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Ralstonia solanacearum pathogen disrupts bacterial rhizosphere microbiome during an invasion

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ABSTRACT

Plant pathogen invasions are often associated with changes in physical environmental conditions and the composition of host-associated rhizosphere microbiome. It is however unclear how these factors interact and correlate with each other in determining plant disease dynamics in natural field conditions. To study this, we temporally sampled the rhizosphere of tomato plants that were exposed to moderate to aggressive *Ralstonia solanacearum* pathogen invasions over one crop season. We found that physiochemical soil properties correlated weakly with the severity of pathogen invasion apart from the water-soluble nitrogen concentration, which increased more clearly during the aggressive invasion. Instead, a much stronger link was found between pathogen invasion and reduced abundance and diversity of various rhizosphere bacterial taxa, simplification of bacterial interaction networks and loss of several predicted functional genes. We further verified our results in a separate greenhouse experiment to show that pathogen invasion causally drives similar changes in rhizosphere microbiome diversity and composition under controlled environmental conditions. Our results suggest that *R. solanacearum* invasion disrupts rhizosphere bacterial communities leading to clear reduction in the diversity and abundance of non-pathogenic bacteria. These changes could potentially affect the likelihood of secondary pathogen invasions during following crop seasons as less diverse microbial communities are also often less resistant to invasions. Strong negative correlation between pathogen and non-pathogenic bacterial densities further suggest that relative pathogen abundance could better predict the severity of bacterial wilt disease outbreaks compared to absolute pathogen abundance. Monitoring the dynamics of whole microbiomes could thus open new avenues for more accurate disease diagnostics in the future.

1. Introduction

Understanding plant pathogen infections in highly variable field conditions is a key challenge for crop protection and future food security. Specifically, it has remained difficult to disentangle the interactive and causal effects between abiotic environmental conditions, soil physiochemical properties, plant development and microbe-microbe interactions under variable field conditions (Chaparro et al., 2014; Huang et al., 2013; Li et al., 2013; Shi et al., 2015; Wei et al., 2011). In the case of *Ralstonia solanacearum* bacterial pathogen, both abiotic and biotic factors have been shown to be important for the infection dynamics (van Elsas et al., 2001; Wei et al., 2011). On a seasonal scale, disease outbreaks have been connected to periods of warm temperatures during the summer crop seasons (Wei et al., 2017, 2011), whereas at more local scale, *R. solanacearum* survival has been linked to low environmental salt concentration and water temperature (Elsas et al., 2001). Also other physiochemical soil properties vary spatially and temporally due to environmental heterogeneity and along with plant development and this variation could be important in explaining the patchiness of bacterial disease dynamics in homogenous agricultural monocultures (Piotrowska-Dlugosz et al., 2016).

In addition to abiotic environmental factors, biotic interactions can affect *R. solanacearum* infection dynamics via resource and interference competition within the host-associated rhizosphere bacterial communities (Wei et al., 2015). For example, microbial community composition and diversity (Chapelle et al., 2016; Elsas et al., 2012; Mendes

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et al., 2011), secondary metabolite activity (Jousset et al., 2014; Wang et al., 2017a), and interaction network architecture (Wei et al., 2015) have been connected to *R. solanacearum* pathogen invasions. Recent evidence also suggests that microbial interactions in the plant rhizosphere can be highly dynamic and shaped by plant development and abiotic environmental conditions (Gu et al., 2016; Wei et al., 2017). For example, plant exudation can change the rhizosphere community composition by recruiting certain beneficial microbes and directly repelling the pathogen (Wei et al., 2017), whereas nutrient availability and temperature has been shown to alleviate diversity-invasion resistance relationships via resource competition (Mallon et al., 2015), and to regulate competitive outcomes between the pathogen and endophytic biocontrol bacterium within the plant stem (Wei et al., 2017), respectively. As a result, disentangling the relative importance of multiple abiotic and biotic factors for pathogen invasion remains a key challenge for understanding the epidemiology of diseases. Here we studied this specifically in the context of *Ralstonia solanacearum* plant bacterial pathogen infecting tomato plants under natural field conditions in China.

Ralstonia solanacearum is a gram-negative plant pathogenic bacterium and a causal driver of global bacterial wilt disease epidemics (Hayward, 1991; Jiang et al., 2017; Yabuuchi et al., 1995). It has an unusually wide host range being capable of infecting more than 200 plant species including many economically important crops (Genin and Denny, 2012; Hayward, 1991; Jiang et al., 2017). The first step of infection is colonization of the plant rhizosphere, where the pathogen must compete with other bacterial taxa (Hibbing et al., 2010). After reaching a threshold density, pathogen switches on its virulence gene expression and invades plant roots (Schell, 2000). Once within xylem vessels, *R. solanacearum* rapidly spreads to aerial plant parts throughout the vascular system (Dalsing et al., 2015; Huang and Allen, 2000; Saile et al., 1997) and blocks the water flow via excessive production of extracellular polysaccharides (Denny and Baek, 1991; Genin and Denny, 2012). Bacterial wilt disease dynamics are often very variable in even seemingly homogenous agricultural monocultures, which raises a question: to what extent does abiotic and biotic environmental factors determine the epidemiology of bacterial wilt? To study this in more detail, we set up a temporal sampling regime where we repeatedly isolated the whole bacterial communities from tomato plant rhizosphere during a Spring crop season in Nanjing, China. Total pathogen and bacterial densities and the soil physiochemical properties (pH, water-soluble carbon, water-soluble nitrogen, nitrate, ammonium and available phosphate) were determined at every sampling and high-throughput sequencing used to determine changes in the rhizosphere bacterial community composition, co-occurrence interaction networks and changes in functional gene abundances with PICRUSt platform (Langille et al., 2013). We then explored the causal effect of pathogen invasion on rhizosphere microbiome composition in separate greenhouse experiment in controlled environmental conditions. We set to study following two key hypotheses: 1) does soil physiochemical properties and rhizosphere microbiome community composition correlate with the aggressiveness of pathogen invasions, and, 2) does the aggressiveness of pathogen invasion affect the diversity and functioning of rhizosphere microbiome communities?

