Cell-free Supernatant of *Pseudomonas aeruginosa* SWUC02 Suppresses *Pythium deliense* Infection in Durian via ROS and Defense Gene Modulation

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***Abstract*—*Durio zibethinus* L. (Durian) production in Thailand is significantly threatened by root and stem rot diseases caused by oomycete pathogens, such as *Phytophthora* spp. and *Pythium* spp. This study evaluated the biocontrol potential of cell-free supernatants derived from *Pseudomonas aeruginosa* SWUC02 (CF-SWUC02) against *Pythium deliense*, a necrotrophic pathogen associated with these diseases. Pathogenicity assays on detached 'Monthong' durian leaves demonstrated that CF-SWUC02 significantly reduced lesion sizes compared to pathogen-only treatments. Histochemical detection using 3,3′-diaminobenzidine (DAB) staining revealed that CF-SWUC02 effectively suppressed pathogen-induced hydrogen peroxide (H_2O_2) accumulation. Furthermore, quantitative real-time Polymerase Chain Reaction (qPCR) analysis indicated that CF-SWUC02 modulated host defense responses by attenuating the overexpression of defense-related genes (*RPP13* and *ZiChi7*) during infection. These combined results suggest that CF-SWUC02 mitigates disease severity by regulating Reactive Oxygen Species (ROS) accumulation and balancing defense gene expression, thereby preventing extensive host tissue necrosis. Ultimately, this study highlights the promising potential of CF-SWUC02 as an environmentally sustainable biocontrol strategy for managing durian root and stem rot disease.**

***Keywords*—biocontrol, Reactive Oxygen Species (ROS), real-time Polymerase Chain Reaction (qPCR), 3,3′-diaminobenzidine (DAB) assay, cell free culture**

I. Introduction

Durio zibethinus L. (Durian) is one of the most economically important fruit crops in Thailand. Among the commercial cultivars, Monthong is the most widely cultivated and exported due to its favorable fruit quality and high market demand [1]. However, durian cultivation is severely constrained by yield losses and tree mortality caused by root and stem rot diseases.

Root and stem rot diseases of durian are primarily caused by oomycete pathogens, including *Phytophthora palmivora* and *Pythium* spp. Infection by these pathogens typically results in dark brown to black necrosis at the root collar and basal stem, eventually leading to plant decline and death [2]. Surveys of diseased durian orchards in Thailand have further shown that *Pythium deliense* is among the *Pythium* species isolated from soils associated with tree decline, indicating that this species forms part of the root and stem rot pathogen complex in durian cultivation [3]. Currently, Chemical fungicides are commonly applied for disease management; however, excessive and prolonged use poses significant risks to environmental sustainability, human health, and long-term agricultural productivity. Consequently, alternative disease management strategies, particularly biological control approaches, have attracted increasing attention.

Antagonistic bacteria represent a promising biocontrol strategy against plant pathogens. These microorganisms suppress disease through multiple mechanisms, including the production of bioactive metabolites, competition for nutrients and ecological niches, and induction of plant defense responses [4]. For example, *Pseudomonas aeruginosa* strain FG106, isolated from the tomato rhizosphere, shows strong *in vitro* and *in vivo* suppression of several fungal and bacterial pathogens of tomato, potato, and taro, and also promotes plant growth [5]. Similarly, the endophytic strain *P. aeruginosa* Ld-08 from *Lilium davidii* exhibits potent antifungal activity against multiple phytopathogenic fungi and enhances plant growth parameters [6]. In addition, Cell-Free supernatant (CF) derived from *P. aeruginosa* strain F2 culture has been

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shown to inhibit the growth of fungal pathogens such as *Rhizoctonia solani* in wheat, and strain RKA5 inhibits the growth of *Fusarium oxysporum* f. sp. *cucumerinum* in cucumber [7, 8].

