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FOOD MICROBIOLOGY

Effect of Plant Systemic Resistance Elicited by Biological and Chemical Inducers on the Colonization of the Lettuce and Basil Leaf Apoplast by *Salmonella enterica*

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ABSTRACT Mitigation strategies to prevent microbial contamination of crops are lacking. We tested the hypothesis that induction of plant systemic resistance by biological (induced systemic resistance [ISR]) and chemical (systemic acquired resistance [SAR]) elicitors reduces endophytic colonization of leaves by *Salmonella enterica* serovars Senftenberg and Typhimurium. *S.* Senftenberg had greater endophytic fitness than *S.* Typhimurium in basil and lettuce. The apoplastic population sizes of serovars Senftenberg and Typhimurium in basil and lettuce, respectively, were significantly reduced approximately 10- to 100-fold by root treatment with microbial inducers of systemic resistance compared to H_2O treatment. *Rhodotorula glutinis* effected the lowest population increases of *S.* Typhimurium in lettuce and *S.* Senftenberg in basil leaves, respectively 120- and 60-fold lower than those seen with the H_2O treatment over 10 days postinoculation. *Trichoderma harzianum* and *Pichia guilliermondii* did not have any significant effect on *S.* Senftenberg in the basil apoplast. The chemical elicitors acidobenzolar-*S*-methyl and DL-β-amino-butyric acid inhibited *S.* Typhimurium multiplication in the lettuce apoplast 10- and 2-fold, respectively, compared to H_2O-treated plants. All ISR and SAR inducers applied to lettuce roots in this study increased leaf expression of the defense gene *PR1*, as did *Salmonella* apoplastic colonization in H_2O-treated lettuce plants. Remarkably, both acidobenzolar-*S*-methyl upregulation and *R. glutinis* upregulation of *PR1* were repressed by the presence of *Salmonella* in the leaves. However, enhanced *PR1* expression was sustained longer and at greater levels upon elicitor treatment than by *Salmonella* induction alone. These results serve as a proof of concept that priming of plant immunity may provide an intrinsic hurdle against the endophytic establishment of enteric pathogens in leafy vegetables.

IMPORTANCE Fruit and vegetables consumed raw have become an important vehicle of foodborne illness despite a continuous effort to improve their microbial safety. *Salmonella enterica* has caused numerous recalls and outbreaks of infection associated with contaminated leafy vegetables. Evidence is increasing that enteric pathogens can reach the leaf apoplast, where they confront plant innate immunity. Plants may be triggered for induction of their defense signaling pathways by exposure to chemical or microbial elicitors. This priming for recognition of microbes by plant defense pathways has been used to inhibit plant pathogens and limit disease. Given that current mitigation strategies are insufficient in preventing microbial contamination of produce and associated outbreaks, we investigated the effect of plant-induced resistance on *S. enterica* colonization of the lettuce and basil leaf apoplast in order to gain a proof of concept for the use of such an intrinsic approach to inhibit human pathogens in leafy vegetables.

KEYWORDS enteric pathogen, foodborne, produce, plant defense, plant immunity, biocontrol, control, herbs, leafy greens, outbreaks

Editor Johanna Björkroth, University of Helsinki

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Received 10 June 2021
Accepted 28 September 2021
Accepted manuscript posted online 6 October 2021
Published 24 November 2021

Salmonella enterica is one of the main causal agents of produce-associated outbreaks (1). Among 12 food categories, 17.6% of salmonellosis outbreaks are linked to produce (2). Leafy greens (including lettuce) and herbs (including basil) ranked among the top six produce vehicles of foodborne outbreaks in the United States from 1996 to 2014 (3). In particular, *S.* Senftenberg has been associated with outbreaks linked to basil in Europe and the United States (4), and large recalls of fresh basil in the United States and of dried basil in Canada and the United States (Canadian Food Inspection Agency, 2012 [http://www.fda.gov/Safety/Recalls/]). Salmonellosis has been linked also to the contamination of lettuce and other leafy greens with *S.* Typhimurium in Europe and the United States (5–7). *S. enterica* has the ability to multiply epiphytically on leaves under warm and wet conditions in the phyllosphere (8, 9). The internalization of the pathogen into leaf tissue may occur in the presence of free water through stomata and other natural openings (10, 11). This provides the bacterial cells access to the plant apoplast, where they may interact with plant cells directly, unimpeded by the cuticle barrier of the phylloplane.

Although in most cases plants do not show any disease symptoms after inoculation with *S. enterica*, leaf chlorosis and wilting in *Arabidopsis thaliana* (12, 13) and plant biomass reduction in lettuce (14) have been reported. Evidence is increasing that *S. enterica* may confront the plant immune system upon interaction with plant cells (13, 15–17). The innate immune system of plants depends on a diverse assortment of cell surface and cytoplasmic receptors that respond to invading pathogens (18). These receptors are commonly classified into a group that recognizes conserved microbial signatures and a group that recognizes highly variable effectors; thus, these two systems are referred to as pathogen- or microbe-associated molecular pattern (PAMP or MAMP)-triggered immunity (PTI or MTI) and effector-triggered immunity (ETI), respectively (19, 20).

PAMP activation in *A. thaliana* by *S. enterica* flagellin has been reported (21). While current knowledge does not support that *S. enterica* can translocate effectors into plant cells via its type 3 secretion system (T3SS) (22), mutants in T3SS components of SPI-1 and SPI-2 displayed reduced endophytic colonization of *A. thaliana* (17, 21) and altered induction of lettuce stomatal response to *S. enterica* ingression (23). Melotto and coworkers provided evidence that both *E. coli* O157:H7 and *S. enterica* Typhimurium modulate stomatal immunity (24) and that the latter pathogen induces stomatal responses depending on plant species and environmental conditions (25). Other mechanisms of plant basal immunity involve the production of reactive oxygen species and nitric oxide, as well as callose deposition, and these also play a role in the leaf apoplast colonization by enteric pathogens (26–28).