2. Materials and methods

2.1. Experimental field site and sampling regime

The field experiment was conducted in Qilin town (118°57′E, 32°03′N), a vegetable production centre for the nearby urban population of Nanjing city, China. The experimental field has been continuously colonized by *R. solanacearum* for more than 10 years (Wei et al., 2011), and hence, bacterial wilt infection will occur naturally during the crop seasons. For this study, we selected one large field (~360 m^2 area) with very high disease incidence of bacterial wilt of tomato (~60% disease incidence during 2012 Autumn crop season) for field sampling during 2013 Spring crop season (from March to June).

Surface-sterilized tomato seeds (*Lycopersicon esculentum*, cultivar "Jiangshu") were germinated on water-agar plates for three days before sowing into seedling plates containing Cobalt-60-sterilized seedling substrate (Huainong, Huaian soil and fertilizer Institute, Huaian, China). On March 14th of 2013, thirty-day aged tomato seedlings were transplanted in the field (~2000 plants transplanted in the beginning of the crop season) and weekly sampling regime started ten days after the transplantation. For the first three weeks, nine healthy plants were randomly collected per week as no visible disease symptoms could be detected. From week four on, tomato plants started to show symptoms of wilting and approximately 50% of plants showed clear signs of bacterial wilt by the end of the crop season (Figs. S1–A). As a result, 6 healthy and 6 diseased plants were randomly collected from week 4 on and only plants with functional root systems were used for further analysis (Figs. S1–B). Sampling was finished 12 weeks after the transplantation.

Samples were classified into three groups based on the sampling time and visibility of bacterial wilt disease symptoms. Samples collected during the first 3 weeks were categorized to the group of initially healthy plants. From week four on, samples were categorized into two groups based on the visibility of bacterial wilt disease symptoms: healthy (no clear disease symptoms regardless of the pathogen presence) and diseased (clear diseases symptoms and high pathogen densities) plants. This classification was used because *R. solanacearum* was also present in the rhizosphere of the healthy plants even though it was not causing visible disease symptoms. Total of 112 rhizosphere soil samples were collected during the 12-week crop season resulting in 27 initial, 35 diseased and 50 healthy plant rhizosphere samples. The following physiochemical soil properties were also measured from each field sample: pH (1: 5 mass ratio of sample and deionized water, PB-10, Sartorius, Germany) (Li et al., 2017), nitrate and ammonium nitrogen (AutoAnalyzer 3, SEAL, Germany), and water-soluble nitrogen and carbon concentrations (Vario TOC cube, Elementary, Germany).

2.2. Greenhouse experiment testing for causality between pathogen invasion and changes in rhizosphere microbiome composition

A separate greenhouse experiment was carried out to test whether pathogen invasion could change bacterial community composition and diversity under controlled environmental conditions. To this end, we used soil that was collected from a riverside in Zhangzhu town of Yixing, China (119°48′ 29″, 31°20′21″" - 160 km away from Nanjing) with no previous *R. solanacearum* infection history. Tomato seedlings were prepared as described above and 80 seedlings at similar growth stage were transplanted into plastic pots with 5 kg of sieved (at 5 mm) and homogenized dry soil. Ten plants were randomly selected and two rhizosphere soil samples per plant pooled together resulting in 5 initial rhizosphere soil samples. Subsequently, 35 plants were inoculated with *Ralstonia solanacearum* QL-Rs1115 strain (isolated from the field experimental site at Nanjing) by root drenching method resulting in final concentration of 5.0×10^6 CFU of bacteria g^{-1} soil (Pathogen present treatment). Another 35 plants were treated with the same amount of heat-killed (autoclaved at 121 °C for 20 min) suspension of dead *R. solanacearum* cells (Pathogen absent treatment). Tomato plants were then grown in a glass greenhouse with natural temperature variation ranging from 25 °C to 35 °C and watered regularly with sterile water for 32 days. The plant growth and disease development was monitored on daily basis. Subset of plants was sampled twenty days after the pathogen inoculation when the infected plants started to show visible disease symptoms. Briefly, three plants with functional root systems were harvested from both treatments at every two days for total of twelve days. Replicate samples within both treatments were then pooled at every sampling to result in 6 samples from the 'infected' and 6 samples from the 'control' treatment. All the final 17 rhizosphere soil