS enzymes do not appear under healthy or non-stress conditions but do appear under stress conditions. The activity and accumulation of CHS enzymes involve PAL enzymes, the synthesis of benzoic acid derivatives, activation of isochorismate synthase, salicylic acid biosynthesis, and the accumulation of tannins and lignin (Chakraborty et al. 2020). CHS enzymes are essential in the phenylpropanoid pathway, which catalyzes the initial step of the flavonoid/isofavonoid pathway, namely the condensation of one molecule of a CoA ester of cinnamic acid or its derivatives, such as coumaric or ferulic acid, with three molecules of malonyl CoA to produce the main product naringenin chalcone, which then diverges into several branches producing various flavonoid-derived metabolites for defense (Akinrinlola and Hansen 2020).

Flavonoid derivatives of infected Cymbopogon nardus *leaves with* Curvularia andropogonis

Healthy plants expressed fewer flavonoid derivatives, followed by wounded and infected plants. It may be because wounded and infected plants experience stress, which leads to the accumulation of flavonoid derivatives. A study by Kaur et al. (2022) demonstrated that plants can grow and develop optimally under healthy conditions, whereas plants under stress trigger stress signaling and activate defense pathways. Solekha et al. (2024b) also stated that plants respond to abiotic stress by producing various secondary metabolites to avoid cell and tissue damage. In *C. nardus*, one flavonoid-derived metabolite, limonene, was identified. According to Bansal et al. (2022), limonene is one of the major bioactive compounds in citronella essential oil. Chen et al. (2018) mentioned that limonene is an abundant monoterpene hydrocarbon. The wounded leaves contained four compounds identified as 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one, 3-deoxy flavonoid, flavan-4-ol, and 6'-deoxychalcone. These metabolites, flavonoid derivatives associated with plant resistance, are synthesized through the phenylpropanoid pathway. The process begins with the condensation of one molecule of p-coumaryl-CoA and three molecules of malonyl-CoA to form chalcone (4',2',4',6'-tetrahydrochalcone), a reaction catalyzed by the CHS enzyme (Solekha et al. 2023). Chalcone is isomerized to flavanone by the Chalcone Isomerase (CHI) enzyme, as a branching point to produce various flavonoid classes, including aurones, dihydrochalcone, flavanonol (dihydroflavonol), isoflavones, flavones, flavonols, leucoanthocyanidin, anthocyanin, and proanthocyanidin (Yin et al. 2020).

Flavan-4-ol in wounded and infected leaves is a precursor of 3-deoxy-anthocyanins and phlobaphenes (Soesanto et al. 2022). The 6-deoxychalchone is a precursor of 5-deoxyflavanone, which is further used to produce flavonoid derivatives such as flavones, dihydroflavones, and isoflavones (Sohn et al. 2021; Zhang et al. 2022). Flavonoids play important roles in plant defense systems and serve as color pigments. Kaur et al. (2022) stated that flavonoids could suppress O2 levels, protect the outer layer of the chloroplast membrane, inhibit virulence factors, and reduce biofilm formation by pathogens. The results of the analysis showed that the infected leaves contained seven flavonoid-derived metabolites, including 5,7,4'-trihydroxyflavonoids; 3-benzopyranes, flavan-3-ol; flavan-3,4-diol, 3,7-dimethyl-1,6-octadien-3-ol; dihydroflavonols, and 4,2',4',6'-trihydroxychalcone). 1,2-diarilpropane is a flavonoid group with a molecular structure that resembles that of an isoflavone (Susanto et al. 2019).

The biochemical response of *C. nardus* to *C. andropogonis* infection involves the suppression of essential oil components and the upregulation of phenolic and flavonoid compounds, as a defense strategy observed in other Poaceae species under fungal attack. *Sorghum bicolor* infected with *Colletotrichum sublineolum* triggers the accumulation of flavonoid-derived phytoalexins such as apigeninidin and luteolinidin, which play a critical role in antifungal defense (Tugizimana et al. 2019). Wheat and barley challenged by *Fusarium graminearum* exhibit an increase in glycosylated flavonoids, including kaempferol, quercetin, and naringenin, which suppress fungal growth and reduce mycotoxin biosynthesis (Gunnaiah et al. 2017). The accumulation of glycosylated flavonoids, such as quercetin, kaempferol, and naringenin, correlates with resistance to fungal pathogens and oxidative stress in *Zea mays* (Balmer et al. 2018). These observations suggest that the flavonoid pathway is a crucial component of basal and induced defense in grasses. These findings indicate that the shift toward flavonoid biosynthesis in *C. nardus* is part of a broader, evolutionarily conserved defense mechanism across the Poaceae family.

