Pseudomonas syringae pv. *tomato* DC3000 Improves *Escherichia coli* O157:H7 Survival in Tomato Plants

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Recently, outbreaks of food-borne diseases linked to fresh produce have been an emerging public health concern worldwide. Previous research has shown that when human pathogens co-exist with plant pathogens, they have improved growth and survival rates. In this study, we have assessed whether *Escherichia coli* O157:H7 benefits from the existence of a phytopathogenic bacterium and the underlying mechanisms were further investigated. When *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) and *E. coli* O157:H7 were co-inoculated by either dipping or infiltration methods, the populations of *E. coli* O157:H7 increased; however, no effect was observed when type three secretion system (T3SS) mutants were used instead, suggesting that *E. coli* O157:H7 benefits from the presence of Pst DC3000. In addition, this study confirmed that the *E. coli* O157:H7 populations increased when they occupied the tomato leaf intercellular space; this colonization of the interior of the leaves was possible due to the suppression of the PAMP-triggered immunity (PTI) by Pst DC3000, in particular with the AvrPto effector. In conclusion, our data support a plausible model that *E. coli* O157:H7 benefits from the presence of Pst DC3000 via AvrPto suppression of the PTI resistance.

Keywords: AvrPto, *E. coli* O157:H7, effector, food-borne disease, *Pseudomonas syringae* pv. *tomato* DC3000, tomato

Introduction

In 1996, *Escherichia coli* O157:H7 infected more than 10,000 Japanese children who had eaten contaminated radish sprouts [1, 2]. In addition, spinach, lettuce, and tomato have been sources of major outbreaks of *E. coli* O157:H7 and *Salmonella*, in the United States [3]. These outbreak reports demonstrate that fresh produce can be a vehicle for pathogen transfer to humans. Thus, fresh produce contaminated with human pathogens has subsequently become a topical issue around the world. Plants may be contaminated with human pathogens during the pre-harvest as well as the post-harvest handling stages. In the pre-harvest stage, irrigation water from manure or animal feces are the major causes of contamination, whereas non-sanitary processing during transportation and bagging are the major sources of contamination in the post-harvest stage [1, 4]. However, the focus of previous research has

been on the risks of food-borne illness and contamination routes rather than the actual relationships between the human and plant pathogens.

Past studies found that when food-borne pathogens internally contaminate a plant, they can survive longer than when they are only on plant surfaces [4, 5]. Furthermore, food-borne pathogens are recognized by the plant immune responses [6, 7], and in cases where they co-exist with plant pathogens, they benefit from the behaviors of these same pathogens [8, 9]. There are relatively few reports that deal with the relationship between human and plant pathogens. Greater understanding of the biological interactions between humans and plant pathogens is indispensable to prevent outbreaks of human pathogens. In the present study, the internalization of *E. coli* O157:H7 in tomato plants, the enhancement of plant immunity, and the mechanisms from which *E. coli* O157:H7 benefits in the presence of a plant pathogenic bacterium have been investigated.

Materials and Methods

Bacterial Strains and Growth Conditions

Bacterial strains used in the current study are described in Table 1. The *E. coli* O157:H7 strain ATCC 43895 and a GFP-tagged version were grown on Luria-Bertani (LB) medium at 37°C for 16 h. For the count of *E. coli* O157:H7 from the inoculated plants, Eosin Methylene Blue (EMB) medium (Difco, USA) was used. *P. syringae* pv. *tomato* DC3000 (Pst DC3000) and its derivatives, and *P. fluorescens* (Pf) were cultured in King’s B (KB) at 28°C for 1 day. Antibiotics were added to the culture medium at the following concentrations: 50 µg/ml kanamycin (Km) for the *E. coli* O157:H7 GFP tagging strain; 20 µg/ml chloramphenicol (Cm) and 50 µg/ml ampicillin (Am) for Pf; 50 µg/ml rifampicin (Rif) for Pst; 50 µg/ml spectinomycin (Sp) for its derivative mutants.