In the present study, we utilized cell-free supernatant from *P. aeruginosa* SWUC02 (CF-SWUC02), a strain previously isolated and characterized [9]. This strain exhibits strong antagonistic activity against *Xanthomonas citri* pv. *citri*, the causal agent of citrus canker, and several fungal phytopathogens, particularly *Colletotrichum truncatum* CFPL01 [9, 10]. Its biocontrol efficacy has been demonstrated through the suppression of disease development in detached-leaf assays and in pot experiments, indicating a strong capacity to inhibit pathogen progression and modulate host defense responses [9], as well as the suppression of anthracnose and induction of defense-related genes in chili seedlings [10]. To evaluate durian responses to root and stem rot pathogens, detached-leaf assays provide a rapid and practical proxy. Lesion development on leaves inoculated with *Phytophthora* or *Pythium* isolates correlates well with cultivar performance and disease severity in the field, making leaf lesion size a reliable indicator of overall susceptibility [11, 12]. Furthermore, real-time Polymerase Chain Reaction (qPCR) with Reactive Oxygen Species (ROS) assays provides a powerful framework to dissect induced resistance [13]. Recent work has shown that CF-SWUC02 suppresses anthracnose and induces defense-related gene expression in chili seedlings [10]. Because early defense against plant pathogens involves rapid modulation of ROS and activation of defense signaling pathways, integrating ROS detection with gene expression analysis provides a robust framework to elucidate induced resistance mechanisms. Therefore, this study aimed to evaluate whether CF-SWUC02 suppresses *P. deliense* SR-S1 infection in durian leaves through modulation of Hydrogen Peroxide (H_2O_2) accumulation and regulation of defense-related gene expression. Histochemical 3,3′-diaminobenzidine (DAB) staining and quantitative real-time PCR (qPCR) were employed to provide complementary molecular and physiological evidence of CF-mediated defense regulation.

II. Materials and Methods

A. Materials

P. deliense SR-S1, the causal agent of durian root and stem rot, was obtained from a previous study by Sarawaneeyaruk *et al.* [9] (unpublished data). The strain was isolated from the rhizosphere of diseased durian plants with root and stem rot symptoms and was identified by ITS nucleotide analysis (Sarawaneeyaruk *et al*. [9] unpublished data). The pathogen was maintained on Potato Dextrose Agar (PDA) for further experiments.

The antagonistic bacterium *P. aeruginosa* SWUC02 was obtained from the culture collection of a previous study by Sudyoung *et al*. [9]. The CF-SWUC02 was prepared according to the method previously described by Ref. [14].

B. Pathogenicity assay

Three healthy grafted durian seedlings were used in this study. The plants were watered daily with alternating applications of tap water and half-strength Murashige and Skoog (half-MS) nutrient solution.

P. deliense strain SR-S1 was cultured on water agar (WA) and incubated at room temperature for 48 h prior to use. Healthy durian leaves were selected, and surface-sterilized with 0.1% (v/v) sodium hypochlorite (NaOCl) for 1 min. The leaves were subsequently rinsed three times with sterile water, then air-dried. Wounds were made on the leaf surface using a sterile needle, after which the treatments are listed in the Table I was applied to the wounded sites. Each treatment was performed with three replicates, and a mock treatment was included as the control. The inoculated leaves were incubated for 12 h, and disease severity was evaluated by measuring lesion size using ImageJ software (version 1.54 p).

TABLE I. Preparation and Inoculation of Durian Leaves

Treatment/Time (h)	Mock	Pathogen	CF-SWUC02	CF-SWUC02 +Pathogen
0	TSB	TSB	CF-SWUC02	CF-SWUC02
12	WA	SR-S1	WA	SR-S1

C. DAB Staining Assay

Inoculated leaves from the previous step were used, with the modification that the leaves were incubated for 6 h before staining. The leaves were subjected to a DAB staining assay, which was performed according to the method described in Ref. [15] with minor modifications. Briefly, a DAB solution was prepared at a concentration of 1 mg mL^{-1} in sterile distilled water. The inoculated leaves were excised at the lesion sites into 1×1 cm segments and transferred into test tubes containing the DAB solution. DAB staining intensity and lesion area were quantified using ImageJ software (version 1.54 p). Non-wounded leaves were included as DAB controls.

