Evaluation of the Hormonal Changes in the Model Plant *Arabidopsis thaliana* as The Consequence of *Pseudomonas aeruginosa* Infection

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Abstract

Plants sense the microbial pathogen by perceiving the microbial signals through their highly specific immune receptors. These immune receptors are located on the plasma membrane and sense all types of microbes. Research over the last twenty years has shown the importance of *Arabidopsis thaliana* for the study of microbe-host interactions. The ability to sequence whole genomes of *Arabidopsis thaliana* has changed our view of biology. Here, the study of hormonal changes under the influence of biological stress of *Pseudomonas aeruginosa* bacteria was investigated. The bacterium *P. aeruginosa* was isolated from oil, cultured in a laboratory environment, and then sprayed onto *Arabidopsis* plants for stress evaluation. 24 hours after the application of stress, the hormones were measured by high-performance liquid chromatography (HPLC). According to the results, all hormones involved in the plant's immune system were significantly altered in response to stress by *P. aeruginosa*. The study revealed that the hormons salicylic, gibberellic acid, and jasmonic acid were the most altered compared to the control plants. While high levels of the hormones salicylic acid, gibberellic acid, and jasmonic acid hormones were observed, in other hormones such as melatonin and abscisic acid did not show asignificant changes. Among the elevated hormones, the levels of the hormones salicylic acid and jasmonic acid were highly statistically significant compared to the controls. These results confirm the specificity of hormone activation and *P. aeruginosa* specifically activates only defense-related hormones. Moreover, the findings of this study can be used for subsequent insvetigations on microbe-host interactions and future microbe infection control programs.

Keywords: *Arabidopsis thaliana*, Biological stress, Hormones, *Pseudomonas aeruginosa*, Immune system

Introduction

Plants are immobile organisms that are exposed to many environmental stresses during their lifetime (Bigeard et al., 2015; Felix et al., 1999; Safaeizadeh and Boller, 2019). Biotic and abiotic stress factors usually

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occur suddenly or simultaneously, and rapid plant responses are crucial for cell survival (DeFalco TA, Zipfel, 2021; Safaeizadeh and Boller, 2019; Signorelli et al., 2019). A fundamental strategy for plant adaptation to environmental challenges posed by biotic and abiotic threats is the regulation of gene expression. At the cellular level, plants regulate gene expression along with their physiological needs to adapt to short- and long-term environmental changes. It was established that plants constantly change under the influence of environmental factors and reprogram their response individually or in combination (Bigeard et al., 2015; DeFalco TA, Zipfel, 2021; Zipfel et al., 2004). Depending on the environmental conditions encountered, the plants activate a specific program of gene expression (Signorelli et al., 2019; DeFalco TA, Zipfel, 2021). In this regard, the specificity of the response is controlled by a variety of "interacting" molecular mechanisms that can interact with each other in a complex regulatory network including transcription factors, kinase cascades, reactive oxygen species, heat shock factors, and small RNAs (DeFalco TA, Zipfel, 2021; Gruszka 2018; Safaeizadeh, 2022; Safaeizadeh and Boller, 2019). The interaction between biotic and abiotic stress factors is regulated by hormonal and non-hormonal signaling pathways which can influence each other positively or negatively. Studies on gene expression in response to biotic and abiotic have shown that genes related to disease resistance in maize may be induced or repressed by abiotic stress (Bigeard et al., 2015; DeFalco TA, Zipfel, 2021). Much research is being conducted around the world to investigate the interactions between plants and pathogens. The choice of plant species depends on the purpose of the study. Knowledge of the basic mechanisms of plant diseases resistance is fundamental for sustainable agriculture and human health (Bigeard et al., 2015; Nobori et al., 2018). The use of the *Arabidopsis thaliana* has led to a rapid growth in information and understanding of disease resistance and susceptibility to pathogens (Bigeard et al., 2015; Safaeizadeh, 2022; Zipfel et al., 2004). *Arabidopsis thaliana* has recently been selected as an organism for a variety of plant science studies (Poupin et al., 2023). Recent research shows that this simple structure can serve as a suitable model not only for plant biology but also for answering fundamental questions about the structure and biological function common to all eukaryotes (Poupin et al., 2023; Safaeizadeh, 2022). While genome projects have proven that all eukaryotic organisms share a common genetic ancestor, Arabidopsis research has made it clear that analyzing plant genomes plays an important role in understanding the basic principles of biology in relation to different species, including humans (Poupin et al., 2023). In the course of this research, several plants have been recognized and introduced as genetic model systems, including maize, tomato, pea, rice, barley, petunia, and snapdragon, but biologists are not sure which species is the best suitable for studying common processes in all plants. They were not in agreement. As a result, our overview and understanding of fundamental aspects of plant growth and development

