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Research Article
(Araştırma Makalesi)

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Anahtar sözcükler: D,L-Pip, bitki savunma cevapları, *Pseudomonas syringae* pv. *tomato* DC3000, sistemik kazanılmış dayanıklılık, stoma savunması

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Exogenous application of pipecolic acid induces stomatal closure in *Arabidopsis thaliana* L.

Ekzojen pipekolik asit uygulaması *Arabidopsis thaliana* L.' da stoma kapanmasını tetikler

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ABSTRACT

Objective: The major objectives of this study were (i) to determine whether exogenous Pipecolic acid treatment triggers the stomatal closure; (ii) to assess how the stomatal response is influenced by the method and concentrations of Pipecolic acid treatment; (iii) to investigate the response of Pipecolic acid-primed plants to the foliar bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 that invades plants through stomata.

Material and Methods: Freshly harvested Arabidopsis leaves were immersed in MES-KCl buffer supplemented with 1 mM of D,L-Pipecolic acid for 2 h. Stomatal aperture was measured in epidermal strips collected from the abaxial side of the leaves. Stomatal aperture in Pipecolic acid-treated plants was also directly quantified after *Pseudomonas syringae* pv. *tomato* DC3000 inoculation.

Results: The treatment with D,L-Pipecolic acid resulted in increased stomatal closure in a concentration-dependent manner. Treatments with 0.1 mM and 1 mM of D,L-Pipecolic acid led to a reduction in stomatal aperture by 32.5% and 54.7%, respectively. Leaves treated with either 1 mM of D,L-Pipecolic acid or L-Pipecolic acid demonstrated similar stomatal apertures corresponding to 2.67 and 2.49 µm, respectively. The stomatal apertures did not exhibit a significant difference between the treatments following the *Pseudomonas syringae* pv. *tomato* DC3000 infection. Pipecolic acid-mediated enhanced defense is independent of stomatal immunity.

Conclusion: Exogenous Pipecolic acid triggers preinvasion stomatal closure in Arabidopsis. There is no difference between pipecolic acid application methods (soil drenching or foliar spray) in terms of affecting stoma closure.

ÖZ

Amaç: Bu çalışmanın ana amaçları şu şekildedir: (i) Ekzojen Pipekolik asit uygulamasının stoma kapanmasını tetikleyip tetiklemediğini belirlemek; (ii) Stoma tepkisinin Pipekolik asit uygulama yöntemi ve konsantrasyonlarından nasıl etkilendiğini saptamak; (iii) Pipekolik asitle uyarılmış bitkilerin, stomalar yoluyla bitkiye giriş yapan bakteriyel patojen *Pseudomonas syringae* pv. *tomato* DC3000'e karşı tepkisini araştırmak.

Materyal ve Yöntem: Taze koparılmış Arabidopsis yaprakları, 2 saat boyunca 1 mM D,L-Pipekolik asit içeren MES-KCl çözeltisine daldırılmıştır. Stoma açıklığı, yaprakların üst yüzeyinden toplanan epidermal şeritlerde ölçülmüştür. Pipekolik asit uygulanmış Arabidopsis bitkilerindeki stoma açıklığı da *Pseudomonas syringae* pv. *tomato* DC3000 inokulasyonundan 0, 2 ve 4 saat sonra alınan epidermal şeritlerde ölçülmüştür.

Araştırma Bulguları: D,L-Pipekolik asit uygulaması, konsantrasyona bağlı bir şekilde stoma kapanmasının artmasına neden olmuştur. 0.1 mM ve 1 mM D,L-Pipekolik asit ile yapılan uygulamalar stoma açıklığında sırasıyla %32.5 ve %54.7 oranında bir azalmaya yol açmıştır. 1 mM D,L-Pipekolik asit veya L-Pipekolik asit ile muamele edilen yapraklar, sırasıyla 2,67 ve 2,49 µm'ye karşılık gelen benzer stoma açıklıkları göstermiştir. Bakteriyel enfeksiyonun ardından stoma açıklıkları uygulamalar arasında önemli bir fark sergilememiştir. Pipekolik asit aracılı gelişmiş savunma, stoma bağışıklığından bağımsızdır.

