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The relative importance of soil moisture in predicting bacterial wilt disease occurrence

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Highlights

- Soil moisture is a key predictor of bacterial wilt disease (BWD) across China.
- Other soil properties have lesser role and are locally associated to BWD.
- Soil moisture can causally drive BWD in greenhouse experiment.
- Water management strategies could potentially be used in BWD control.

Title

The relative importance of soil moisture in predicting bacterial wilt disease occurrence

Running Title

Soil moisture predicts wilt disease

Authors

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Summary

Soil-borne plant diseases cause major economic losses globally. This is partly because their epidemiology is difficult to predict in agricultural fields, where multiple environmental factors could determine disease outcomes. Here we used a combination of field sampling and direct experimentation to identify key abiotic and biotic soil properties that can predict the occurrence of bacterial wilt caused by pathogenic *Ralstonia solanacearum*. By analysing 139 tomato rhizosphere soils samples isolated from six provinces in China, we first show a clear link between soil properties, pathogen density and plant health. Specifically, disease outcomes were positively associated with soil moisture, bacterial abundance and bacterial community composition. Based on soil properties alone, random forest machine learning algorithm could predict disease outcomes correctly in 75% of cases, with soil moisture being the most significant predictor. The importance of soil moisture was validated causally in a controlled greenhouse experiment, where the highest disease incidence was observed at 60% of maximum water holding capacity. Together, our results show that local soil properties can predict disease occurrence across a wider agricultural landscape, and that management of

soil moisture could potentially offer a straightforward method for reducing crop losses to *R. solanacearum*.

Keywords

Bacterial wilt disease; Soil moisture; Soil physicochemical properties; Rhizosphere bacterial communities; *Ralstonia solanacearum*; Random forest algorithm

1 Introduction

Multiple physicochemical and biotic environmental factors have long been known to be important for plant disease outbreaks, as suggested by the conceptual Disease Triangle model (McNew, 1960). For example, soil moisture (Aung et al., 2018), pH (Rahman and Othman, 2020), nutrient availability (Berg and Koskella, 2018) and microbial communities (Trivedi et al., 2020) all influence the severity of soil-borne diseases. Furthermore, temperature can directly affect pathogen densities (Wei et al., 2015a; Pimentel and Ayres, 2018) or the strength of interactions between pathogen and its competitors (Wei et al., 2017; Velásquez et al., 2018) with important consequences for the disease occurrence. However, while the significance of environment has been long recognised (Cheng et al., 2019), we still poorly understand the relative importance of different factors, or their combinations, for the disease outcomes. Furthermore, the effects of soil properties are often studied locally within one specific agricultural area making it difficult to extrapolate results up to a level of a country or a continent (Janvier et al., 2007; Orr and Nelson, 2018). To study this, we used China-wide ~~(area of 1.3 million Km^2)~~ sampling of tomato plant rhizosphere to identify key abiotic and biotic soil properties associated with bacterial wilt disease occurrence, and experimentally tested if one of the most important factors, soil moisture, could causally drive bacterial wilt disease incidence in a greenhouse experiment.

Ralstonia solanacearum bacterium is a causative agent of notorious bacterial wilt disease that leads to a systemic wilting of plants (Hayward, 1991). It can infect multiple important crops belonging to the *Solanaceae* family (e.g. potato, tomato and tobacco) and has a global distribution (Mansfield et al., 2012). Previous studies have identified associations with multiple soil physicochemical factors and *R. solanacearum* infections both in the field and greenhouse experiments (Hayward, 1991; Jiang et al., 2017; Wei et al., 2018; Siregar et