Plant innate immune responses may be activated chemically or biologically by inducing systemic signaling cascades that effect resistance in distal plant parts not infected or colonized by a phytopathogen or microbe. This systemic plant resistance relies on phytohormone signaling pathways. Systemic acquired resistance (SAR) is mediated by salicylic acid (SA)-dependent processes and is associated with the production of PR proteins; on the other hand, induced systemic resistance (ISR) is a response to colonization of plant roots by plant growth-promoting bacteria and fungi and is generally mediated by the jasmonic acid (JA) and ethylene pathways (29–31). Because they are rooted in broad immunity to plant disease, SAR and ISR exhibit long-lasting nonspecific resistance effective against a wide range of organisms.

The effect of SAR and ISR on the growth and survival of human enteric pathogens in plants remains unknown. In this study, we tested whether induction of systemic resistance can reduce colonization of leafy vegetables by human pathogens. We investigated this as-yet-unexplored aspect of plant-enteric pathogen interactions by treating lettuce and basil plants with inducers of SAR and ISR and testing their effect on the apoplastic population levels of *S. enterica*. We also assessed the expression of marker genes belonging to defense signaling pathways to further our understanding of *S. enterica*-plant interactions upon elicitation.

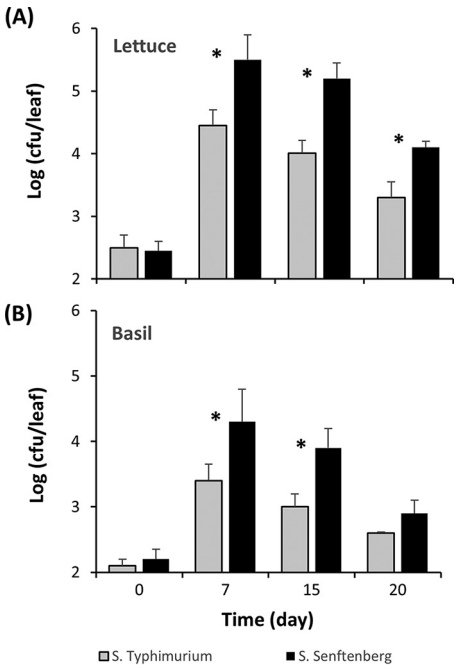

FIG 1 Endophytic population change of *S.* Typhimurium (gray bars) and *S.* Senftenberg (black bars) in leaves of lettuce (A) and basil (B) plants over time after passive apoplast inoculation. Bars represent the means of the $\log_{10}$-transformed population sizes for three replicate leaves sampled from three replicate pots, and SEM. *, Significant difference in population sizes between the two serovars (Student *t* test, $P < 0.05$).

RESULTS

***Salmonella* endophytic fitness in lettuce and basil leaves.** Various *Salmonella* serovars were tested for their ability to colonize lettuce and basil plants endophytically. Serovar Senftenberg strain 127468 colonized leaves of lettuce and basil plants better than serovar Typhimurium strain 14028S, displaying significantly greater endophytic population sizes over a period of 15 to 20 days after inoculation (Student *t* test; $P < 0.05$) (Fig. 1A and B). Serovar Senftenberg also colonized the basil leaf apoplast at significantly greater densities at 10 and 20 days postinoculation than did serovars Montevideo strain 125473, Enteritidis strain 124655, Infantis strain 122798, and Dublin strain 135157 (Tukey's multiple-comparison test; $P < 0.05$). *S.* Senftenberg population sizes were 16.0 and 13.0 times greater than those of *S.* Dublin at 10 and 20 days postinoculation, respectively (Fig. 2).

Effect of ISR and SAR inducers on endophytic colonization of lettuce and basil leaves. Plants were treated by soil drench with inducers prior and after *Salmonella* inoculation and their effect on endophytic populations of the human pathogen in leaves was assessed. In lettuce, all four ISR inducers had a significant effect on *S.* Typhimurium colonization 10 days postinoculation (Tukey's multiple-comparison test, $P < 0.05$) (Fig. 3A). *R. glutinis* Y13-treatment inhibited *S.* Typhimurium in lettuce the most effectively, with a 120.2-fold decrease in density over that in control (H_2O-treated) plants, compared to 7.1-, 10.7-, and 28.2-fold decreases by treatment with *Trichoderma harzianum* T39, *Pichia guilliermondii* S2, and *B. subtilis* Mel16, respectively. In basil, treatment with *R. glutinis* Y13 and *B. subtilis* Mel16 caused the lowest *S.* Senftenberg population sizes of all four ISR inducers, as revealed by a 63.1- and 10.0-fold differences compared to that in the control plants (Tukey's multiple-comparison test, $P < 0.05$) (Fig. 3B).

Lettuce plants were also pretreated with the SAR inducers Bion and BABA. The effect of the inducers on *S.* Typhimurium endophytic populations was measured in a short colonization time course to assess whether they may inhibit *Salmonella* multiplication. Due to this short colonization period, vacuum infiltration was used rather than passive internalization in order to obtain apoplast inoculum densities that were sufficiently high to prevent stochastic effects, thus reducing leaf-to-leaf variability, but also

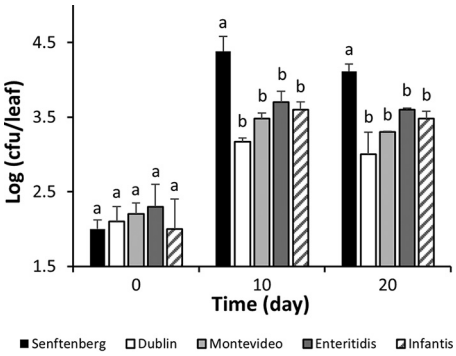

FIG 2 Endophytic population change of *S. enterica* serovars Senftenberg, Dublin, Montevideo, Enteritidis, and Infantis in the leaves of basil plants immediately and 10 and 20 days after passive apoplast inoculation. Bars represent the means of the $\log_{10}$-transformed population sizes for five replicate leaves sampled from five replicate pots, as well as the SEM. Different letters above the bars indicate significant differences in *Salmonella* population size within each incubation time by Tukey's multiple-comparison test ($P < 0.05$).

sufficiently low to enable for amplification of bacterial multiplication. Endophytic multiplication after vacuum infiltration of lettuce plants (without the aid of a surfactant) with low inoculum levels in the mesophyll of approximately 1×10^4 CFU/leaf was evidenced by an increase in *S.* Typhimurium population sizes in the control plants (H_2O treatment) of as much as 53-fold (Fig. 4). Population sizes of this human pathogen showed little or no further increase in the apoplast of the control plants by 48 h. While *S.* Typhimurium multiplied endophytically in all treatments, the population sizes were significantly lower already at 24 h postinoculation in the leaves of plants treated with low concentrations of SAR inducers (Bion [0.01%] and BABA [0.8 mg/ml]), than in the