such as flowering, root development, hormonal function, and response to environmental signals is limited. Twenty years ago, botanists, used a combination of genetic and molecular tools, to search for a new organism as a suitable model for analyzing the data obtained. Plants such as petunia and tomato were logical candidates for plant models, especially for studies on *Agrobacterium* sp., but attention gradually focused on *A. thaliana*, a small plant from the Brassicaceae family, which was selected as the first genetic organism (Gruszka 2018; Poupin et al., 2023; Safaeizadeh, 2022; Safaeizadeh and Boller, 2019).

Research over the last twenty years has shown similarities between Arabidopsis and cultivated species (Poupin et al., 2023; Zipfel et al., 2004). The ability to sequence whole genomes in *Arabidopsis* has changed the life in biology (Provart et al., 2021). The *A. thaliana* was one of the first eukaryotes to have its genome sequenced. Recent studies have shown that pathogens are identified by specific receptors known as pattern recognition receptors (PRR) located in the plasma membrane of plant cells (Poupin et al., 2023; Safaeizadeh and Boller, 2019; Saijo et al., 2018). These receptors identify specific molecules of microbes produce internal cellular messages and initiate defense responses. Specific molecules of microorganisms that are recognized by these receptors are called PAMP (Pathogen Associated Molecular Pattern; Bigeard et al., 2015; Saijo et al., 2018). These molecules are specific to the pathogen and are not found in the host plant. The identification of PAMPs triggers several cellular response processes such as the production and secretion of ethylene hormones, salicylic acid, jasmonic acid, and other plant hormones, the alteration of ion flux between the cytoplasmic membrane, causing alkalinization of the intercellular space. In addition, an increase in cytoplasmic calcium ion concentration is observed as a result of the perception of defense signals by pattern recognition reports (Bagautdinova et al., 2022; Ruan et al., 2019; Safaeizadeh and Boller, 2019). Another response is the induction of mitogen-activated protein kinases (MAPK), which activate transcription factors (Bigeard et al., 2015; DeFalco TA, Zipfel, 2021). Proteins such as defensins, lytic enzymes, or enzymes required for the synthesis of phytoalexins (antimicrobial metabolites) are copied from coding genes (Bigeard et al., 2015; Saijo et al., 2018). Other defense responses include the deposition of callose on the surface of the cell wall and the production of reactive oxygen species (ROS), which can be toxic to pathogens and cause the formation of cross-links in the plant cell wall (DeFalco TA, Zipfel, 2021; Szechyńska-Hebda et al., 2022; Torres and Dangl, 2016).

Bacteria, viruses, and fungi are the most important pathogens that infect plants and have a broad host range can cause various serious diseases worldwide (Bigeard et al., 2015; Felix et al., 1999; Gautam, 2023). Plant pathogenic bacteria cause significant damage to agricultural products. the main symptoms include leaf spot, burns, chancre, vascular wilt, and gall formation. Pathogenic bacteria cause disease symptoms by invading plant tissue through natural pores, wounds, or insects. Although most bacteria

are endophytes, some of them can also live on the surface of the plant. Most pathogenic bacteria are limited to the intercellular space and some of them are active in the xylem or plant sap (Bigeard et al., 2015; Torres and Dangl, 2016). There are compatible and incompatible interaction between bacteria and plants. In the compatible interaction, the bacterium is in contact with a susceptible host and the result of this type of interaction is the occurrence of diseases.