Bacterial Inoculations and Population Tests

Fully expanded leaves from 5-week-old tomato (*Solanum lycopersicum* ‘Prime Alexander’) were used for the analysis of survival rates, the population size over time, and internal colonization by the *E. coli* O157:H7 strain. To prepare bacterial inoculum, the *E. coli* O157:H7 strain was incubated on solid LB medium and the cells were then suspended in 10 mM MgCl₂ at the concentration of 1 × 10⁷ CFU/ml with 0.02% (v/v) Silwet L-77 surfactant (Lehle Seeds Co., USA). Two leaflets on the third branch of each tomato plant were dip-inoculated in the bacterial suspension for 2 min. For infiltration, a bacterial suspension of 1 × 10⁵ CFU/ml was infiltrated into tomato leaves of the third branch using blunt syringes. Inoculated plants were kept at 26°C for 10 h of illumination. Leaf disks were sampled 10, 20, and 30 days post inoculation (DPI) from both dipped and infiltrated inoculations using a 1 cm-diameter cork borer. The samples were then ground in 1 ml 10 mM MgCl₂ using a plastic pestle. The samples were then diluted tenfold and 100 µl aliquots were plated on EMB medium and grown at 37°C overnight, after which the bacterial colonies were measured. To investigate *E. coli* O157:H7 populations in plants over time, bacterial suspensions of

1 × 10⁴ CFU/ml were directly infiltrated into tomato leaves and the bacterial colonies were then counted at 1, 2, 3, 4, and 5 DPI. To determine the internalization of *E. coli* O157:H7, one of the two leaves from each of the dip-inoculated plants described above, was sterilized with 70% ethanol for 20 s and then 0.5% sodium hypochlorite for 10 s and then washed with sterilized distilled water thrice for 5 min each time. Leaf disks were punched out using a 1 cm-diameter cork borer. The leaf disks were macerated with a plastic pestle, grown on medium and the bacterial colonies were counted as described above. The non-sterilized leaf was considered the negative control. The experiments were repeated three times with similar results.

For stomatal integration, the *E. coli* O157:H7 strain tagged with the green fluorescent protein (GFP) gene was grown to an OD₆₀₀ of 0.6 in LB broth and incubated for 12 h after the addition of 0.5 mM IPTG, then pelleted from the broth, and the cells were resuspended in 10 mM MgCl₂ to an OD₆₀₀ of 0.6 supplemented with 0.02% of Silwet L-77. Tomato leaves were dip-inoculated and then incubated for 2 h at room temperature, after which leaf pieces were excised and mounted with 70% glycerol in PBS buffer. GFP-labeled bacteria were visualized using a super sensitive high resolution confocal laser scanning microscope (Zeiss LSM 880, Germany). Fluorescent bacterial scanning using an excitation wavelength of 488_{nm} and a 502-534_{nm} emission filter were conducted.

Callose Deposition

A callose deposition assay for *Nicotiana benthamiana* was modified as follows from a previous report [10].

Co-Inoculation of *E. coli* O157:H7 with *P. syringae* pv. *tomato* DC3000

To test whether *E. coli* O157:H7 benefits from the co-inoculated plant pathogenic bacteria, we inoculated *E. coli* O157:H7 with Pst DC3000 and its derivatives that lack a type three secretion system (T3SS) and effectors. Concentrations of 1 × 10⁵ CFU/ml for each strain were mixed with 10 mM MgCl₂ and the mixture was infiltrated directly into the tomato leaves. At 1 DPI, disks were collected and *E. coli* O157:H7 was grown on an EMB plate as mentioned above. Besides the leaves, 1.0–1.5 cm immature fruits of tomato were also used for co-existence analysis. The immature fruits were cut into two halves and the round surfaces placed on sterilized glass slides above filter paper in glass petridishes, and 20 µl of mixture with both *E. coli* O157:H7 and Pst DC3000 at 1 × 10⁵ CFU/ml concentrations were dropped onto the upper and sliced surfaces. Immature fruits (0.1 g) were sectioned and used to assess bacterium populations using the same method described above.

The experiments were performed in three replications with 20 leaves in the leaf assay and 18 immature fruits in the fruit assay per replication. The experiments were repeated at least three times. Statistical significance was considered by analysis of variance (ANOVA) and Duncan’s multiple range test for *p* values = 0.05.

Table 1. Bacterial strains used in this study.