D. qPCR Analysis

The inoculated leaves that were incubated for 6 h were used. The leaves were excised and immediately transferred into microcentrifuge tubes. The total RNA was extracted using the TRIzol–chloroform method, followed by DNase I treatment to remove genomic DNA contamination. RNA concentration and purity were assessed by measuring the absorbance at A260 and the A260/A280 ratio, respectively. RNA integrity and quality were further evaluated by 1% agarose gel electrophoresis.

qPCR analysis was performed using the KAPA SYBR® One-Step qRT-PCR Kit. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method using expression of housekeeping gene *Ef1α* as a internal control. Gene-specific primers targeting the elongation factor 1-alpha gene (*Ef1α*), putative disease resistance RPP13-like protein 1 gene (*RPP13L*), and endochitinase 1-like gene (*ZiChi7*), which are involved in plant defense responses, were used. All primer sequences were derived from Ref. [10] and are shown as follows:

Ef1α-f (5′- ACA TGA TTA CCG GTG CCT CAC A -3′)
Ef1α-r (5′- ACA CCA AGG GTG AAA GCA AGC A-3′)
*RPPL13*f (5′- CAC CAT CTT CCT TCG CAT TT -3′)
*RPPL13*r (5′-CCC ACT GCC CAA ATC TTC TA -3′)
ZiChi7-f (5′- GAC CGC ATC GGC TAC TAC AAG -3′)
ZiChi7-r (5′- CTG GTT GTA GCA GTC GAG GTT -3′)

E. Statistical Analysis

All experiments were performed with three biological replicates. Pathogenicity and DAB staining data were analyzed by one-way Analysis of Variance (ANOVA), followed by the Least Significant Difference (LSD) test. qPCR data were analyzed using Student's t-test, and relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Differences were considered significant at $p < 0.05$.

III. Result and Discussion

A. Antagonistic Activity of CF-SWUC02 against P. deliense SR-S1 on Durian Leaves

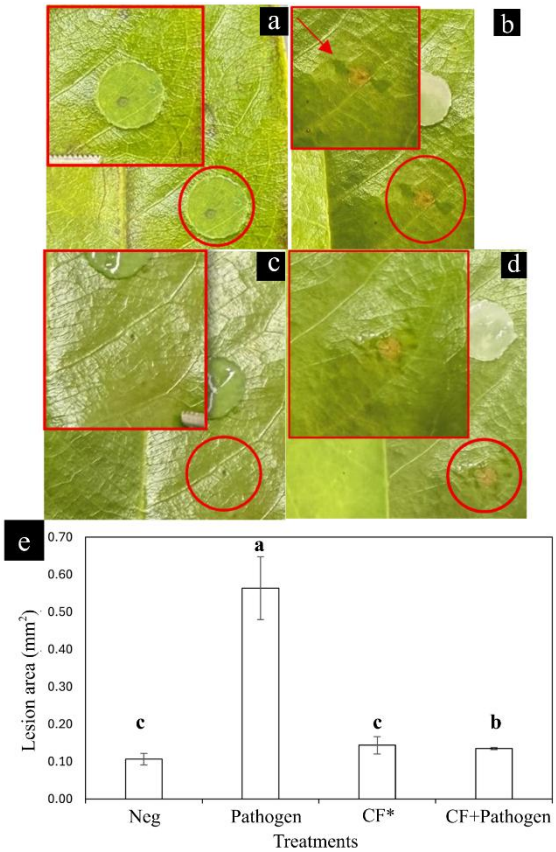

Fig. 1. Pathogenicity assay of *P. deliense* SR-S1 on durian leaves. Symptoms on durian leaves under different treatments: (a) negative control, (b) pathogen inoculation, (c) CF-SWUC02 treatment, and (d) CF-SWUC02 with pathogen. Red circles indicate lesion areas, which are magnified in the inserts. CF indicates CF-SWUC02. (e) Lesion diameter (cm) following different treatments. Bars represent mean ± SD ($n = 3$). Different letters indicate significant differences among treatments ($p < 0.05$).

