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Plant-mediated restriction of *Salmonella enterica* on tomato and spinach leaves colonized with *Pseudomonas* plant growth-promoting rhizobacteria

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ABSTRACT

Reducing *Salmonella enterica* association with plants during crop production could reduce risks of fresh produce-borne salmonellosis. Plant growth-promoting rhizobacteria (PGPR) colonizing plant roots are capable of promoting plant growth and boosting resistance to disease, but the effects of PGPR on human pathogen-plant associations are not known. Two root-colonizing *Pseudomonas* strains S2 and S4 were investigated in spinach, lettuce and tomato for their plant growth-promoting properties and their influence on leaf populations of *S. enterica* serovar Newport. Plant roots were inoculated with *Pseudomonas* in the seedling stage. At four (tomato) and six (spinach and lettuce) weeks post-germination, plant growth promotion was assessed by shoot dry weight (SDW) and leaf chlorophyll content measurements. Leaf populations of *S.* Newport were measured after 24 h of leaf inoculation with this pathogen by direct plate counts on Tryptic Soy Agar. Root inoculation of spinach cv. 'Tyee', with *Pseudomonas* strain S2 or S4 resulted in a 69% and 63% increase in SDW compared to non-inoculated controls ($p < 0.005$ and $p < 0.01$, respectively). Similarly, Romaine lettuce cv. 'Parris Island Cos' responded positively to S2 and S4 inoculation (53% and 48% SDW increase, respectively; $p < 0.05$), and an increase in leaf chlorophyll content ($p < 0.001$), compared to controls. Tomato cv. 'Nyagous' yielded significantly greater SDW (74%, $p < 0.01$ and 54%, $p < 0.05$ for S2 and S4, respectively), and also higher leaf chlorophyll content (19% and 29%, $p < 0.001$, respectively) relative to controls. Leaf chlorophyll content only increased in S4-inoculated tomato cv. 'Moneymaker' plants (27%, $p < 0.001$), although both S2 and S4 promoted plant growth by over 40% compared to controls ($p < 0.01$ and $p < 0.05$, respectively). No significant growth promotion was detected in tomato cv. 'BHN602', but S2-inoculated plants had elevated leaf chlorophyll content (13%, $p < 0.01$). Root inoculation with *Pseudomonas* S4 restricted *S.* Newport populations inoculated on leaves of spinach ($p < 0.001$) and all three tomato cultivars ($p < 0.05$), compared to controls, 24 h post *Salmonella* inoculation. Impairment of *S.* Newport leaf populations was also observed on spinach when plant roots were inoculated with S2 ($p < 0.01$). With an initial leaf inoculum of approximately 6.0 log CFU of *S.* Newport/plant, the significantly greater reduction of *S.* Newport populations on *Pseudomonas*-treated plants than those on non-inoculated control plants after 24 h was modest with differences of one log or less. By contrast, the survival of *S.* Newport on the leaves of Romaine lettuce was not influenced by *Pseudomonas* root colonization. These findings provide evidence that root inoculation of certain specialty crops with beneficial *Pseudomonas* strains exhibiting PGPR properties may not only promote plant growth, but also reduce the fitness of epiphytic *S. enterica* in the phyllosphere. Plant-mediated effects induced by PGPR may be an effective strategy to minimize contamination of crops with *S. enterica* during cultivation.

1. Introduction

The human enteric pathogen *Salmonella enterica* can survive in agricultural environments and use plants as alternative hosts (Schikora et al., 2012). If kill steps are insufficiently applied during food processing and preparation, edible plants contaminated with *S. enterica* during cultivation can transmit the pathogen to consumers, and may cause salmonellosis outbreaks (CDC, 2011). On-farm Good Agricultural Practices (GAPs) are implemented to reduce contamination risk during field production, since some outbreaks can be traced back to the pre-harvest stage (CDC, 2008; Greene et al., 2008). Contamination continues to occur during production (Angelo et al., 2015; CDC, 2016), and there is a need to develop cost-effective interventions to curb the persistence of *Salmonella* on pre-harvest crops.