al., 2020). For example, *R. solanacearum*-infected plants have previously been associated with increased soil moisture (Jiang, 2016), acidic pH (Li et al., 2017a) and high nitrogen availability (Dalsing et al., 2015; ~~Y. Gu et al., 2020~~Gu et al., 2020a). These environmental factors could affect bacterial wilt occurrence directly by favouring the growth of the pathogen, as *R. solanacearum* needs to reach certain threshold density in the soil to express key virulence factors that are triggered by quorum sensing signalling (Genin and Denny, 2012; Peyraud et al., 2016, 2018). Alternatively, soil properties could have indirect effects on the pathogen via plants or associated plant rhizosphere microbiome. Plants have evolved sophisticated defence mechanisms against pathogens, and recent evidence suggests that environmental factors can directly affect plant immunity and defence hormone pathways (Velásquez et al., 2018). Rhizosphere microbiome also plays a crucial role in forming the first line of defence against invading pathogens, often considerably shaping the disease severity (Kwak et al., 2018; Wei et al., 2019, 2020). In general, diverse microbial communities can limit pathogen growth due to intense competition for nutrients, space and other resources (Wei et al., 2015b; ~~S. Gu et al., 2020~~Gu et al., 2020b), or because they are likely to contain highly antagonistic species that can directly inhibit the pathogen for example by secreting antimicrobial molecules (Raza et al., 2016a, 2016b). Crucially, soil properties often determine the composition and diversity of rhizosphere microbiome and could hence indirectly affect the likelihood of *R. solanacearum* infections.

Understanding the potential role of soil properties in *R. solanacearum* infections is especially important because bacterial wilt dynamics often show high temporal and spatial variability both between and within fields (Wei et al., 2017, 2018). Previous work has shown that between-field variability could be driven by local fluctuations in temperature and humidity (Wei et al., 2017), while within-field variation could be explained by spatial

differences in soil physicochemical properties or the composition of microbial communities, which both have been associated with disease outcomes previously (Wei et al., 2018, 2019; Lee et al., 2021). However, it is unclear which soil properties are relatively more important than the others, and if the previously observed patterns hold across a wider geographical area with varying local environmental conditions. To study this, we focused on six geographically separated tomato fields in China (area of 1.3 million Km^2) to explore the role of within- and between-field variation in abiotic and biotic soil properties for bacterial wilt disease occurrence. We first collected and analysed 139 rhizosphere soil samples originating from healthy and diseased plants at every field and identified significant associations between the disease outcome, pathogen densities and different soil properties. Second, machine learning algorithm was used to identify the relatively most important soil properties associated with the bacterial wilt disease, whose importance was directly tested in a greenhouse experiment. It was found that despite considerable between-field variation, healthy and diseased plants were consistently associated with certain soil properties, which could predict bacterial wilt disease occurrence with 75% accuracy. Soil moisture, bacterial community composition and bacterial abundances were the most important predictors of disease ~~by~~ incidence based on~~a~~ random forest model~~, and~~. Furthermore, soil moisture content treatment at 60% of maximum water holding capacity led to the highest levels of disease incidence in a controlled greenhouse experiment. Together, our findings suggest that local variation in abiotic and biotic soil properties can reliably predict bacterial wilt disease outcomes across large agricultural area.

2 Experimental Procedures

2.1 Sampling sites and collection of plant soil samples

Soil samples were collected from tomato fields at six locations in Changsha of Hunan province (112°58'E, 28°11'N), Ningbo of Zhejiang province (121°67'E, 29°91'N), Nanchang of Jiangxi province (115°51'E, 28°41'N), Nanjing of Jiangsu province (18°57'E, 32°03'N), Nanning of Guangxi province (108°21'E, 22°49'N) and Wuhan of Hubei province (114.31'E 30.52'N) during tomato bacterial wilt disease outbreaks in the summer 2015. The sampled fields in Central (Hubei and Hunan), Eastern (Jiangsu, and Zhejiang) and Southern (Guangxi) China recurrently experience *R. solanacearum* outbreaks (Jiang et al., 2017) and had suffered from bacterial wilt disease epidemics between 3 to 15 years based on communication with the local farmers. Within each sampling site, common local tomato cultivars were used: *Solanum lycopersicum* cv. "Hengkang #1" in Changsha, "CTX 201" in Nignbo, "Hezuo 906" in Nanachang, "Guihong #1", "Jipin" in Nanjing and "Huafan #13" in Wuhan. At each sampling site, around 12 symptomatic (diseased) and 12 asymptomatic (healthy) tomato plants were chosen randomly based on the presence and absence of visible disease symptoms ~~and randomly sampled~~ at the early fruiting stage ~~resulting in(~~ a total of 139 rhizosphere samples). Excess root soil was discarded by gently shaking and the remaining soil attached on the root surfaces was collected and considered as the rhizosphere soil (Wei et al., 2011). Around 10 g of fresh rhizosphere soil per plant was sampled and divided into two sealed 5 mL Eppendorf tubes to retain natural soil properties. One tube was cryopreserved in 5 mL of 30% glycerol at –80 °C to analyse biotic properties of rhizosphere samples (pathogen and total bacteria densities and microbial community diversity and composition). Another tube was used for determining soil physicochemical (abiotic) properties as described in the following section.