The immune systemof plants consists of various highly specific and well-established immune receptors which are localized on the plasma membrane at the cell surface. These immune receptors can recognize and monitor all danger signals from different invaders. Recent findings may improve our knowledge and understanding of the highly complex properties of signals perceived by immune receptors. However, the nature, and specificity of these danger signals is different for each invader. We still, do not know exactly how they activate the defense hormone and what amounts of the hormone they produce. We do not even know, at what levels these hormones are needed to activate the robust immune system. In view of the above points and the importance of microbe-host interaction in plant biology, in this study, we investigated the hormonal changes in the model plant *A. thaliana* in response to infection with *Pseudomonas aeruginosa.*

P. aeruginosa is a gram-negative microbial bacterium belonging to the Pseudomonadaceae family (Lee et al., 2020; Qin et al., 2022). *P. aeruginosa* is commonly found in soil and water, but also in plants and humans (Jurado-Martín et al., 2021; Lee et al., 2020; Qin et al., 2022). *Pseudomonas* is believed to be one of the few true plant pathogens (Lee et al., 2020). *Pseudomonas aeruginosa* is a common human pathogen that causes chronic lung infections in patients with cystic fibrosis, people with burns, and other immunocompromised individuals. The pathogenesis of *P. aeruginosa* is related to a combination of factors (Jurado-Martín et al., 2021; Lee et al., 2020; Qin et al., 2022). These factors include flagella that support motility, and systems involved in the release of proteins that cause infection in host cells (Jurado-Martín et al., 2021; Lee et al., 2020; Qin et al., 2022). Despite the importance of this microbe, little is known about the interaction of *P. aeruginosa* and the model plant *A. thaliana.* Since, as this research focuses on the model plant *A. thaliana,* result of this research will be of great benefit expanding our understanding of important crops. Therefore, it is necessary to evaluate the hormonal changes of *A. thaliana* as a consequence of infection with *P. aeruginosa*.

Material and methods

Cultivation of Arabidopsis

The *Arabidopsis* seeds were grown in pots and covered with cellophane after watering, cellophane was spread over the pots. The pots were placed in the refrigerator for 48 hours. After 48 hours, the pots were transferred to the growth room. Two weeks after cultivating of *Arabidopsis*, when the seeds germinated, some holes were made in the cellophane because less moisture was needed. Two days after cellophane pores were made, it was completely removed from

the trays. Two days later, the *Arabidopsis* sprouts germinated and were planted in culture dishes. The trays were placed on cellophane-covered shelves in the growth room. After two weeks, the Arabidopsis seedlings were transferred from the seedling tray to the pot.

Preparation of bacteria culture and growth of the bacteria

Preparation of the bacterial culture supplemented with the following materials: $MgSO_4.7H_2O_2$, M=1/2324 g/mol, beef extract=2/5 g/mol, peptone meat=2/5 g/mol; sucrose=2.5 g/mol; yeast extract=0/5 g/mol; agar=4 g/mol. It was prepared in the volume of 500 ml. All the above ingredients were weighed and added to 500 ml of distilled water and the pH was measured with a pH meter. At an acidity of 7.2, agar was added to the solution and the pH was measured again. The pH of the solution reached 5.6, which was adjusted to 7 by adding drop-by-drop NAOH. The solution was placed in an autoclave for twenty minutes. After autoclaving, it was stored in the refrigerator. *Pseudomonas aeruginosa* bacteria were cultured in YEB culture medium under the hood and placed in an incubator for 24 hours before treatment.

Treatment of bacteria and infection test

The bacteria were sprayed on *A. thaliana* leaves at the four-leaf stage at an optical density 600 ($OD_{600\ nm}$ =0.02). The samples were transferred to the comprehensive research laboratory to measure the hormones after 24 hours, distilled water was used as the control.