Previous research on the variation of *C. nardus* flavonoid derivatives in infections with *C. andropogonis* or other fungi and pathogens is not available, so it cannot be compared with previous research regarding the number of flavonoid derivatives expressed. Flavonoids in plants are effective for antifungal activity (Al Aboody and Mickymaray 2020). The study's findings showed that flavonoid-derived compounds in infected plants are higher than in both wounded and healthy plants. Flavonoids possess antifungal properties that disrupt the integrity of the fungal cell membrane, altering the biosynthesis of ergosterol and modulating it through transduction signals, which occurs at the last stage of the growth to pathogenicity transition in fungi (Rodriguez et al. 2023). Maize produces diverse blend of O-methylflavonoids with antifungal activity upon attack by a broad range of fungi (Forster et al. 2022). The essential oil content in *C. nardus* provides antifungal activity against *C. albicans* (Mapuranga et al. 2020). This research indicates a correlation between the number of flavonoid derivatives and the resistance of *C. nardus*, which is more resistant than *C. citratus* against *C. andropogonis* due to its different compound profile. It is in accordance with the hypothesis that a higher number of flavonoid derivatives in infected *C. nardus* is a defense mechanism against *C. andropogonis*. Flavonoids have various functions based on their structure, one of which is defense against fungal attack (Mathesius 2018). Under abiotic stress, flavonoids play a role in reducing ROS production by inhibiting enzymes that produce Reactive Oxygen Species (ROS) like cyclooxygenase, lipoxygenase, monooxygenase, and xanthine oxidase (Okazaki and Saito 2016).

The disease severity, expression of defense proteins, content of flavonoid derivatives, and CHS enzyme activity have not been previously studied in *C. nardus* infected with *C. andropogonis*, thus making it a novel study. Moreover, a new 110 kDa protein with an unknown function was noted in infected *C. nardus* that has not been specifically identified. Seedlings of *T. aestivum*, *S. bicolor*, *Z. mays* *Pisum sativum*, and *Brassica juncea*, and the mycelium of *Neurospora crassa,* accumulated an immunologically homologous polypeptide of 104 kDa under drought stress. However, analysis using anti-rice HSP 104 and anti-yeast HSP 104 antibodies showed that rice HSP 104 differs from rice HSP 110, indicating that the 110 kDa protein was not immunologically detected (Okazaki and Saito 2016). In another study, western blot analysis of root tissues from *Z. mays*, *H. vulgare*, and *Allium cepa* identified two protein bands (180 and 110 kDa), one of which was suggested to be a myosin-like protein (Radford and White 1998). The precise identity and function of the 110 kDa protein remain uncertain. However, when anti-rice HSP 104 and anti-yeast HSP 104 antibodies were examined, rice HSP 104 was found to be different from rice HSP 110, so that 110 kDa was not immunologically included (Okazaki and Saito 2016). A study of myosin-like protein in plasmodesmata in root tissue of *A. cepa, Z. mays*, and *H. vulgare* revealed the presence of two proteins via western blotting, (180 and 110 kDa bands), of which one may be myosin-like protein (Radford and White 1998), thus, the function and name of the 110 kDa is still unclear.