Sonuç: Eksojen Pipekolik asit, Arabidopsis'te enfeksiyon öncesi stoma kapanmasını tetikler. Pipekolik asit uygulama metotları arasında (toprağı ıslatma ya da yaprağa püskürtme) stoma kapanmasını etkilemesi bakımından fark yoktur.

INTRODUCTION

Stomata, natural apertures formed by specialized guard cells situated in the leaf epidermis, serve a crucial physiological role in regulating the processes of transpiration and carbon dioxide (CO_2) uptake essential for photosynthesis. Various plant pathogens from diverse taxonomic origins, including bacteria, oomycetes, and fungi, penetrate the inner tissues of leaves by utilizing stomatal pores (Melotto et al., 2006; Solanki et al., 2019; Yang et al., 2021a).

Stomata play a prominent role in the regulation of plant innate immunity response, predicated on the perception of pathogen associated-molecular patterns (PAMPs). This phenomenon is scientifically elucidated as stomatal defense (Melotto et al., 2006, 2017). Numerous scientific inquiries have demonstrated that plants have evolved to sense and respond to the PAMPs to restrict pathogen entry by closing the stomata efficiently (Melotto et al., 2006; Lozano-Durán et al., 2014; Judelson & Ah-Fong, 2019; Ye et al., 2020). In return, certain pathogens possess the capacity to inhibit plant-mediated stomatal closure (Gudesblat et al., 2009; Lozano-Durán et al., 2014) or induce stomatal openings (Schellenberg et al., 2010) to gain access to the interior.

Stomatal movement is regulated by the influx and efflux of water and solutes such as K^+ into and out of guard cells. This regulation is governed by sophisticated signaling networks that respond to environmental stimuli or endogenous signals (Jia & Zhang, 2008; Kollist et al., 2014). Reactive oxygen species (ROS), along with their detoxifying enzymes, ion pumps, channels, plant hormones and transport proteins for H^+, K^+, Ca^{2+} in the plasma membrane, collectively contribute to the orchestration of stomatal movement (Jia & Zhang, 2008; Kollist et al., 2014; Sierla et al., 2016; Qi et al., 2018). Among these regulatory elements, abscisic acid (ABA) plays pivotal role in mediating stomatal closure, and its functions have been extensively elucidated in the literature (Munemasa et al., 2015; Hsu et al., 2021). The functions of plant defense related hormones, such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) in the regulation of stomatal closure have also been highlighted (Acharya & Assmann, 2009). Notably, the phenolic hormone SA emerges as a crucial signal in the plant's defense against pathogens, assuming a direct role in stomatal defense (Zeng et al., 2011; Miura & Tada, 2014; Melotto et al., 2017). Additionally, it has been observed to play a secondary role in downstream signaling, contributing to the regulation of stomatal closure in response to pathogenic threats (Wang et al., 2020).

A non-protein amino acid pipecolic acid (Pip) was identified as a one of the key mediators of systemic resistance against (hemi)biotrophic pathogens (Návarová et al., 2013, Bernsdorff et al., 2016; Hartmann et al., 2017). Systemic acquired resistance (SAR) is a phenomenon wherein plants exhibit an enhanced defense response to a diverse range of pathogens in distal tissues following a localized pathogen attack (Tosun & Onan, 2020). The establishment of SAR requires the systemic translocation of signaling molecules after a local infection. SA and the *N*-hydroxylated derivative of pipecolic acid (Pip), *N*-hydroxy-pipecolic acid (NHP), are the two main regulatory molecules of SAR (Shan & He, 2018; Wang et al., 2018b). NHP coordinates the establishment of SAR in conjunction with the immune signal SA. The activation of pathogen-inducible pipecolate and salicylate pathways involves both shared and distinct regulatory elements. Additionally, reciprocal interactions between these metabolic branches are evident (Bernsdorff et al., 2016; Hartmann & Zeier, 2019). A set of Calmodulin-binding Transcription Activator (CAMTA) transcription factors, which exhibit some degree of overlap, regulate specific gene sets involved in the accumulation of SA and NHP through a positive-feedback loop (Kim et al., 2020).