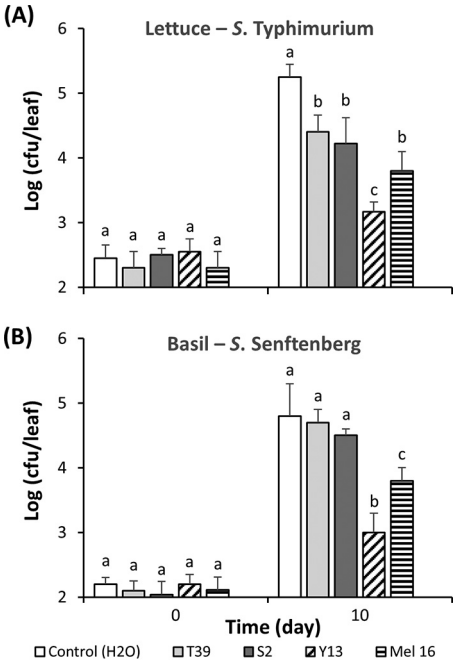

FIG 3 Effect of ISR-inducing microorganisms on endophytic colonization of lettuce leaves by *S.* Typhimurium (A) and of basil leaves by *S.* Senftenberg (B). Soil drench treatments were performed with H_2O (control) (white bars), *T. harzianum* T39 (light gray bars), *P. guilliermondii* S2 (dark gray bars), *R. glutinis* Y13 (crosshatched bars), and *B. subtilis* Mel 16 (striped bars). Assessment of endophytic population sizes was performed immediately and 10 days after passive apoplast inoculation with *Salmonella*. Bars represent the means of the $\log_{10}$-transformed population sizes of three replicate leaves sampled from three replicate pots, as well as the SEM. Different letters above bars illustrate significant difference in population sizes as per Tukey's multiple-comparison test ($P < 0.05$).

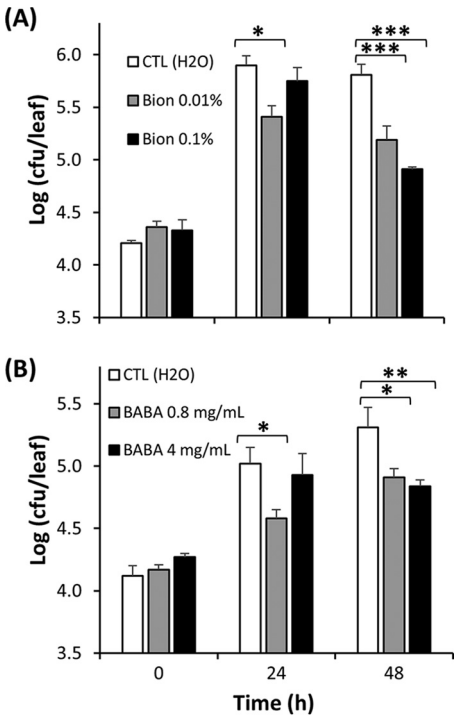

FIG 4 Effect of Bion (A) and BABA (B) at different concentrations as 24-h preinoculation treatments by root drench on the endophytic multiplication of *S.* Typhimurium in leaves of young potted romaine lettuce plants inoculated by vacuum infiltration. Data represent the means of the log-transformed population sizes of the pathogen for two, three, and four replicate leaves sampled from three replicate pots immediately, and 24 and 48 h after inoculation, respectively, and SEM. *, **, and *** indicate significant differences ($P < 0.05$, 0.01, and 0.001, respectively) in *Salmonella* population sizes between elicitor- and H_2O-treated plants, as determined by Dunnett's multiple-comparison test.

control plants treated with H_2O (Dunnett's multiple-comparison test; $P < 0.05$) (Fig. 4). At 48 h postinoculation, *S.* Typhimurium population sizes in the inducer-treated plants were significantly lower than in control plants, irrespective of the inducer concentration (Dunnett's multiple-comparison test); at the highest concentrations of Bion and BABA, the population sizes were 0.11- and 0.55-fold, respectively, that observed in control plants. However, no significant effect of inducer dosage was observed (Fig. 4).

Expression of plant defense genes in lettuce treated with SAR and ISR inducers. Table 1 shows the expression of genes involved in SA and JA signaling pathways in lettuce leaves 24 h after plants were treated with 0.1% Bion or 4 mg/ml BABA compared to that in control plants (H_2O treated). Bion induced expression of SAR marker genes

TABLE 1 Comparative transcript abundance of defense genes in romaine lettuce leaves following plant treatment with Bion and BABA treatment relative to the control treatment (H_2O)

	Mean comparative expression (SEM)[a]	
Gene	**Bion**	**BABA**
PR1	3.27 (1.05)	3.89 (1.11)
PR2	5.27 (1.23)	ND
EDS1	5.20 (2.00)	ND
PAL2	3.07 (1.42)	ND
FLS2	2.28 (1.03)	ND
ERF	BD	3.73 (1.19)
DEF	BD	4.99 (1.06)

[a]Bion and BABA were applied by soil drench at 0.1% and 4 mg/ml, respectively, and leaves sampled for gene expression 24 h after treatment. Comparative expression was measured by RT-qPCR and is shown as fold change over expression in H_2O-treated plants. The data represent the means for three replicate leaves. BD, below detection level; ND, not determined.