Measuring the concentration of phytohormones

The hormones were measured using an HPLC device or Liquid Chromatography, which stands for high-performance liquid chromatography and is also called high-pressure liquid chromatography, is a device for identify, and quantify the components of a compound. Chromatography consists of two parts: chromato means color and graphi means drawing, and refers to the old method of separating substances based on color. Due to the use of high-pressure solvents, the word high-pressure liquid chromatography can also be used. In this study, the protocol of Huot et al. (2017) was used as a reference method. The ultra-pure HLPC of each hormone was used as a standard control. Three biological replicates were analyzed in each experiment.

Results

Understanding the underlying mechanisms in plantdevelopment of plant diseases is considered as the crucial step to development an effective disease control strategy. Although there is many research on *Arabidopsis thaliana* and virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), little is known about the interaction of *A. thaliana* and *Pseudomonas aeruginosa.* The amount of jasmonic acid in the *Arabidopsis thaliana* plant increased significantly ($p \leq 0.01$) under the influence of treatment with *Pseudomonas aeruginosa* bacteria (Figure 1). The amount of trans-zeatin in the *A. thaliana* plant increased significantly ($p \leq 0.05$) when treated with *Pseudomonas aeruginosa* bacteria (Figure 2). The bacterial treatment resulted in a 28.101% increase in

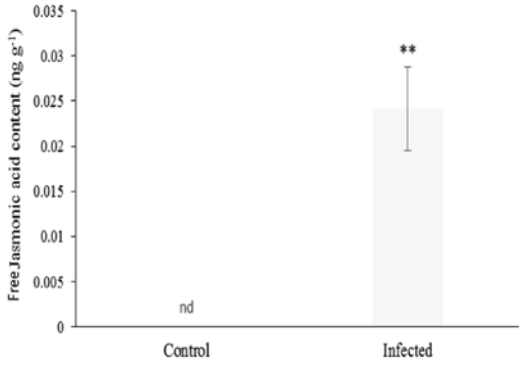

Fig. 1. Effect of *Pseudomonas aeruginosa* bacteria treatment on jasmonic acid content in *Arabidopsis thaliana*, ** indicates the significance at 0.01 level in the t-test

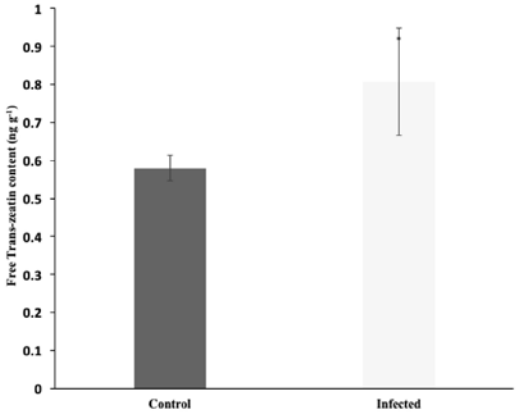

Fig. 2. Effect of *Pseudomonas aeruginosa* bacteria treatment on trans-zeatin content in *Arabidopsis thaliana.* * indicates the significance at the 0.05 level in the t-test

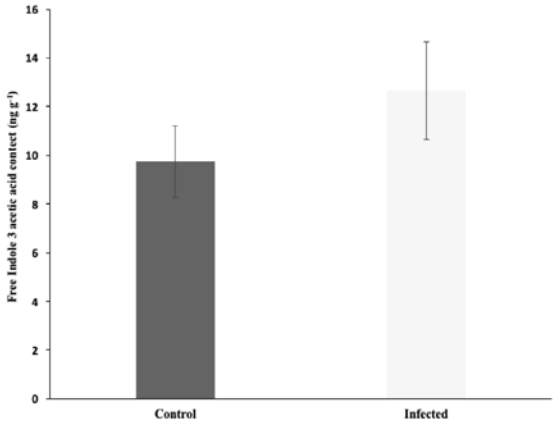

Fig. 3. Effect of *Pseudomonas aeruginosa* bacteria treatment on indole-3-acetic acid content in *Arabidopsis thaliana*

the amount of zeatin in the *A. thaliana* plant. As shown in Figure 3, indole-3-acetic acid in *A. thaliana* increased when treated with *Pseudomonas aeruginosa*.