Clear differences in disease symptoms were observed among treatments (Fig. 1). Leaves inoculated with *P. deliense* SR-S1 (Fig. 1b) exhibited severe disease symptoms characterized by necrotic and water-soaked lesions. In contrast, leaves receiving the CF-SWUC02 with pathogen treatment (Fig. 1d) showed only much smaller water-soaked and necrotic symptoms, while minimal or no lesions were observed in the negative control (Fig. 1a) and in the CF-SWUC02 treatment alone (Fig. 1c).

Quantitative analysis of lesion diameter using ImageJ software confirmed these observations (Fig. 1e.). The pathogen treatment produced the largest lesions (0.86 ± 0.12 cm), which were significantly larger than those in all other treatments ($p < 0.05$). Lesion size in the CF-SWUC02 with pathogen treatment (0.27 ± 0.01 cm) was significantly reduced, being approximately 3.2-fold smaller than that of the pathogen treatment. However, it remained significantly larger than lesions in the negative control (0.12 ± 0.02 cm) and in the CF-SWUC02 alone treatment (0.04 ± 0.02 cm), which did not differ significantly from each other, indicating the absence of pathogenic effects of CF-SWUC02. Overall, these results demonstrate that CF-SWUC02 markedly reduces disease severity caused by *P. deliense* SR-S1 on durian leaves.

B. Detection of Hydrogen Peroxide by DAB Staining

The DAB staining assay revealed significant differences in Hydrogen Peroxide (H_2O_2) accumulation among treatments (Fig. 2), consistent with the lesion development observed in the pathogenicity assay (Fig. 1). Leaves inoculated only with *P. deliense* SR-S1 exhibited the largest stained areas at 6 h post-inoculation (hpi), indicating strong H_2O_2 accumulation at the infection sites. Intense brown precipitates, indicative of high H_2O_2 accumulation, were clearly detected in pathogen-inoculated leaves (Fig. 2b). In contrast, markedly weaker DAB staining was observed in leaves treated with CF-SWUC02 alone (Fig. 2c) or CF-SWUC02 combined with the pathogen (Fig. 2d). Minimal or no DAB staining was detected in the negative control (Fig. 2a) and in the non-wounded DAB control (Fig. 2e). Together, these results indicate that CF-SWUC02 treatment mitigates pathogen-induced ROS accumulation and alleviates disease symptom development in durian leaves.

Leaves inoculated with *P. deliense* SR-S1 exhibited intense DAB staining, indicating substantial accumulation of Hydrogen Peroxide (H_2O_2) at infection sites. As a major ROS generated during early plant immune responses, H_2O_2 plays a central role in defense signaling. However, in interactions involving necrotrophic pathogens, excessive oxidative accumulation can promote host cell death and accelerate disease progression [16]. In contrast, leaves treated with only CF-SWUC02 or in combination with the pathogen showed markedly weaker DAB staining, suggesting that CF-SWUC02 attenuated pathogen-induced ROS accumulation. This attenuation of ROS is likely linked to bioactive metabolites present in CF-SWUC02, particularly rhamnolipids. Glycolipid biosurfactants commonly produced by *P. aeruginosa*, display strong antimicrobial activity against diverse fungal pathogens. Rhamnolipid-enriched fractions such as PA3 have been shown to suppress pathogen growth while modulating plant immune responses, including the regulation of ROS homeostasis and defense signaling pathways [10].