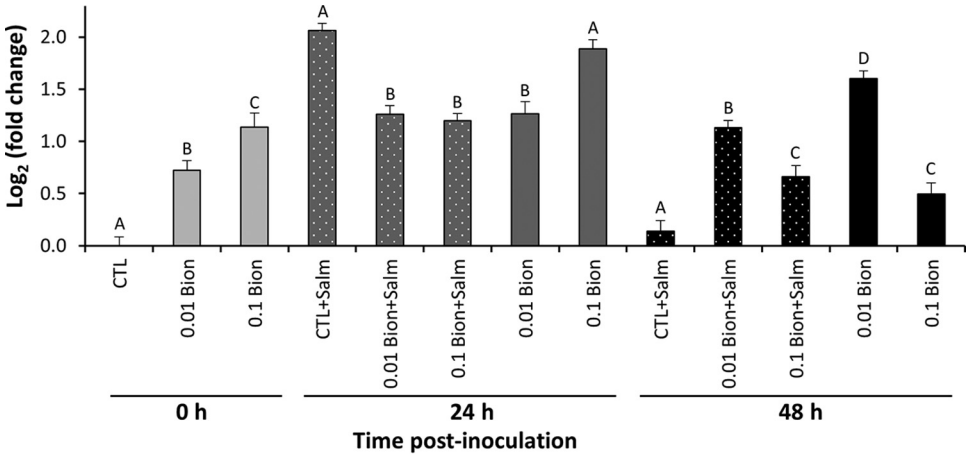

FIG 5 Expression of the SAR marker *PR1* in leaves of lettuce plants pretreated with 0.01 or 0.1% Bion, or H_2O (control), as a root drench 24 h before vacuum infiltration with *S.* Typhimurium or with H_2O (0 h). *PR1* expression at 24 h and 48 h is shown as $\log_2$-fold change compared to that in leaves of mock-inoculated control (H_2O-treated) plants at 0 h. Expression in inoculated leaves is denoted by +Salm. Solid bars, plants treated with Bion or H_2O but noninoculated. Solid bars with dots, plants treated with Bion or H_2O and inoculated with *S.* Typhimurium. Data represent the means for three replicate leaves sampled from three replicate pots, as well as the SEM. Different letters above bars illustrate significant difference within each sampling time as determined by Tukey's multiple-comparison test ($P < 0.05$).

PR1, *PR2*, and *EDS1* greater than 3-fold relative to the control, while PAL2 and FLS2 were also upregulated. In contrast, the expression of *ERF* and *DEF*, markers for the ethylene and JA signaling pathways, was below the detection limit in Bion-treated plants but was induced 3.73- and 4.99-fold, respectively, upon BABA treatment compared to the control. BABA-treated plants also had 3.89-fold greater expression of *PR1* than control plants. The mechanism of BABA-induced plant immunity is broader than that of Bion-mediated SAR and may result from the interplay of several signaling pathways (32, 33).

Figure 5 illustrates the comparative transcription of *PR1* in leaves of lettuce plants treated with 0.01 and 0.1% Bion versus an H_2O-treated control at various times after infiltration with *S.* Typhimurium or with H_2O, as measured by RT-qPCR. *PR1* increased in expression 24 h after treatment with Bion in a dose-dependent manner since its transcription at that time (0 h) was greater after SAR induction with 0.1% than 0.01% Bion (Tukey's multiple-comparison test; $P < 0.05$). After infiltration with H_2O (mock-inoculated plants), *PR1* expression was the highest 48 h after treatment with 0.1% Bion (24 h after inoculation), with a difference of 3.7-fold compared to control plants prior to infiltration. Inoculation with *S.* Typhimurium and subsequent endophytic multiplication increased *PR1* expression 4.1-fold 24 h postinoculation compared to transcriptional levels in control plants prior to inoculation, thus to a similar extent as treatment with 0.1% Bion at that time. Remarkably, the expression of *PR1* was 1.8- and 1.6-fold lower in leaves of plants pretreated with 0.1% Bion and colonized endophytically by *S.* Typhimurium for 24 h than in leaves of untreated plants inoculated with the pathogen and in leaves of plants treated with 0.1% Bion that were mock inoculated, respectively. Thus, *PR1* upregulation by either the pathogen or Bion appeared to be suppressed by the activity of the other. However, the high expression of *PR1* triggered by *S.* Typhimurium colonization (in the control H_2O-treated plants) was not sustained and at 48 h postinoculation returned to preinoculation levels (0 h). In contrast, the enhanced transcriptional activity of *PR1* persisted in Bion-treated plants for up to 48 h postinoculation (72 h posttreatment; Tukey's multiple-comparison test; $P < 0.05$).

The ability of *S.* Typhimurium to suppress *PR1* transcription in lettuce plants primed for basal defense was further tested by elicitation with *B. subtilis* Mel16 and *R. glutinis* Y13. While ISR is commonly attributed to the activity of the JA pathway, additional involvement of *PR1* has been described in lettuce and other species (34, 35), and SAR is

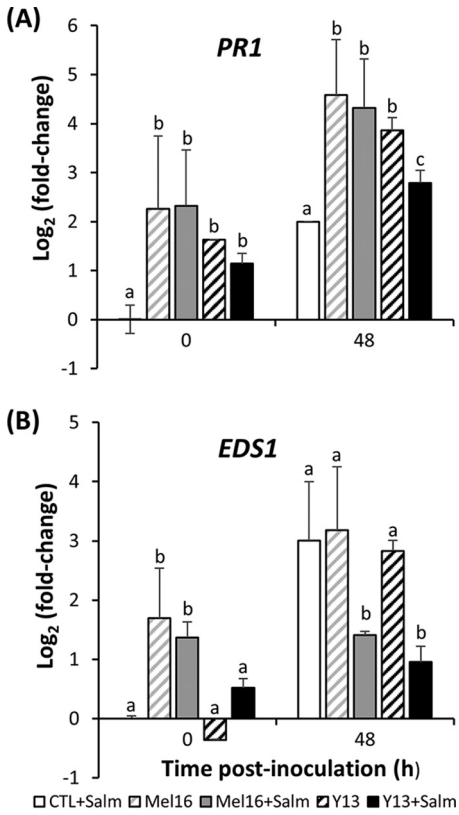

FIG 6 Relative expression of SA-pathway genes in lettuce leaves in response to root treatment with H_2O (control) (white bar), *B. subtilis* Mel16 (gray bars, crosshatched or solid), or *R. glutinis* Y13 (black bars, crosshatched or solid), followed by leaf apoplast mock inoculation with H_2O (crosshatched bars) or inoculation with *S.* Typhimurium (solid bars). Gene expression in inoculated leaves is denoted by +Salm. *PR1* (A) and *EDS1* (B) transcript quantification by RT-qPCR was carried out in five replicate leaves sampled from five replicate pots 3 days after root treatment at 0 h and 48 h after mock inoculation or inoculation with *S.* Typhimurium. Expression is shown relative to the transcriptional level in mock-inoculated control (H_2O treated) plants at 0 h. Different letters above bars illustrate significant difference in relative gene expression within each time point as per Tukey's multiple-comparison test ($P < 0.05$).