The exogenous application of Pip confers protection across a diverse spectrum of plant species, encompassing both monocotyledonous and dicotyledonous plants, against biotrophic pathogens. Application of Pip/NHP to *Arabidopsis thaliana* L. roots has been observed to result in reduced *Pseudomonas syringae* pv. *maculicola (*Návarová et al., 2013, Chen et al., 2018; Schnake et al., 2020) and *Pseudomonas syringae* pv. *tomato* (*Pst* DC3000) colonization (Wang et al., 2018a). Furthermore, exogenous application of Pip and/or NHP has demonstrated systemic protection against *Pseudomonas*

syringae pv. *lachrymans (*Schnake et al., 2020; Pazarlar et al., 2021) and *Podosphaera xanthii* in *Cucumis sativus* L. (Pazarlar et al., 2021) and as well as towards *Phytophthora infestans* (Schnake et al., 2020) and *Pst* (Zhang et al., 2020) in *Solanum lycopersicum* L. Additionally, an increased plant protection against *Blumeria graminis* f. sp. *hordei* and *Xanthomonas translucens* pv. *cerealis* was demonstrated through Pip treatment in *Hordeum vulgare* L. plants (Lenk et al., 2019).

Despite the accumulating data showing that exogenous application Pip confers protection to various (hemi)biotrophs, the contribution of stomatal defense in regulating this enhanced protection remains unexplored. The major objectives of this study were (i) to determine whether exogenous Pip treatment triggers the stomatal closure; (ii) to assess how the stomatal response is influenced by the method and concentrations of Pip treatment; (iii) to investigate the response of Pip-primed plants to the foliar bacterial pathogen *Pst* that invades plants through stomata.

MATERIALS and METHOD

Plant materials and growth

Arabidopsis thaliana ecotype Col-0 seeds were surface-sterilized by 2% sodium hypochlorite for 5 min followed by a 5 min treatment with 70% ethanol. The seeds were sown in the growth substrate (Klasmann TS1, Germany), subjected to a 3-days stratification at 4°C, and plants were grown in a growth chamber under conditions of 22°C with a 16/8 h day/night cycle (Li et al., 2011).

Chemicals

MES-KCl buffer, comprising 50 mM KCl, 0.1 mM $CaCl_2$, and 10 mM Mes-KOH (pH 6.15), was used to induce stomatal opening. The D,L-Pip (CAS number 535-75-1) and L-Pip (CAS number 3105-95-1) (TCI Chemicals, India), and H_2O_2 (Merck Millipore, Germany) were used in this study.

Pathogen inoculation

As a model pathogenic bacterium, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), received from the Department of Plant and Environmental Sciences, Copenhagen University, Denmark was used in this study. *Pst* DC3000 was grown in liquid King's medium B (10 g/l proteose peptone, 1.5 g/l K_2HPO_4, 1.5 g/l $MgSO_4 \cdot 7H_2O$, and 10 ml glycerol), for 24 h and bacterial cells were harvested into sterile dH_2O. The bacterial suspension was adjusted to the OD_{600}=0.2 (PG Instruments-T60 spectrophotometer). Inoculation was performed by foliar spraying (Ziphel et al., 2004).

Stomatal bioassays

Stomatal closure was measured according to the method as described by Li et al. 2013. The fully expanded youngest leaves of 4-week-old Arabidopsis plants were detached and floated on MES-KCl buffer for 2 h with adaxial surfaces facing upwards in a plastic Petri dish (⌀ 90 mm) in a growth chamber at 22°C. The leaves were then transferred to fresh MES-KCl buffer, either alone (as a control) or supplemented with pipecolic acid (0.1 mM or 1 mM) or 100 μM of H_2O_2, followed by further incubation for 2 h. Peels (epidermal strips) collected from the abaxial side of the leaves by using forceps were mounted on a glass slide and observed under light microscope. Images of epidermal peels were captured under the microscope and the stomatal apertures were measured following the calibration of length based on an external scale used during taking the pictures by using Image J software. At least 10 stomata from 5 different leaves were counted per treatment.