As a result of bacterial treatment, the content of indole-3-acetic acid in *A. thaliana* increased by 23.045%. A significant increase ($p \leq 0.01$) of kinetin was observed in the *Arabidopsis thaliana* by treatment with *Pseudomonas aeruginosa* bacteria (Figure 4). The bacterial treatment increased the amount of kinetin in *A. thaliana* by 36.990%. Figure 5 shows that gibberellic acid significantly increased ($p \leq 0.01$) under the influence of treatment with *Pseudomonas aeruginosa* bacteriaand caused a 61.117% increase in gibberellic acid content compared to the control. The content of salicylic acid increased significantly ($p \leq 0.01$) in *A. thaliana* under the influence of treatment with *Pseudomonas aeruginosa* bacteria (Figure 6). Indole-3-butyric acid has increased by 21.693% under the effect of treatment with *Pseudomonas aeruginosa* compared to the control (Figure 7). Melatonin levels decreased in response to *Pseudomonas aeruginosa* (Figure 8). The bacterial treatment caused a 31.79% decrease in melatonin levels compared to the control. Abscisic acid levels decreased by 11.0752% in response to *Pseudomonas aeruginosa* (Figure 9).Previous studies have shown that defense responses to microbial pathogens are coordinated by the circadian clock. Bhardwaj et al., (2011) investigated that clock-mediated defense plays an important role in resistance to the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). They showed an increased resistance to *Pst* DC3000 in the early morning hours. This response is mediated by immunity triggered by pathogen-associated molecular patterns (PAMP)-. Furthermore, they showed that in response to the perception of defense signals, the expression of defense genes was increased in the early morning. Even, the deposition of callose in response to microbial infection was significantly higher in the early morning than at other times of their experiments according to their results. In their different assessment of defense responses in *A. thaliana* afterinfection with *Pst* DC3000, they concluded that the plant cells responded more effectively to bacterial pathogens during the day. Therefore, for subsequent studies to measure the hormone changes in response to microbial infection, it is suggested to observe monitor the defense-regulated hormones especially salicylic acid, gibberellic acid, and jasmonic acid hormones during the day and at night, and compare whether the hormone levels are various or the same in response to microbial infection.

Recently Solis et al. (2019) observed cyclodipeptides from *Pseudomonas aeruginosa* in *A. thaliana.* Although they investigated the expression of defense genes such as pathogenesis-related-1 proteins (PR-1) and lipoxygenase 2 (LOX2), they did not monitor the concentration of the defense hormones salicylic acid, gibberellic acid, and jasmonic acid in response to infection with *Pseudomonas aeruginosa*.

Previous research has shown that Zinc plays an important role in activating plant defenses against pathogens and herbivores

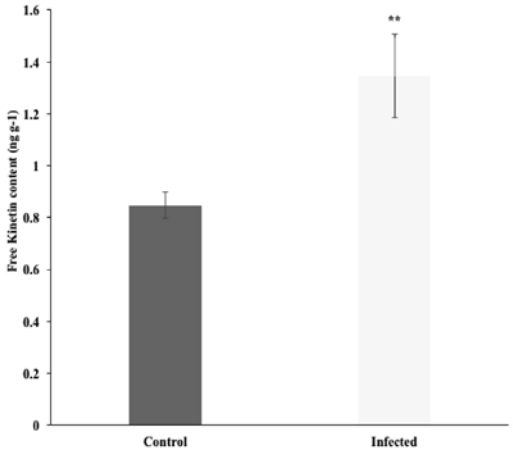

Fig. 4. Effect of *Pseudomonas aeruginosa* bacteria treatment on kinetin content in *Arabidopsis thaliana*. **, indicates the significant at the 0.01 level in the t-test