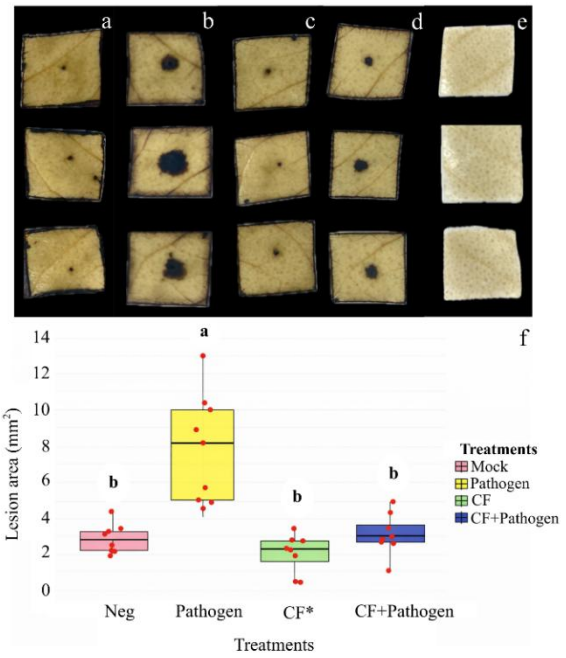

Fig. 2. Detection of H_2O_2 accumulation in durian leaves by DAB staining under different treatments. Representative DAB-stained leaf discs: (a) negative control, (b) pathogen inoculation, (c) CF-SWUC02 treatment, (d) CF-SWUC02 with pathogen, and (e) non-wounded leaves used as the DAB control. Brown precipitates indicate H_2O_2 accumulation at infection sites. (f) Lesion area (mm²) of DAB-stained regions under each treatment; CF* indicates CF-SWUC02. Boxplots show the median, interquartile range, and distribution of lesion area (n = 9), with points representing individual observations. Different letters (a–c) indicate significant differences among treatments ($p < 0.05$).

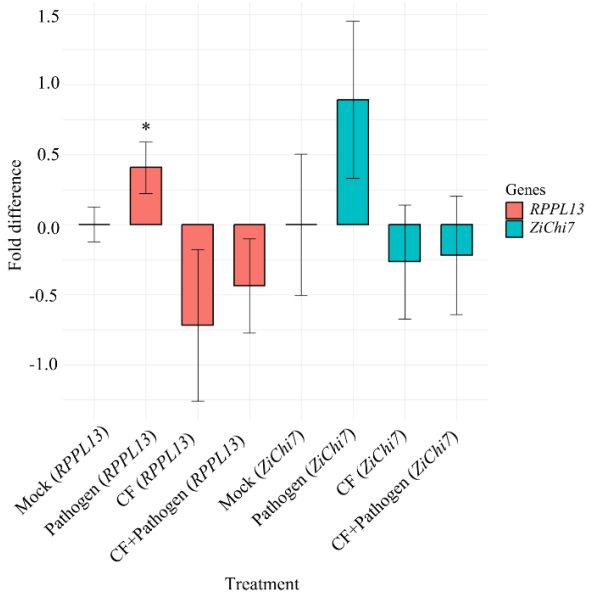

Fig. 3. Relative expression of defense-related genes in durian leaves under different treatments. Fold differences in the expression of *RPP13L*, and *ZiChi7* were determined. Expression levels were normalized to EF1α and are presented relative to the mock treatment. Bars represent mean ± SD (n = 3). Asterisks (*) indicate statistically significant differences compared with the mock treatment ($p < 0.05$).

C. qPCR Analysis of Plant Defense Response Gene Expression

Quantitative real-time PCR analysis revealed differential expression patterns of the defense-related genes *RPP13L*, and *ZiChi7* in durian leaves under different treatments (Fig. 3). Compared with the mock control treatment, leaves inoculated with *P. deliense* SR-S1 showed a significant upregulation of both genes, indicating activation of host defense responses upon pathogen infection. In contrast, treatment with CF-SWUC02 alone resulted in downregulation of both genes relative to mock control, suggesting that CF-SWUC02 did not induce a stress-related defense response. Notably, leaves treated with CF-SWUC02 together with the pathogen exhibited significantly reduced expression levels of *RPP13L*, and *ZiChi7* compared with the pathogen-only treatment.