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The contamination of fresh produce crops with *S. enterica* constitutes a plant-microbe interaction. Plants are able to recognize *S. enterica*. *Arabidopsis* responded to flagellin from *S. enterica* by inducing the expression of genes similar to what is seen in pathogen-associating molecular pattern (PAMP)-triggered immunity (PTI) responses (Meng et al., 2013). *S. enterica* serovar Typhimurium has also been shown to elicit the release of reactive oxygen species (ROS) in tobacco (Shirron and Yaron, 2011), and stomatal closure in *Arabidopsis* and lettuce (Roy et al., 2013). These responses raise the possibility that plants can defend themselves against *S. enterica* proliferation in the phyllosphere.

Plant growth-promoting rhizobacteria (PGPR) are known for their ability to promote growth through phytohormone production or improving nutrient acquisition, and enhance plant immunity through induced systemic resistance (ISR), whereby colonization of plant roots by PGPR confers resistance to a variety of phytopathogen to non-colonized plant structures (Beneduzi et al., 2012; Pieterse et al., 2014; Somers et al., 2004). Concerns about the environmental and public health risks of synthetic agrochemicals used in crop production have diverted interest to biocontrol and biofertilizer agents, and a number of microbial formulations have been commercialized (Stockwell and Stack, 2007). *Pseudomonas* spp. are abundant in rhizosphere soil and many strains of these soilborne bacteria have been characterized as PGPR due to their ability to influence plant development and health. The notable beneficial properties of *Pseudomonas* strains to plants include facilitation of mineral nutrient uptake, production of or enhanced response to phytohormones, vitamin biosynthesis, direct antagonism of plant pathogens (Ahemad and Kibret, 2014; Kloepper et al., 1980; Patten and Glick, 2002; Zamioudis et al., 2013) and induction of systemic disease resistance (Matilla et al., 2010; Meziane et al., 2005; Ongena et al., 2008). Despite the extensive body of work documenting induced systemic resistance to plant pathogens, little is known about the effect of PGPR on enteric pathogens associating with edible plants. A recent study reported that inoculation of lettuce and spinach roots with a *Bacillus subtilis* strain capable of inducing ISR, primed stomata to close in response to *S. enterica* Newport on spinach leaves and *Listeria innocua* on Romaine lettuce leaves, and also reduced populations of *L. innocua* on lettuce leaves (Markland et al., 2015).

In light of the possibility of PGPR-mediated ISR against human pathogens, the objective of this study was to investigate the effect of two *Pseudomonas putida* strains in our collection on human pathogen viability on aboveground portions of vegetable crops. These strains of *Pseudomonas* have been shown to enhance soybean nodulation (Zhang et al., 1996; Dashti et al., 2000), and may influence the growth development and health of vegetable crops. Root inoculations of tomato, spinach and lettuce with two unique *Pseudomonas* strains were conducted to assess plant growth promotion as well as proliferation of *S. enterica* Newport inoculated onto leaf surfaces.

2. Materials and methods

2.1. Bacterial strains and vegetable cultivars

Two rifampicin-resistant *Pseudomonas* spp. strains S2 and S4 were obtained from Dr. Brian Klubek (Southern Illinois University Carbondale), identified as strains of *Pseudomonas putida*. The *Salmonella enterica* serovar Newport used in this study is a tomato outbreak strain (Greene et al., 2008) adapted for rifampicin resistance. To grow the rifampin-resistant bacterial strains, culture media (broth and agar plates) were supplemented with 50 μg/ml rifampin (Tokyo Chemical Industry Co. LTD., Japan).