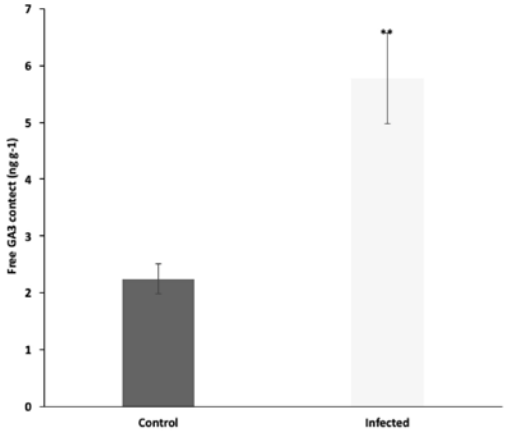

Fig. 5. Effect of *Pseudomonas aeruginosa* bacteria treatment on GA3 gibberellic acid content in *Arabidopsis thaliana* plant. **, indicates the significant at the 0.01 level in the t-test

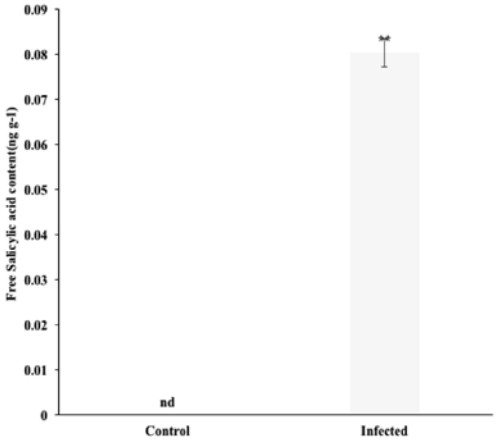

Fig. 6. Effect of *Pseudomonas aeruginosa* bacteria treatment on salicylic acid content in *Arabidopsis thaliana*. **, indicates the significant at the 0.01 level in t-test

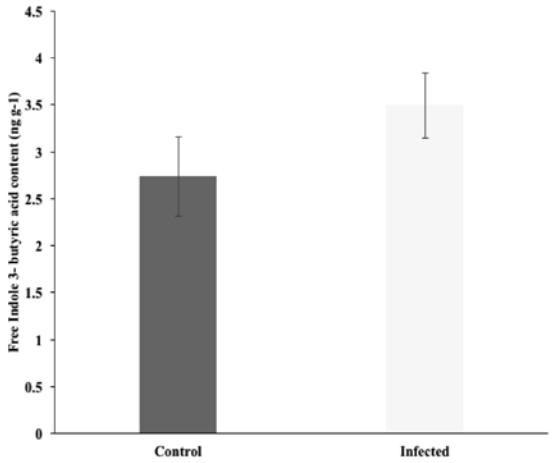

Fig. 7. The effect of *Pseudomonas aeruginosa* bacteria treatment on the content of indole-3-butyric acid in *Arabidopsis thaliana*

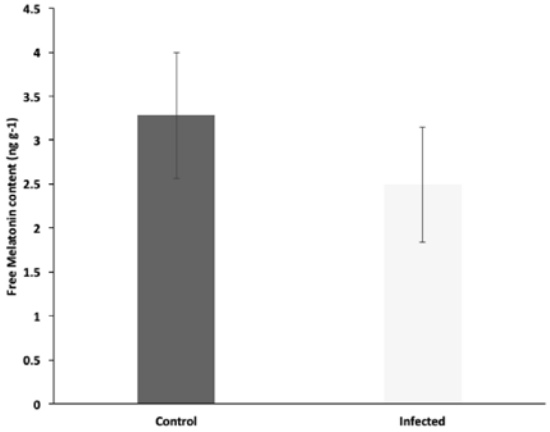

Fig. 8. The effect of *Pseudomonas aeruginosa* bacteria treatment on melatonin content in *Arabidopsis thaliana*