known to be triggered by both biological and chemical inducers (36). Figure 6A illustrates the relative expression of *PR1* in lettuce plants at 3 days after root treatment and before apoplast inoculation with *S.* Typhimurium or mock inoculation with H_2O (0 h), as well as 48 h thereafter. Microbial elicitation caused an increase in PR1 expression in lettuce leaves compared to the case in H_2O-treated plants by the time of inoculation (0 h), and this induction was sustained 48 h later. Endophytic colonization of the leaves by *S.* Typhimurium in the absence of treatment with an elicitor of plant resistance increased *PR1* expression 4-fold by 48 h postinoculation compared to preinoculation levels (0 h) (Fig. 6A), as we observed also in experiments with Bion (Fig. 5). However, this transcriptional increase was lower than that observed in plants that had been treated with *B. subtilis* Mel16 and *R. glutinis* Y13, whether these were inoculated with the human pathogen or mock inoculated (Tukey's multiple-comparison test; $P < 0.05$). Furthermore, in *R. glutinis*-treated plants, colonization by *S.* Typhimurium significantly decreased *PR1* transcription levels by 48 h postinoculation, whereas no effect of the pathogen was detected in *B. subtilis*-treated plants.

As observed for *PR1*, the expression of *EDS1* was increased significantly by the presence of *S.* Typhimurium in the leaf apoplasts of unelicited plants (control) (Fig. 6B). While the leaves of plants elicited with *B. subtilis* Mel16 and *R. glutinis* Y13 showed enhanced *EDS1* transcription at 48 h after mock inoculation and inoculation with *S.* Typhimurium, this transcriptional activity was lower in leaves colonized by the

pathogen compared to that observed in mock-inoculated leaves (Tukey's multiple-comparison test; $P < 0.05$).

DISCUSSION

While good agricultural practices and postharvest sanitization contribute to improving produce safety, mitigation strategies to inhibit foodborne pathogens on produce are greatly lacking. Approaches based on the enhanced capability of plants to respond to the presence of enteric pathogens and inhibit their colonization may provide an inherent *in planta* first step to minimize preharvest contamination of crops. The use of plant breeding to enhance microbial produce safety has been proposed (37). This approach is based on the premise that plant traits related to the physicochemical nature of plants as a bacterial habitat or to plant innate immune responses that inhibit enteric pathogens may be selected for in breeding programs to lower microbial contaminant loads in produce and reduce the risk of human infection.

For their presence to be sensed by the plant immune system, microbes must breach or bypass the cuticle barrier of the leaf to come into direct contact with plant cells in the apoplast. We show here that the ability of *Salmonella* to get established and survive in the plant apoplast varies among strains of different serovars. In both lettuce and basil plants, *S.* Senftenberg strain 127468 colonized leaves endophytically at greater population densities than *S.* Typhimurium 14028s. *S.* Senftenberg also displayed greater apoplastic population sizes than strains of serovars Montevideo, Enteritidis, Infantis, and Dublin in basil leaves 10 and 20 days after inoculation. Infiltration of *Arabidopsis thaliana* leaves with *S.* Senftenberg and *S.* Typhimurium strain SL1344 failed to reveal any difference in colonization between the two serovars since their population sizes remained stable after inoculation, although the former strain caused leaf chlorosis and wilting, while the latter did not (12). Plant disease symptoms were not observed in basil or lettuce in our study. On the other hand, we observed in this study, increases in endophytic population sizes of approximately 10- to 50-fold within 24 to 48 h after infiltration into lettuce leaves for *S.* Typhimurium and also for *S.* Senftenberg (data not shown), indicating that active *Salmonella* population growth can occur in the leaf apoplast when inoculum levels are low. Such active bacterial multiplication may allow for differences in the interaction of various *Salmonella* strains with plants during colonization. Delaquis and coworkers demonstrated differences in the colonization of two lettuce genotypes by 43 *S. enterica* strains from 29 serovars (38), indicating that bacterial and plant factors may affect the outcome of *Salmonella*-plant interactions.

Mechanisms of plant immunity rely broadly on phytohormone signaling pathways. These signals can effect long-distance resistance away from the infection site and be triggered by chemical elicitors (SAR) or biological inducers (ISR) (39, 40). Although enteric pathogens by themselves have the ability to elicit plant defense responses, induced plant resistance via SAR and ISR may potentiate inhibition against these microbial contaminants and hence, crop safety. Of the four microbial strains inoculated onto roots prior to inoculation of the leaves with *Salmonella*, the yeast *R. glutinis* Y13 caused the greatest inhibition of plant colonization in both serovars, with reductions of 63- and 120-fold in *Salmonella* endophytic population sizes in basil and lettuce apoplasts, respectively, compared to H_2O-treated plants. *R. glutinis* suppresses fungal diseases on a variety of crops (41, 42). Increased activity of the PAL, PPO, and POD enzymes, which are involved in the production of plant secondary metabolites that have antimicrobial activity, was reported in tomato wounds upon treatment with *R. glutinis* (43). Increased PPO expression, as well as the production of phenolic compounds, was observed after exposure of basil plants to methyl-JA, an analog of the JA signal that is involved in ISR (44). Furthermore, treatment with *B. subtilis*, an effective ISR-inducing microorganism, similarly enhanced PAL expression in rice cell cultures (45), and significantly reduced *Salmonella* colonization on basil and lettuce in this study. One could hypothesize that enhanced production of phenolic compounds via increased PAL and related enzymatic activity (PPO and POD) as a result of root colonization by *R. glutinis* and *B. subtilis* inhibited endophytic *Salmonella* cells in the aerial

parts of basil and lettuce. In addition, the production of cyclic lipopeptides by *B. subtilis* and other species is involved in the establishment and maintenance of ISR, thereby protecting many plant species from a wide variety of pathogens, including leaf-spot bacterial pathogens (34, 45, 46).