To directly measure stomatal aperture on Arabidopsis plants, four-week-old plants treated with either 1 mM of D,L-Pip or water (control) by soil drenching or foliar spraying. Subsequently, the leaves were inoculated with *Pst* DC3000 at 2 h post-treatment. At least three leaves per plant were peeled and more than 50 stomatal aperture was measured at 0, 2 and 4 hours after *Pst* DC3000 inoculation.

Statistical analysis

The data presented in this study were analyzed statistically with GraphPad Prism v10. Shapiro-Wilk test was used to test the normality and homogeneity of the data. The comparisons between treatments were made using Student's *t*-test (***P*<0.01, ****P*<0.001, *****P*<0.0001, ns non-significant).

RESULTS and DISCUSSION

The role of stomatal closure in plant innate immunity, often referred to as the stomatal defense response, has become a prominent and actively discussed topic in recent years. A substantial amount of recent data indicates that Pip and NHP, serving as endogenous signals in plant immunity, predominantly regulate SAR in a SA-dependent manner, with a partial involvement in SA-independent pathways (Bernsdorff et al., 2016; Hartmann & Zeier, 2019). Nitric oxide (NO) and ROS have been postulated to play a role in Pip-mediated SAR and priming responses (Lenk et al., 2019; Wang et al., 2018a; Pazarlar et al., 2021). Considering that some components such as SA and ROS regulated by Pip treatment are also associated with stomatal closure, experiments were conducted to assess whether exogenous Pip treatment could induce stomatal closure in Arabidopsis. To investigate the effects of Pip on stomatal closure in Col-0 plants, freshly harvested leaves were immersed in MES-KCl buffer supplemented with 1 mM of D,L-Pip for 2 h. The treatment with D,L-Pip resulted in increased stomatal closure in a concentration-dependent manner. Treatments with 0.1 mM and 1 mM of D,L-Pip led to a reduction in stomatal aperture by 32.5% (*P*<0.0001) and 54.7% (*P*<0.0001), respectively. As a positive control H_2O_2, known to induce stomatal closure, was included in the experiment. H_2O_2 induced a significant increase (58.9%) in stomatal closure (*P*<0.0001), confirming the success of the experimental setup (Figure 1A, 1B).

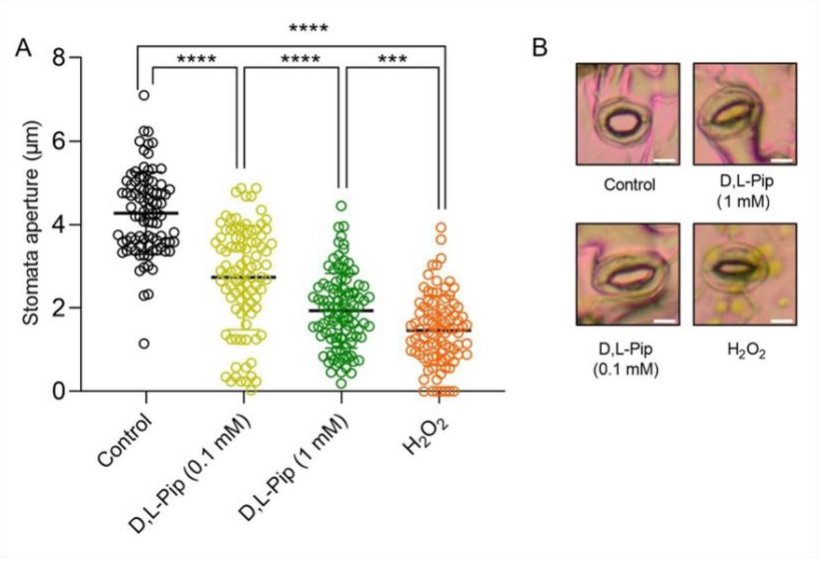

Figure 1. Exogenous D,L-Pip treatment induces stomatal closure. A. Stomatal aperture of D,L-Pip and H_20_2 treated leaves, B. Representative images of stomatal openings (****P*<0.001, *****P*<0.0001).