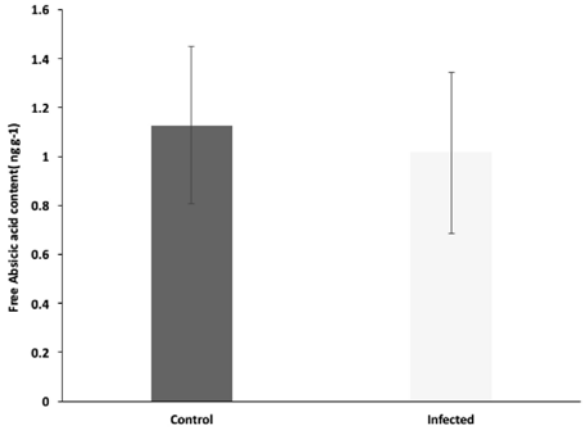

Fig. 9. The effect of *Pseudomonas aeruginosa* bacteria treatment on abscisic acid content in *Arabidopsis thaliana*

(Cabot et al., 2019). Zinc has been shown to play a direct role in the activation of defense hormones and a deficiency reduce susceptibility to microbial infections (Cabot et al., 2019). Therefore, it proposed to investigate the role of zinc after infection with *P. aeruginosa* in *A. thaliana.*

Discussion

Animal and plant cells have highly specific immune receptors, which are innternal and intracellular sensors of innate immunity (Jones et al., 2016; Mermigka et al., 2020). These receptors are known as pattern-recognition receptors, NOD-like receptors, nucleotide-binding domain, and leucine-rich repeat receptors. By specifying forming of macromolecular structures, they can recognize a variety of microbial pathogens. The function of these receptors, their mode of action, how they activate the immune system, and how hormone levels are change as a result of microbial infection has been an open question for researchers for almost two decades (Hout et al., 2017; Jones et al., 2016). Plant hormones are the most important regulators of plant growth and immunity (Hout et al., 2017; Safaeizadeh and Boller, 2019). Over the past few decades, researchers have gathered a wealth of information on the role of various plant hormones in immunity and their ability to influence the outcome of plant-pathogenic interactions (Hout et al., 2017). Plant hormones, also known as phytohormones, are naturally occurring small organic molecules that are not only important for plant growth processes, but also play an important role as signaling substances. Schroeder et al. 2019 found a connection between natural or developmental processes. It is also a key component of the immune response of plants (Schroeder et al., 2019). Hormones like salicylic acid, jasmonic acid, and ethylene help the body fight off pathogens. But new research shows that abscisic acid, gibberellic acid, cytokinin, auxin, and brassinosteroids, which are usually linked with non-stress. Furthermore, hormone biosynthesis is a key component of the immune response of plants (Schroeder et al., 2019). Plant hormones tend to interact through complex antagonistic or synergistic interactions (Hout et al., 2017; Schroeder et al., 2019; Safaeizadeh and Boller, 2019). These interactions result in changes in the plant's physiology, which end with a proper defense against the attack of the pathogen or, in the case of pathogenic agents, changes that benefit the invading pathogenic organism (Schroeder et al., 2019). Anyhow, it is a complex and interconnected combination of hormonal actions that modulate plant immunity.

Salicylic acid (SA) is a phenolic compound with plant hormone activity that is known as an important endogenous signaling molecule in plant immunity (Bagautdinova et al., 2022; Ding et al., 2023; Wang et al., 2020). However, studies have documented SA's indirect involvement in germination, flowering, mitochondrial electron transport, and resistance to abiotic stress, including heat resistance (Ding et al., 2023; Wang et al., 2020). Also, the level of SA accumulated in the places of pathogen infection, and a relationship between SA accumulation and resistance to pathogen attack were observed. The similarity between the effects of SA

application and pathogen attack on plant physiology led to the suggestion that SA is a signal for the activation of defense against invading agents (Ding et al., 2023; Wang et al., 2020). It is interesting to note that, SA is derived from the primary metabolite chorismate, through two main enzymatic pathways, one involves the phenyl-alanine ammonia lyase pathway, and the other involves a two-step process metabolized by the enzymes isochorismate synthase (ICS), which converts chorismate to isochorismate, and Isochorismate pyruvate lyase (IPL), which catalyzes the combined version. isochorismate to SA (Garcion et al., 2008).