The ISR-inducing microorganisms *T. harzianum* T39 and *P. guilliermondii* S2 had a smaller, but significantly inhibitory effect on *S.* Typhimurium apoplastic colonization of lettuce. Various species of *Trichoderma* have been shown to protect crops against fungal diseases and this endophytic fungus has been implicated in induced resistance through a spectrum of strategies (47, 48). In lettuce, the biocontrol of leaf spot diseases by *T. asperellum* T1 was mediated by the enhanced activity of defense-related enzymes such as PPO and POD (49). The induced resistance by *Pichia* spp. has been poorly described but may proceed via several signals, including ethylene, JA, and SA signal transduction pathways (50). Although ISR has been investigated in great part for the control of fungal diseases, its commonly observed enhanced synthesis of phenolic and oxidative species in plants suggests a mode of action in the inhibition of endophytic leaf colonization by *Salmonella* observed here.

Bion and BABA, two common chemical inducers of SAR that are effective against a wide range of plant diseases (36, 51), were also tested as soil drenches in our system, but their effect was investigated over a shorter time after *S.* Typhimurium vacuum infiltration of lettuce leaves. Both elicitors significantly inhibited multiplication of the pathogen after infiltration compared to the control treatment (soil drench with H_2O), although only the lower concentrations exerted a more immediate inhibition at 24 h postinoculation. Bion has been shown to protect lettuce from bacterial infection by the leaf spot pathogen, *Xanthomonas campestris* pv. vitians (52) and basil from downy mildew disease (53). In our preliminary experiments, 0.03% Bion as soil drench lowered the population sizes of *S.* Senftenberg in the basil apoplast by 6.2-fold compared to H_2O (control) treatment by 10 days postinoculation (data not shown). While BABA provided good control of the oomycete pathogen causing downy mildew in lettuce (54), it also reduced disease symptoms by the bacterial pathogens *Pseudomonas syringae* pv. tomato (55) and *Pectobacterium carotovorum* in *A. thaliana* leaves (56). In our system, *S.* Typhimurium inhibition by BABA and Bion was approximately in the same range as BABA inhibition of bacterial pathogens in *A. thaliana* leaves (55, 56). It is unclear if their effect would have been greater, perhaps in the same range as the effect caused by some ISR strains that we report herein, had longer periods of *S.* Typhimurium colonization been tested.

The time course of *PR1* expression in Bion-treated lettuce plants revealed that this SAR marker gene was induced in lettuce leaves in a dose-dependent manner 24 h after soil drench (as assessed at the time of inoculation), thus confirming that our system was functional. Expression continued to increase in mock-inoculated plants compared to that before inoculation. However, in elicited plants that were also inoculated with *S.* Typhimurium, the change in *PR1* expression was smaller at 24 h postinoculation than the change in *S.* Typhimurium-inoculated plants that were not elicited (H_2O treatment), as well as compared to the change in leaves induced with 0.1% Bion in the absence of *S.* Typhimurium (mock inoculation). This apparent antagonism in *PR1* expression suggests that the occurrence of cross talk between defense signaling activities triggered by *S.* Typhimurium colonization of the lettuce apoplast and by Bion elicitation. Notably, this suppressive effect by *S.* Typhimurium colonization on SA signaling was evidenced also by a decrease in *PR1* expression in leaves of lettuce roots inoculated with the ISR elicitor *R. glutinis*, the most effective ISR inducer among the four agents that we tested. Likewise, *EDS1* transcription in the leaves of lettuce plants elicited with *B. subtilis* and *R. glutinis* was suppressed when leaves were colonized by this enteric pathogen. These common observations with different induced plant resistance approaches and different lettuce cultivars in our study strongly suggest the occurrence of interference in plant signaling by different concomitant defense pathways in the presence of *S.* Typhimurium.

The upregulation of *PR1* in lettuce leaves during apoplastic colonization by *S.* Typhimurium is in line with previous reports that plant basal defenses, as reflected

partly by enhanced transcription of *PR1* and other components of the SA signaling pathway, are elicited by this human pathogen, e.g., in *A. thaliana* (13, 24, 27). Of note is our observation in this study that *PR1* upregulation triggered solely by *S.* Typhimurium did not persist at levels comparable to those triggered by chemical elicitors and microbial inducers. Such a transient induction of *PR1* by *S.* Typhimurium was observed similarly in *A. thaliana* (13). This sustained defense response upon chemical or microbial elicitation of systemic resistance may underlie its inhibitory effect on *Salmonella* colonization in lettuce and basil leaves in our study. However, given its pattern of expression in Bion- and Y13-treated plants and the potential occurrence of defense signaling cross talk in the presence of *S.* Typhimurium, the production of PR1 cannot fully explain the inhibition of *Salmonella* colonization observed in this study, so additional defense-related mechanisms or pathways are very likely at play. *Salmonella* flagellin is recognized via FLS2 in plants, and its flagellin peptides, as well as *Salmonella* colonization itself, induce ROS production in *A. thaliana* and tomato (21, 28, 57). Furthermore, activation of the SA signaling pathway by elicitor treatment increased flg22-triggered oxidative burst and callose deposition in *A. thaliana* (58). Bion treatment also enhanced FLS2 transcription in lettuce in our study, while BABA and ISR-inducing microorganisms augmented callose deposition to control plant pathogens infection in lettuce and other species in previous studies (54, 59, 60). Since differential colonization of *S.* Typhimurium in lettuce cultivars and *A. thaliana* is partly a function of ROS production and callose deposition upon apoplast inoculation (26, 27), these plant defense responses are worthy of investigating further to enhance our understanding of the inhibitory effect of SAR and ISR on endophytic *Salmonella*.

Plant defense priming has been described by Mauch-Mani and collaborators as the induction of a plant physiological readiness to mount a faster, stronger or more sustained attack on a target by exposure to low doses of stimulus (61). In the primed state, the plant is conditioned to respond to an invader with minimal activation of defenses, which are then potentiated upon the presence of the invader, thus minimizing allocation costs to the plant (62), which would otherwise be incurred by a persistent heightened state of defense (63). The potential for plant defense priming to minimize microbial contamination of crops could be of significant interest, particularly since it may concomitantly reduce the incidence of certain plant diseases. Future plant technologies may provide the means to engineer or exploit intrinsic physiological traits for an enhanced and timed defensive capability against microbial contaminants on plants. The results we described here serve as a proof of concept that enhanced plant immunity through elicitation may contribute to a spectrum of plant-based tools to weaken the probability of enteric pathogen survival and multiplication on crops.