Spinach (*Spinacia oleracea*) cultivar 'Tyee', Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) cultivar 'Parris Island Cos', and three tomato (*Solanum lycopersicum*) cultivars 'Moneymaker', 'Nyagous' and 'BHN 602', were chosen for this study. Two plant seeds were sown in each 6.4 cm^2 plastic pot filled with sunshine professional growing mix #1 LC1 (Sungro Horticulture, Agawam, MA) and thinned to one seedling per pot after germination for subsequent root inoculation. All plants were grown at a constant temperature of 22 °C, relative humidity of 88%, and controlled light condition (16 h light:8 h dark photoperiod) with regular drip irrigation in a growth chamber for the duration of experiments.

2.2. Preparation of Pseudomonas inoculum for plant inoculation

Frozen stock of *Pseudomonas putida* strains S2 or S4, maintained at -20 °C in Brucella Broth (Becton Dickinson, Sparks, MD) containing 15% glycerol, were streaked on Trypticase Soy Agar (TSA) (BD) plates and incubated at 28 °C for 48 h. Single colonies were transferred to Nutrient Broth No.3 (Sigma-Aldrich, St. Louis, MO) and grown to late-log phase to CFU of $> 10^9$ cells/ml for plant root inoculation. PGPR-treated plants received 3 ml of *Pseudomonas* broth culture of $> 10^9$ CFU/ml, and non-inoculated control plants received 3 ml of fresh Nutrient Broth No.3, dripped onto the base of each plant. Two separate root inoculations were carried out one week and two weeks post-germination. Successful root colonization by each strain of *Pseudomonas* was confirmed by colony enumeration of whole root rinsates on TSA-rifampicin (TSA-rif) plates upon the completion of experiments (data not shown).

2.3. Measurements of plant growth and leaf chlorophyll content

PGPR-treated and control plants were maintained for a total of 6 weeks (spinach and lettuce) and 5 weeks (tomato) under the same growth chamber conditions described above. All aerial biomass was clipped off the base of each plant, placed in a paper bag, transferred to a 70 °C oven and incubated for 48 h before recording shoot dry weight (SDW) in g. Chlorophyll content of mature leaves was measured in triplicate with a SPAD-502 chlorophyll meter (Konica Minolta, Japan).

2.4. Preparation of Salmonella inoculum for leaf inoculation

Root-inoculated spinach and lettuce were grown for 6 weeks, and tomato for 4 weeks post-germination, prior to leaf inoculation with *S.* Newport. To perform leaf inoculations, the frozen stock of *S.* Newport was streaked on TSA-rif plates and incubated at 35 °C overnight. One single colony of *S.* Newport from the overnight culture was streaked on another fresh TSA-rif plate and grown again at 35 °C overnight. *S.* Newport inocula were made by suspending bacterial colonies in 0.1% Peptone Water (PW) (BD) to an OD_{600} of 0.5, to obtain a cell density of approximately 1×10^9 CFU/ml. A 100-fold dilution of this *S.* Newport suspension was made with 0.1% PW for leaf inoculation and the actual inoculum level was determined on TSA-rif plates. Leaves to be inoculated were gently marked with a black marker pen on the leaf stalk. An aliquot of 10 μl of *S.* Newport inoculum was pipetted as five separate droplets on the adaxial side of one fully-extended mature leaf, and a total of two leaves with a combined inoculum dose of 1×10^6 CFU were inoculated per plant.

2.5. S. Newport retrieval from inoculated leaves

Inoculated leaves were harvested 24 h post-inoculation. The two inoculated leaves per plant were clipped off the stem with aseptic utensils and placed in a sterile Whirl-Pak bag (Nasco, Forth Atkinson, WI). Leaves were immersed in 30 ml of 0.1% PW, hand massaged for 30 s, and sonicated in a Branson Ultrasonic Cleaner (Branson Ultrasonics Corporation, Danbury, CT) for 1 min, to dislodge the attached *Salmonella* cells from the leaf surfaces. The sonicated samples were shaken at 150 rpm for 10 min and serial dilutions were prepared from the leaf rinsates for *S.* Newport enumeration on TSA-rif plates.