Şekil 1. *Ekzojen D,L-Pip uygulaması stoma kapanmasını tetikler. A. D,L-Pip ve H_20_2 uygulanan bitkilerde stoma açıklığı, B. Stoma açıklıklarının temsili fotoğrafları (***P<0.001, ****P<0.0001).*

Additional experiments were conducted to test whether the stomatal-inducing effect of Pip is enantiomer-dependent. Leaves treated with either 1 mM of D,L-Pip or L-Pip demonstrated similar stomatal aperture, indicating that the effect is independent of the Pip enantiomer (Figure 2). Some other studies also demonstrated that the responses of plants to pipecolic acid remain independent of its enantiomers (Návarová et al., 2013; Pazarlar et al., 2021).

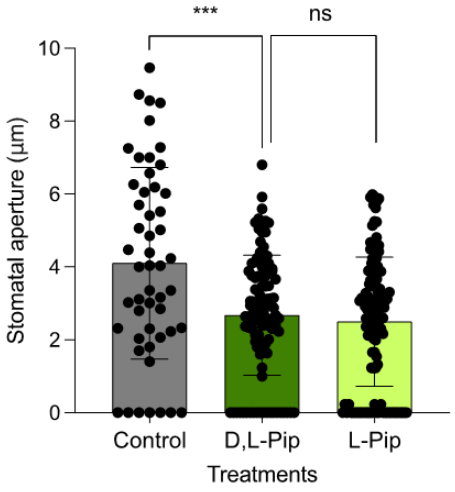

Figure 2. The stomatal closure inducing effect is independent of the Pip enantiomer (***P<0.001, ns non-significant).

Şekil 2. *Pip'in stoma kapanması üzerine etkisi enantiyomerinden bağımsızdır (***P<0.001, ns önemli değil).*

This dataset depicted in Figures 1 and 2 represents the initial observation in the literature that the exogenous application of Pip leads to a significant increase in stomatal closure. The studies indicate that SA, functioning as a SAR signal, modulates stomatal defense, potentially triggering the generation of ROS and NO, alongside calcium signaling (Joon-Sang, 1998; Hao et al., 2010; Hao et al., 2011; Khokon et al., 2011). Pip-triggered stomatal closure may similarly occur via ROS accumulation or Ca^{+} signaling via indirect salicylic interaction. Furthermore, considering the relationship of SAR components such as SA with ABA, which serves as a key regulator of stomatal movements (Prodhan et al., 2018), it is important to note the potential involvement of ABA in Pip-mediated stomatal closure.

As most studies conducted so far have focused on examining the SAR-related effects of exogenous Pip applications, the preferred method for Pip application has been soil drenching. In this study, the effects of soil drench and foliar spray of 1 mM of D,L-Pip on stomatal closure were evaluated. The results obtained indicated no significant difference in stomatal aperture between Pip treatments with soil drench and foliar spray (Figure 3A).