Strain	Genotype or relative phenotype
<i>Escherichia coli</i>	
O157:H7 ATCC 43895	Wild-type
GFP labeling	pBAV1K-t5-gfp; Km ^R
<i>Pseudomonas fluorescens</i> 55	
	Wild type Cm ^R , Am ^R
<i>P. syringae</i>	
pv. <i>tomato</i> DC3000	Wild-type Rif ^R
CUCPB5112	Δ <i>hrcQ</i> _b ::FRT; Sp ^R
CUCPB5535	Δ <i>avrPtoB</i> ; Rif ^R Ap ^R
CUCPB5543	Δ <i>avrPtoB/avrPto</i> ; Rif ^R Ap ^R
CUCPB5585	Δ28 effectors; Rif ^R Sp ^R

Results

E. coli O157:H7 Can Colonize in Tomato Leaves

Previous reports [11, 12] confirmed that *E. coli* O157:H7 can colonize and survive on the plant surface and in the interior of plants, including tomato skins, spinach, and lettuce leaves. We analyzed how long *E. coli* O157:H7 can survive in tomato leaves using both dip and infiltration-contamination methods. The study showed that *E. coli* O157:H7 populations were maintained for 30 days reaching 4.75 and 3.92 log CFU/disk, for each method respectively, which were similar to the initial concentrations from the dip and infiltration inoculation methods (Fig. 1A). These

results signify that *E. coli* O157:H7 is able to inhabit and survive for more than one month in a plant. Therefore, the infiltration inoculation was deemed suitable for further experiments to observe the populations of *E. coli* O157:H7 in plants. We infiltrated leaves with bacteria to analyze the population increases over time. As shown in Fig. 1B, the population increased at 1 DPI and the level achieved was maintained until 5 DPI, thus, 1 DPI was sufficient to have confidence of colonization of *E. coli* O157:H7 in the interior of leaves. In order to examine the internalization of *E. coli* O157:H7, we prepared non-disinfected and disinfected samples using ethanol and sodium hypochlorite. The populations of *E. coli* O157:H7 on the non-disinfected

Fig. 1. Populations of *E. coli* O157:H7 in tomato (*Solanum lycopersicum* 'Prime Alexander') leaves.

(A) Survival ratio of *E. coli* O157:H7 based on the time course until 30 days post inoculation for the dip and infiltrated-inoculations. (B) Increasing time points and the population of *E. coli* O157:H7 by dip-inoculation. (C) Populations of *E. coli* O157:H7 on the surface and in the interior of tomato leaves with and without surface disinfection. (D) Confocal image of GFP-tagged *E. coli* O157:H7 cells colonizing a tomato leaf with numerous proliferated cells around a stomate.

leaves were 4.05 log CFU/disk compared to the disinfected leaves at 3.59 CFU/disk (Fig. 1C). After 1 day, however, the populations on the disinfected leaves were found to be similar to the non-disinfected ones, suggesting that *E. coli* O157:H7 had colonized the interior of the tomato leaves. In addition, the GFP-labeled *E. coli* O157:H7 strain was observed near and seemed to be associated with the leaf stomata (Fig. 1D). The internal colonization of *E. coli* O157:H7 in the tomato leaves was subsequently thought to have entered through the stomata.

E. coli O157:H7 Induces the Immune Response in Plant

To test whether *E. coli* O157:H7 elicits immune responses in plants, we used callose deposition assays to assess for PAMP-Triggered Immunity (PTI) in leaves of *N. benthamiana*. The *P. fluorescence* 55 strain induced numerous calloses in *N. benthamiana*, but 10 mM MgCl₂ did not as indicated in Fig. 2A. *E. coli* O157:H7 induced a similar number of calloses to that of *P. fluorescence* (Fig. 2B), which was the representative strain used to induce callose deposition for PTI responses in plants [10].

Fig. 2. Callose deposition in *Nicotiana benthamiana*.

Leaves were syringe-infiltrated with *Pseudomonas fluorescence* 55, *E. coli* O157:H7, or 10 mM MgCl₂ buffer and assayed at the indicated times using aniline blue and fluorescence microscopy. A, Representative callose deposits of 1 mm² in field were captured using a Leica DMI 3000B. B, The mean number of callose deposits from three replicates.