Detection of glutathione reductase in *Arabidopsis thaliana* revealed two protein bands at 53 kDa and 110 kDa. The 110 kDa band appeared weaker than the 53 kDa band, suggesting that glutathione reductase corresponds to the 53 kDa protein. Therefore, further investigation is needed to confirm the identity of the 110 kDa, as the results were not statistically significant (Marty et al. 2019). Based on previous research, the protein band with a relative molecular mass of 110 kDa is a novelty that warrants further investigation. These findings indicate that functional proteins are involved in regulating the response mechanism of *C. nardus* to *C. andropogonis* infection. *C. andropogonis* secretes cell wall-degrading enzymes such as endo-β-(1→4)-glucanase to break down glycosidic linkages in the plant cell wall. *C. nardus* responds by secreting PAMPs to the cell surface to protect itself from the fungus. The PAMP signal activates the gene expression of plant defense-related proteins such as PR-17, a globulin-like protein; PR-3; 1,3-glucanase and chitinase, along with a 110 kDa PR protein. However, the function of this PR protein remains unidentified. Regulation of gene expression also produces several flavonoid derivatives such as 5,7,4'-trihydroxy flavonoids; 3-benzopyranes; flavan-3-ol; flavan-3,4-diol; 3,7-dimethyl-1,6-octadien-3-o; dihydroflavonols; and 4,2',4',6'-trihydroxy-chalcone. Plants have evolved both passive and active defense mechanisms, with Systemic Acquired Resistance (SAR) being a key component of active defense (Klessig et al. 2018). The activity of CHS mRNA increases early during incompatible plant-pathogen interactions, preceding the accumulation of phytoalexins and the hypersensitive response. Different pathogens elicit distinct defense responses; virulent and avirulent strains can induce varied patterns of defense gene expression, including CHS (Kaur et al. 2022). Chalcone Synthase (CHS) plays a crucial role in plant defense by catalyzing the biosynthesis of flavonoid and isoflavonoid phytoalexins (Zhang et al. 2017). Its expression is induced under various stress conditions, including pathogen infection (Ahmad et al. 2023).

Salicylic Acid (SA) is a critical signaling molecule in SAR, promoting the expression of Pathogenesis-Related (PR) genes and enhancing the accumulation of reactive oxygen species (Saleem et al. 2021). Methyl Salicylate (MeSA), a volatile derivative of SA, has been identified as a mobile signal for SAR. The esterase activity of Salicylic Acid-Binding Protein 2 (SABP2), which converts MeSA back into SA, is essential for the perception of SAR signals in systemic tissues (Singewar et al. 2021). The SAR signaling pathway involves complex interactions between SA, jasmonic acid, auxins, and ethylene, with NPR1 and PR1 proteins playing key regulatory roles (Roychowdhury et al. 2024). These findings underscore the complexity of molecular interactions that underlie plant defense responses.

Recent studies have highlighted the potential of Essential Oils (EOs) in enhancing plant defense and disease resistance. Rose essential oil, rich in β-citronellol, has been shown to activate defense-related genes in tomato plants, thereby reducing herbivore damage while attracting natural predators (Kaneko et al. 2024). Essential oils derived from *Origanum compactum* and hydrolates of *Citrus × aurantium* have demonstrated effectiveness against bacterial pathogens and induced resistance in tomatoes against *Xanthomonas vesicatoria* (Proto et al. 2022). Linalool, a major component of *Cinnamomum camphora* oil, has been reported to induce resistance against tobacco mosaic virus and other plant pathogens by triggering salicylic acid-mediated immune responses (Jiang et al. 2023).

These findings suggest that Essential Oils (EOs) have potential as alternatives to chemical pesticides, with potential applications as bioinsecticides, biofungicides, and bioherbicides (Bolouri et al. 2022). Essential oils from citronella and other aromatic plants have demonstrated eco-friendly pesticide properties with lower environmental impact (Raveau et al. 2020). Recent advances in transcriptome sequencing have enabled the identification of genes involved in terpene biosynthesis and other secondary metabolite pathways in citronella, providing valuable genomic resources for crop improvement and biotechnological applications (Devi et al. 2016). However, further research is necessary to optimize the application methods, efficacy, and stability of EOs for widespread agricultural use.

Breeding plants with genetic resistance remains crucial for developing crop systems that can withstand both biotic and abiotic stresses (Paul et al. 2025). Plants rely on Pattern Recognition Receptors (PRRs) to detect external threats and initiate immune responses (Boutrot and Zipfel 2017). In grassland ecosystems, the ecological benefits of plant-soil interactions, especially those influenced by root traits, are increasingly recognized. Breeding programs are now focusing on enhancing these beneficial traits while maintaining forage productivity for livestock (Marshall et al. 2016). The integration of genetic tools, ecological principles, and collaborative efforts offers a promising pathway toward more sustainable and resilient agricultural systems (Paul et al. 2025).

In conclusion, the activity of Chalcone Synthase (CHS) in *C. nardus* increased significantly following infection by *C. andropogonis* (0.435 U/mL), accompanied by the expression of PR-17, while PKS Type III remained unexpressed. This infection also triggered metabolic changes, notably the rise in flavonoid derivatives to seven compounds, reflecting an active defense response. Additionally, leaf extracts from healthy *C. nardus* showed the highest antifungal activity against *C. andropogonis*, with an inhibition rate of 37.06%, supporting its potential as a natural antifungal agent.

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