Pathogen invasion makes a dramatic change in the transcriptional and metabolic modifications, and the transcriptional changes allow the invaded cells to establish robust and appropriate defense responses (Li et al., 2016; Liu et al., 2015). In innate immunity, transcription factors are regarded as the key elements in the plant immune system. Furthermore, Jasmonic acid signaling mediates resistance to necrotrophic pathogens, such as the bacterial pathogen *Pectobacterium atrosepticum* (Ruan et al., 2019; Truman et al., 2007). Studies showed that jasmonic acid has an important role in stomatal opening, inhibition of Rubisco biosynthesis, and change in the uptake of nitrogen and phosphorus, and it has a role in the transport of organic matter such as glucose (Ruan et al., 2019; Truman et al., 2007).

Considering that the biosynthesis pathway of ABA (like other hormones SA and JA) takes place in the chloroplast, it plays an important role in microbial defense (Kumar et al., 2022). This could be the reason for insufficient production of ABA or callose in response to leaf infection.

Gibberellins, or gibberellic acid (GA) are a large family of tetracyclic ditropenoid hormones that help regulate plant growth and immune responses (Hedden, 2020; Tian et al., 2017; Xu et al., 2020). GA was first identified when rice infected with a necrotrophic pathogen *Gibberella fujikuroi* showed abnormal elongation. which was later found to be caused by a compound similar to GA secreted by the pathogens, and the most common biologically active forms of GA are GA1, GA3, and GA4 derivatives (Hedden, 2020; Tian et al., 2017; Xu et al., 2020).

One limitation of this experiment was monitoring and evaluating the levels of the phytohormone ethylene (Collins et al., 2020; Guan et al., 2015; Huang et al., 2015). This hormone is regarded as the key phytohormone, which involves several plant-pathogen interactions (Collins et al., 2020; Huang et al., 2015). Ethylene plays a crucial and positive role in plant resistance against all microbial pathogens and in defense responses to damage-associated molecular patterns (Hander et al., 2019). Ethylene biosynthesis is highly elevated in response to both prokaryote and eukaryote pathogens, and as a consequence of their elicitor perception by pattern recognition receptors, the production of ethylene is initiated (Collins et al., 2020; Huang et al., 2015).

As the measurement of the phytohormone ethylene needs gas chromatography, it is suggested to evaluate and monitor the levels of ethylene for the same experiment and determine the levels of ethylene changes in response to *P. aeruginosa* infection.

Studies have shown that ethylene response factors turn on of hormonal pathways that control the expression of defense genes that respond to jasmonate. Anyhow, despite the importance of the ethylene phytohormone, we could monitor other plant hormones, such as salicylic acid (SA) and jasmonic acid (JA). Many research experiments have confirmed the role of SA and JA biosynthesis in plant innate immunity and plant bacterial resistance.

In conclusion, for the first time, we evaluated and monitored the hormonal changes in the model plant *A. thaliana* and the bacterial pathogen *Pseudomonas aeruginosa* after 24 hours post infection. The results of our study clearly showed that *Pseudomonas aeruginosa* enhances and activates the components of innate immunity in the model plant, *A. thaliana.* We discovered that, the perception of *Pseudomonas aeruginosa*, led to an elevation in the levels of important defense hormones salicylic acid and jasmonic acid. We recommend conducting this experiment at various time intervals, such as 36, 48, and 72 hours after treatment, and using various ratios of *Pseudomonas aeruginosa* for infection to assess and monitor hormonal changes. Moreover, in light of the results that we found in this study, we can treat crop plants with attenuated *P. aeruginosa* to activate their immune system and defense hormones. Once the invading microbes activate the defense hormones in these plants, they will exhibit resistance to infection. Furthermore, it is recommended to utilize different infection methods such as syringe infection assays, deep inoculation methods, and flower inoculation, to evaluate the levels of hormonal changes and defense gene expression after the bacterial infection.

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