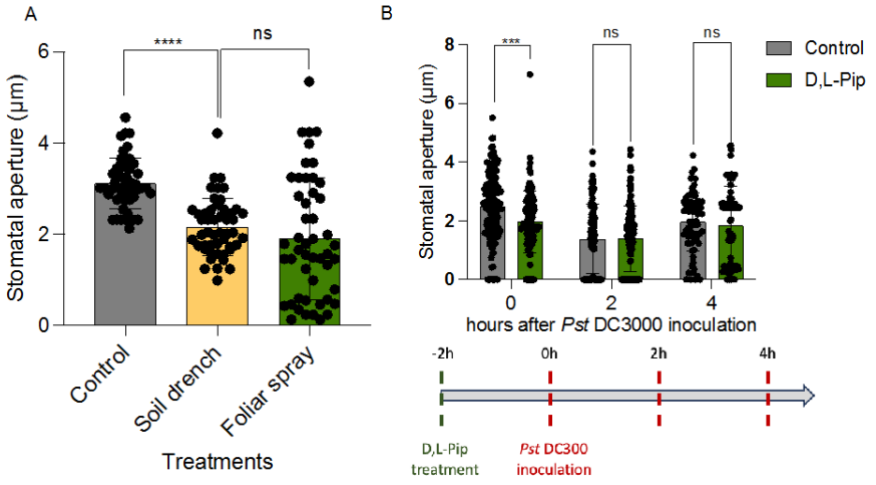

Figure 3. D,L-Pip triggered stomatal closure is not affected differently by Pst DC3000 (***P<0.001, ****P<0.0001, ns non-significant).

Şekil 3. *D,L-Pip ile tetiklenen stoma kapanması Pst DC3000'den farklı şekilde etkilenmez (***P<0.001, ****P<0.0001, ns önemli değil).*

Stomata represent the main sites of entry for foliar pathogens mainly plant pathogenic bacteria. Restricting pathogens at entry points into plants constitutes a highly effective mechanism for plant protection. In this context, stomatal aperture in Pip-treated plants was directly quantified to examine how plants pre-treated with Pip respond to *Pst* DC3000 in terms of stomatal closure. The pretreatment with 1 mM D,L-Pip led to a reduction in stomatal aperture, as illustrated in Figure 3B. Subsequent inoculation with *Pst* DC3000 resulted in a noteworthy closure of stomata observed in both control and Pip-treated plants 2 hours post inoculation (hpi) However, the stomatal apertures did not exhibit a significant difference between the two treatments. The infection-induced stomatal closure observed at 2 hpi partially disappeared in plants at 4 hpi, regardless of whether they were pretreated with Pip or not (Figure 3B). The exogenous application of Pip has been shown to prime plants for enhanced resistance against *Pst* DC3000 (Wang et al., 2018a). Here, it was speculated whether stomatal defense is involved in the enhanced defense triggered by exogenous application of Pip. Even though D,L-Pip by itself caused a reduction in stomatal aperture, the observed difference in inoculated plants was statistically insignificant when compared to the control treatment. This suggests that Pip-mediated enhanced defense is independent from stomatal immunity. Coronatine (COR), a virulence factor employed by *Pst*, is known to induce the reopening of closed stomata as a strategy to overcome stomatal immunity (Zeng & He, 2010; Panchal et al., 2016; Toum et al., 2016; Uddin et al., 2022). Thus, the presence of *Pst*-originated COR could hinder the Pip-triggered stomatal closure. These findings indicate that Pip triggers preinvasion stomatal immunity. Similar observations were reported in various studies involving different signaling molecules, such as ABA (Liu et al., 2019) melatonin (Yang et al., 2021b).

CONCLUSIONS

The mechanism behind the enhanced protection in Pip applied plants have been under investigation in the last decade. It has now well established that NHP is a mobile signal takes function in signaling during SAR. Nevertheless, a comprehensive elucidation of the mechanisms governing the action of molecules that activate the plant defense system, including Pip, remains imperative. In this study, it has been demonstrated that stomatal closure is triggered following exposure to exogenous Pip, regardless of the treatment method employed. Similarly, the presence of *Pst* did not impact stomatal closure, suggesting that Pip-triggered plant protection is independent of stomatal defense in model plant Arabidopsis.

Data Availability

Data will be made available upon reasonable request.

Author Contributions

Conception and design of the study: SP sample collection: SP; analysis and interpretation of data: SP; statistical analysis: SP; visualization: SP; writing manuscript: SP.

Ethical Statement

I declare that there is no need for an ethics committee for this research.

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