P. deliense is classified as a necrotrophic oomycete pathogen that infects host plants by inducing host cell death, and subsequently exploiting dead tissues for nutrient acquisition [17]. Necrotrophic pathogens typically trigger strong oxidative bursts, and activation of defense-related genes during early infection, often culminating in tissue necrosis, and disease development. In this study, the necrotrophic lifestyle of *P. deliense* was reflected in both the molecular and physiological responses of durian leaves.

qPCR analysis demonstrated a significant upregulation of the defense-related genes RPP13L, and ZiChi7 in leaves inoculated with *P. deliense* compared with the mock control. This transcriptional activation suggests that pathogen infection induced host defense signaling pathways, which is consistent with reports that necrotrophic pathogens can strongly induce plant immune responses, including pathogenesis-related proteins and resistance-associated genes [13].

These findings were further supported by the pathogenicity assay. Leaves inoculated with only *P. deliense* developed larger lesions characterized by water-soaking and necrotic symptoms, whereas leaves treated with CF-SWUC02 together with the pathogen exhibited significantly smaller lesions, predominantly showing limited necrosis. The reduction in lesion size, and severity indicates that CF treatment attenuated disease development, likely by modulating host defense responses and restricting excessive oxidative damage. This modulation may reduce host cell death, thereby limiting the necrotrophic advantage of *P. deliense*.

Despite these promising findings, a potential limitation of this study lies in the origin of the biocontrol agent. Because *Pseudomonas aeruginosa* is widely recognized as an opportunistic human pathogen, its use may raise biosafety concerns, potentially affecting the acceptance of agricultural products associated with this bacterial species. To mitigate these concerns, the present study employed a sterile cell-free culture filtrate rather than live bacterial cells. The absence of viable microorganisms in this formulation substantially reduces biosafety risks and eliminates concerns related to opportunistic infection or environmental persistence [14]. By utilizing only extracellular bioactive metabolites, this approach retains antagonistic efficacy while avoiding regulatory and safety challenges commonly associated with live microbial applications. Beyond disease suppression, the culture

filtrated contains beneficial secondary metabolites, including siderophores and other bioactive compounds, which may contribute to enhanced plant growth and stress tolerance, thereby strengthening the practical applicability of CF-SWUC02 as a sustainable biocontrol strategy [14].

Taken together, the integrated results from qPCR, DAB staining, and pathogenicity assays suggest that CF-SWUC02 plays a protective role by regulating pathogen-induced defense responses in durian leaves. These findings highlight the potential of CF-SWUC02 as an effective and environmentally friendly biocontrol strategy for managing durian root and stem rot disease. This approach aligns with the Bio-Circular-Green (BCG) economic model and the UN (Sustainable Development Goals) SDGs, particularly SDG 12 (Responsible Consumption and Production), by reducing reliance on synthetic fungicides, and promoting more sustainable durian production.

IV. Conclusion

This study demonstrated that *P. deliense* SR-S1 infection induces necrotic lesions, elevated Hydrogen Peroxide (H_2O_2) accumulation, and upregulation of defense-related genes in durian leaves. DAB staining and qPCR analyses confirmed that the pathogen infection strongly activated oxidative bursts and host defense responses. In contrast, the application of CF-SWUC02 significantly reduced ROS accumulation, lesion development, and pathogen-induced gene expression. These results indicate that CF-SWUC02 suppresses necrotrophic disease progression by modulating host oxidative and defense responses. Therefore, CF-SWUC02 shows strong potential as a biocontrol agent for managing durian root and stem rot disease.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

T.C.-I. performed the pathogenicity assays and wrote the manuscript. N.J. carried out DAB staining and qPCR experiments. N.S. supervised all experimental work. O.P. reviewed and edited the manuscript. S.S. supervised the overall study, secured funding, and revised the manuscript, and served as the corresponding author. All authors have read, and approved the final version of the manuscript.

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