P. syringae pv. *tomato* DC3000 Enhances the Ability of *E. coli* O157:H7 to Colonize in Tomato

Since *E. coli* O157:H7 was able to colonize the interior of tomato plants, we tested whether *E. coli* O157:H7 may benefit from the co-inoculated plant pathogenic bacteria. When *E. coli* O157:H7 was co-inoculated in leaves with mutants of Pst DC3000, which lacked the T3SS and 28 effectors, respectively, *E. coli* O157:H7 populations were not increased at 1 DPI (Fig. 3A). Whereas, *E. coli* O157:H7 co-inoculated with Pst DC3000 had significantly increased

Fig. 3. Population dynamics of *E. coli* O157:H7 inoculated with Pst DC3000 and its mutants on tomato leaves (A) and fruits (B).

Results shown are the mean number of counted *E. coli* O157:H7 cells from three replicates and the standard deviation from 20 leaves from three samples for each treatment $\pm$ standard error. Significant differences in populations compared with *E. coli* O157:H7 are indicated with an asterisk ($p = 0.05$).

populations (Fig. 3A). These findings were similar to those when the two bacteria were inoculated in immature fruits of tomato. Moreover, the recovered populations of *E. coli* O157:H7 from immature fruits were higher than the population in leaves. These results suggest that the survival ability of *E. coli* O157:H7 is enhanced when associated with the plant pathogenic bacterium, Pst DC3000. The plausible explanation is likely due to the suppression of the plant immune system evoked by *E. coli* O157:H7 and Pst DC3000. Additionally, *E. coli* O157:H7 may be more easily adopted into fruits than leaves, suggesting that contact with contaminated sources during the pre-harvest season must be avoided for food safety.

E. coli O157:H7 Can Propagate in Plants by Avoiding PTI Via the *avrPto* of *P. syringae* pv. *tomato* DC3000

In this study, Pst DC3000 had a key role in enhancing the capability of *E. coli* O157:H7 to survive in tomato plants via avoiding the plant immunity response. However, it was still unclear which mechanism was helping *E. coli* O157:H7 to survive in the plant. The mutants of T3SS and those with the 28 effector deletions did not result in increased populations of *E. coli* O157:H7 and it was thought that the roles of the deleted effectors may be to suppress plant immunity. To examine this hypothesis, we used the effectors *AvrPto* and *AvrPtoB*, which are known to suppress PTI. When *E. coli* O157:H7 was co-inoculated with the wild type of Pst DC3000 and $\Delta avrPtoB$ in tomato leaves and fruits, *E. coli* O157:H7 populations were significantly increased but not with $\Delta 28$ effectors and $\Delta avrPtoB/avrPto$ double mutants (Figs. 4A and 4B). Thus, we concluded that *E. coli* O157:H7 benefitted from *AvrPto* suppression of PTI.

Discussion

In this study, survival periods of *E. coli* O157:H7 were estimated for 30 DPI for both the dip and infiltration-inoculation methods. It was previously found that human pathogenic bacteria, particularly *E. coli* O157:H7 and *S. enterica* serovar Typhimurium, were able to survive for 30 and 25 days when inoculated on lettuce and tomato plants using dip and spray inoculation methods, respectively [5, 13]. Thus, the survival rate of 30 days in tomato leaves was considered reasonable in our inoculation regime. The ability of *E. coli* O157:H7 to enter into the tomato plant via the stomata and colonize it internally was confirmed by confocal observations and disinfection analysis with 70% ethanol and 0.5% hypochlorite, in agreement with previous studies on entero-aggregative *E. coli* [11] and *S. enterica* [5].

Fig. 4. Population dynamics of *E. coli* O157:H7 inoculated with Pst DC3000 and its *AvrPtoB* and *AvrPtoB/AvrPto* double mutants on tomato leaves (A) and fruits (B).

Results shown are the mean number of counted *E. coli* O157:H7 cells from three replicates and standard deviations from 18 fruits from three samples for each treatment, $\pm$ standard error. Significant differences in populations between the *E. coli* O157:H7 and mutants of Pst DC3000 are indicated with asterisks ($p = 0.05$).

Therefore, we concluded that the stomata may be the critical site for *E. coli* O157:H7 to enter the tomato plant and further grow in the apoplast space. This allows the bacteria to avoid external stresses such as UV irradiation and desiccation; this behavior is similar to that of *S. enterica* [14] whose population was maintained in this manner for more than one month, which was enough to lead to contamination of a subsequent host.