MATERIALS AND METHODS

Microbial strains and growth conditions. *Salmonella enterica* serovar Typhimurium strain 14028s was obtained from M. McClelland (University of California, Irvine, CA). *S. enterica* serovars Senftenberg strain 127468 (clinical isolate, 2010, Israel), Montevideo strain 125473 (clinical isolate, 2009, Israel), Enteritidis strain 124655 (clinical isolate, 2009, Israel), Infantis strain 122798 (chicken isolate, 2009, Israel), and Dublin strain 135157 (milk isolate, 2010, Israel) were obtained from L. Valinsky (Central Laboratories, Ministry of Health, Israel). These *Salmonella* strains are here referred to as *S.* Typhimurium, *S.* Senftenberg, *S.* Montevideo, *S.* Enteritidis, *S.* infantis, and *S.* Dublin. *S.* Typhimurium 14028s was marked with rifampin resistance by selection of a spontaneous rifampin-resistant mutant on growth medium containing rifampin at 100 μg/ml and used in SAR experiments. All ISR and *Salmonella* serovar comparison experiments were carried out with wild-type strains. All strains were cultured in Luria-Bertani half-salt broth (5 g of NaCl/liter; LBHS) by agitation at 28°C overnight to early stationary phase. Inoculum suspensions were prepared by washing the cells first in 10 mM potassium phosphate buffer (pH 7; KPB) and again in 1 mM KPB using centrifugation (17,000 $\times$ *g*) for 3 min at 24°C and then resuspending them in distilled deionized (DDI) H_2O for inoculation at concentrations detailed below.

The biological inducers of ISR used in this study were isolated in the laboratory of Y. Elad and as described previously (41, 42, 48, 64). *Trichoderma harzianum* strain T39 was grown on potato dextrose agar (PDA) for 10 days, while inducers *Pichia guilliermondii* strain S2 and *Rhodotorula glutinis* strain Y13 were grown on PDA for 4 days. *Bacillus subtilis* strain Mel16 was cultured on nutrient agar for 2 days. All biological inducers were cultured at 28°C, washed, and resuspended in DDI H_2O to reach the inoculum cell concentration described below in "Plant Treatment and Leaf Apoplast Inoculation." The inoculum concentration was adjusted based on *T. harzianum* conidium counts using a hemocytometer and based on the optical density at 600 nm (OD_{600}) for bacterial and yeast strains.

Plant material and growth conditions. Lettuce plants (*Lactuca sativa*, romaine cv. Parris island [USA] and cv. Noga 936 [Israel]) were grown for 2 months in 500-ml pots to the four- to six-leaf stage in a plant growth chamber at 22°C (USA) and in a greenhouse at 20 to 25°C (Israel), with 14-h daylight period. The mean leaf weight and the standard errors of the mean (SEM) were 1,197 ± 20 and 578 ± 4 mg for lettuce and basil, respectively. Basil plants (*Ocimum basilicum* cv. Peri) were obtained from a commercial nursery (Hishtil, Israel), cultivated in a greenhouse at 20 to 25°C, and grown in 750-ml pots for 1 month to the four- to six-leaf-stage. All plants were cultivated in greenhouse soil composed of peat moss and perlite (approximate ratio, 80:20).

Plant treatment and leaf apoplast inoculation. (i) Passive apoplast inoculation. The effect of ISR strains on endophytic colonization of basil by *S.* Senftenberg and of lettuce cv. Noga 936 by *S.* Typhimurium was tested as follows: basil or lettuce plants were treated by soil drench with 20 ml/plant of cell suspension of the biological inducers *T. harzianum* T39 (10^6 conidia/ml), *B. subtilis* strain Mel16 (10^8 CFU/ml), *R. glutinis* strain Y13 (10^8 CFU/ml), and *P. guilliermondii* strain S2 (10^8 CFU/ml) in DDI H_2O. The control treatment consisted of a soil drench with sterile DDI H_2O. Plants were then kept at 20 to 25°C in a greenhouse. After 3 days, all plants, including the control plants, were inoculated by submerging the upper part of the plants in a suspension (10^8 CFU/ml DDI H_2O) of *S.* Senftenberg or *S.* Typhimurium for 30 min. The plants were then rinsed thoroughly by sequential immersions in sterile DDI H_2O to enrich for inoculant in the apoplast. Application of the inducer agents was repeated as soil drench immediately after inoculation before the plants were returned to the greenhouse, as previously described by Elad and Rav-David (64). Inoculation for comparative lettuce and basil apoplast colonization by various *Salmonella* serovars was performed using the approach described above, except that the plants did not receive any soil drench treatment.

(ii) Apoplast inoculation by vacuum infiltration. The effect of the SAR on *S.* Typhimurium multiplication in the leaf apoplastic space was investigated using the compounds acibenzolar-*S*-methyl (ASM) and DL-β-amino-butyric acid (BABA; Sigma) in lettuce cv. Parris Island. Acibenzolar-*S*-methyl, a functional SA analog that is commercially available as Bion 50 WG (or Actigard 50 WG), was kindly provided by Syngenta Crop Protection, Inc. (Greensboro, NC). Plants were treated by soil drench with 25 ml of 0.01 and 0.1% Bion, as well as 0.8 and 4 mg/ml BABA, 24 h before inoculation. Control treatment consisted of soil drench with DDI H_2O. The aerial part of the plants, including that of H_2O-treated plants, was then inoculated upside down in the inoculum suspension by vacuum infiltration. Briefly, cotton was placed tightly on the soil surface around the potted plant to prevent soil from falling into the suspension. The lettuce leaves were immersed in 1.8-liter suspension of *S.* Typhimurium at 10^4 CFU/ml DDI H_2O that did not contain any surfactant, such as Silwet L-77; for infiltration of the suspension into the leaves, the beaker with the suspension and the plant were placed in a vacuum bell jar (Abbess Instruments, Holliston, MA), and a vacuum was applied for 1 min to remove air from the leaf mesophyll. The vacuum was then quickly released to replace the air in the apoplast with the cell suspension (65). After inoculation, the aerial part of the plant was rinsed three times by successive immersion in 1 liter of sterile DDI H_2O to remove the inoculum from the leaf surfaces to the greatest extent possible. The plants were placed in a chamber at 28°C and 65% relative humidity, thus ensuring that the surfaces of the leaves were macroscopically dry and that *S.* Typhimurium multiplication during incubation was limited to the leaf apoplast.