In earlier studies, *E. coli* O157:H7 did not induce plant

immunity [15]. However, on *Arabidopsis*, the population of the *E. coli* O157:H7 flagella mutant strain was higher than the wild type population, indicating that the flagella of the *E. coli* O157:H7 wild type were recognized by *Arabidopsis* [7]. In our study, *E. coli* O157:H7 induced numerous callose depositions, on a level similar to those induced by *P. fluorescens* 55. Callose depositions were considered to indicate cell wall-based defense activation following the perception of PAMPs (Pathogen-Associated Molecular Patterns) including flagellin, elongation factor Tu, lipopolysaccharides, and glycoproteins as a preliminary defense of the PTI in plants [16, 17]. The population of *E. coli* O157:H7 on dip-inoculated leaves declined gradually over time, which may be attributed to plant responses triggered by perceptions of PAMPs, in particular with flagella of *E. coli* O157:H7 (Fig. 1A). However, we could not detect stomatal closure to prevent bacteria entering the plant due to *E. coli* O157:H7 exposure, as has been previously reported [6]. The population tests and callose depositions observed in the current study supported the idea that *E. coli* O157:H7 is capable of entering the plant epidermis via the stomata and growing endophytically, and PTI is triggered in the process of penetration by bacteria like *Salmonella* [8]. However, *E. coli* O157:H7 that multiplied or was sustained in the tomato leaves did not cause any symptoms after its populations were detected at concentrations of at least log 4 and 6 CFU/ml in the case of infiltrated and dip-inoculated leaves, suggesting that *E. coli* O157:H7 triggers PTI, not ETI (effector-triggered immunity) in agreement with previous reports that human pathogens did not have T3SS (type III secretion system) to inject effectors into plant cells [4].

When PTI was triggered by *E. coli* O157:H7, the population of *E. coli* O157:H7 in the tomato declined slightly over time, whereas *E. coli* O157:H7 populations were significantly increased when they were co-inoculated with Pst DC3000. Similarly, previous studies reported the key role of phytopathogens for the survival of human pathogens. Meng and colleagues [8] reported that Pst DC3000 was able to significantly enhance the growth of *Salmonella* in *N. benthamiana* and also co-inoculations with *Xanthomonas perforans* influenced *Salmonella* colonization in tomato phyllospere [18]. Furthermore, populations of *E. coli* O157:H7 and *Salmonella* were increased in necrotic lesions on leaves of romaine lettuce caused by the downy mildew pathogen, *Bremia lactucae* [9]. In addition, the finding that populations of *E. coli* O157:H7 were not increased by co-inoculation with Δ T3SS and Δ 28E mutants supports the hypothesis that human pathogens may benefit from

association with plant pathogenic bacteria via the suppression of immune responses by the T3SS effectors, which were delivered into plant cells by plant pathogens [8].

AvrPto and AvrPtoB effectors were chosen for this study because of their modes of action to circumvent plant defenses [19–22]. Two effector proteins can share the same host targets and also manipulate multiple host factors [23]. For example, AvrPto suppresses the kinase activity of FLS2 and EFR, and AvrPtoB that includes an E3 ubiquitin ligase domain can inhibit many PRRs such as FLS2, FEN, CERK1, and Bti9 [24]. The results showed that the growth of *E. coli* O157:H7 co-inoculated with Δ avrPtoB was similarly increased to that of the wild type but not Δ avrPtoB/avrPto, suggesting that the mechanism of AvrPto is important for *E. coli* O157:H7 to colonize plants. Although we did not perform a complementation test for these effectors in the preset study, AvrPto helps survival of *E. coli* O157:H7 in tomato plant; therefore, AvrPto and AvrPtoB must be complementary to those deletion mutants and the 28 effectors deletion mutant in future study. Additionally, the growth of *E. coli* O157:H7 in fruits was higher than in leaves. The pH of immature and mature fruits is 6.7 and 4.4 respectively [25] but, does not seem to affect the growth of *E. coli* O157:H7 as it was found to be able to survive in epidermal fruit tissues in which the pH was neutral. This highlights the contamination risks present with tomato fruits. Therefore, food-borne illness outbreaks are possible if raw fresh produce contaminated with human pathogens is consumed.

Based on our data, *E. coli* O157:H7 can induce PTI, an early immune response in plants, in the process of penetration and colonization of the plant and can propagate by avoiding the PTI with the assistance of Pst DC3000, via its effector, AvrPto. Thus, a better understanding of the relationships between plant and human pathogens will help to develop effective strategies in reducing food-borne illnesses and maintaining human health.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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