Bacterial cell recovery and quantification of apoplastic population sizes. Basil leaves (the third or fourth leaf) and lettuce leaves (the fourth or fifth leaf) were sampled at random from different plants and pots immediately after inoculation (0 h) and at various times of incubation after inoculation. The number of replicate leaf samples and replicate pots is provided in each figure legend and varied slightly depending on the number of available plants of very similar age and size. The leaves were surface sterilized by immersion in 2% sodium hypochlorite containing 0.1% Tween 20 for 30 s. Each leaf was rinsed five times in five different beakers containing 250 ml of DDI H_2O. The leaves were homogenized with a mortar and pestle in 2 ml of DDI H_2O to release the *Salmonella* cells from the apoplast, and aliquots of the homogenate were dilution plated for colony plate counts. For SAR experiments, which were carried out with the rifampin-resistant *S.* Typhimurium 14028s, the homogenate was plated onto LBHS agar containing rifampin at 100 μg/ml (LBHS-rif agar). For ISR experiments and comparative *Salmonella* serovar colonization experiments, the homogenates were plated onto XLD agar. Plate counts were performed after incubation of the plates at 37°C for at least 20 h. No colonies were detected on LBHS-rif agar or XLD agar from homogenates obtained from leaves infiltrated with sterile DDI H_2O only, indicating that the plate counts were specific for *Salmonella*.

In addition, homogenates from leaves that were surface inoculated by rapid immersion of the plant aerial part, rinsed in sterile DDI H_2O five times, and surface sterilized after plant incubation and then, prior to bacterial recovery, were plated on LBHS-rif agar. The plates were incubated at least 24 h at 28°C and did not yield any bacterial colonies. This demonstrated (i) the efficacy of our surface sterilization treatment and (ii) the efficacy of our approach in enriching for *Salmonella* populations in the apoplast; that is, by thoroughly rinsing leaf surfaces after inoculation and then incubating the plants under conditions that do not promote free water on the leaves, the number of cells on the leaf surface immediately after inoculation was restricted, and *Salmonella* multiplication in the phyllosphere was minimized, respectively.

Measurement of relative plant gene expression. Expression of select basal plant defense genes that are part of the pathways involved in SAR and ISR was quantified in lettuce leaves at the indicated times after inoculation with *Salmonella* or mock inoculation (H_2O only). Leaves were harvested, frozen in liquid nitrogen, and stored in a −80°C freezer until used for RT-qPCR. Portions (50 mg) of leaf tissue from three or five replicate leaves were ground using a mortar and pestle in liquid nitrogen before RNA was extracted using

Ambion TRIzol reagent and a PureLink RNA kit (Life Technologies). The RNA was DNase treated a second time with Turbo DNase I (Ambion), and the absence of DNA was confirmed by RT-qPCR in the absence of reverse transcriptase using primers for ACT7. Gene expression in lettuce treated with Bion and BABA was analyzed by one-step RT-qPCR with the Stratagene Brilliant II SYBR green 1-Step kit (Stratagene) on a MxPro 3000P cycler (Stratagene). Gene expression in lettuce treated with ISR microbial inducers was analyzed by two-step RT-qPCR using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) in which 1 μg of purified RNA was reverse transcribed to cDNA. The obtained cDNA served as the template for PCR amplification using the Fast SYBR green Master Mix (Applied Biosystems, Foster City, CA). Real-time detection was performed in triplicates with a 7300 Real-Time PCR System (Applied Biosystems). Expression of ACT7 was used to normalize the data between samples. The sequences of the primers were as follows: EDS1-F, 5′-ACCTTGAGGAATACACGCGATCCA; EDS1-R, 5′-AAGGCGTGTGTGAATATCCGGTCA (66); ERF-F, 5′-CCGTTTGATT GTTCCGATTT; ERF-R, 5′-TTCGGCTTCTTCACTGGATT (34); DEF1-F, 5′-GCCATCTTCTCTGCTTTTGAA; DEF1-R, 5′-ACACAAGACACTGCGACGAC (34); FLS2a-F, 5′-ATTCCGGCGTCTATTTTCTGTA; FLS2a-R, 5′-ATTAGTCAGCCA CAAAGGGAAA; PAL2-F, 5′-CACCCTCCTTCAAGGTTACTCCGG; PAL2-R, 5′-GGGACGAGATCGCCGGAGGCGG; PR1-F, 5′-TCGCCACAAGACTTTGTTAATG; PR1-R, 5′-GAGGCAAGATTTTCACCATAGG (67); PR2-F, 5′-TTGAGTGGA TCCAACATTGAAG; PR2-R, 5′-TCATGGATATTGGTCAAAGCAG; ACT7-F, 5′-GCAATTCAAGCCGTTCTTTC; and ACT7-R, 5′-GATCCAAACGGAGGATAGCA (67). Primers targeting the defense genes FLS2a, PAL2, and PR2 were designed with the Primer3 software. The efficiencies of all primers used in our systems were tested by PCR.

Statistical analysis. Statistical analysis of the data was performed with Prism version 7.05 (GraphPad Software). The bacterial population sizes were log transformed before analysis. Differences among population sizes and gene expressions were tested with Student *t* test or one-way analysis of variance, followed by Dunnett's or Tukey's multiple-comparison test ($P < 0.05$). All experiments were replicated at least twice.

ACKNOWLEDGMENTS

We are grateful to Julio Martinez and Li Hui at the USDA, ARS, for technical assistance, and to Lea Valinsky at the Central Laboratories, Ministry of Health, Jerusalem, Israel, for providing *Salmonella* strains.

This research was supported by grant IS-4862-15 R from the United States-Israel Binational Agricultural Research and Development Fund and by USDA Agricultural Research Service CRIS project 2030-42000-052-00D.

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