2.2 Determination of abiotic and biotic soil properties

2.2.1 Abiotic properties

Abiotic physiochemical properties included soil moisture content (Moisture, %), pH, available phosphorus (P, $mg \cdot kg^{-1}$), available potassium (K, $mg \cdot kg^{-1}$), water-soluble carbon (C, $mg \cdot kg^{-1}$) and total nitrogen (N, $mg \cdot kg^{-1}$). The difference in fresh and air-dried soil sample weight was used as a proxy of soil moisture for each rhizosphere sample. Soil pH was measured in a 20% water (w/w) suspension (Li et al., 2017a) using a pH meter (PB-10, Sartorius, Germany). Available P and K were extracted with hydrochloric acid and ammonium fluoride and measured using molybdenum blue method (Pansu and Gautheyrou, 2006). The water-soluble carbon and total N were determined by following a previous protocol (Pansu and Gautheyrou, 2006) using a multi C/N analyzer 3000 (Analytik Jena AG, Germany).

2.2.2 Biotic properties

The total DNA was exacted from ~0.25 g of cryopreserved rhizosphere soil using PowerSoil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA) following the manufacturer´s protocol. DNA quality and concentration were checked using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Soil DNA was subjected to 16S ribosomal RNA (rRNA) Illumina amplicon sequencing to determine the diversity and composition of bacterial communities at Shanghai Biozeron Biological Technology Co. Ltd. The V4 hypervariable region of the 16S rRNA gene was amplified with the primer pair 563F (5'-AYTGGGYDTAAAGVG-3') and 802R (5'-TACNVGGGTATCTAATCC-3'). All sequences were processed with QIIME (Caporaso et al., 2010). The OTU similarity cut-off was assigned at 97% identity level using USEARCH (Edgar, 2010). OTUs were assigned to corresponding bacterial taxa using the Ribosomal Database Project (RDP) database with the online version of the RDP

classifier (Cole et al., 2014). The microbial community diversity was determined as Shannon diversity index (Shannon) and Chao1 richness index (Shannon) using the vegan R package (Dixon, 2003) after removing *R. solanacearum* OTUs (Wei et al., 2018). Microbial community composition was quantified as a dissimilarity index (Bray-Curtis) based on average Bray-Curtis distance of each sample from each other at the OTU level. The pathogen and total bacterial densities were examined with qPCR using *R. solanacearum*-specific primer Rsol_*fliC* (Schönfeld et al., 2003) and general bacterial primer pair Eub338/Eub518 (Fierer et al., 2005). SYBR Premix Ex Taq Kit (TaKaRa Biotech. Co, Japan) was used following the manufacturers' protocol, and each sample was measured in triplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA).

2.3 Establishing causality between soil moisture and bacterial wilt incidence in tomato

A greenhouse experiment was conducted in Yixing of Jiangsu province to test whether soil moisture can predictably drive the development of bacterial wilt disease under controlled environmental conditions. Experimental soils that were free of *R. solanacearum* were collected from a riverside of Zhangzhu town in Yixing: no *R. solanacearum* growth was detected using semi-selective agar medium (Wei et al., 2018). Tomato seeds (*S. lycopersicum* cv. "Jipin") were surface-sterilized with NaClO (3%; v:v) and germinated on moist filter paper for 2 days before sowing in sterilized nursery substrate (Huaian Agricultural Technology Development Ltd). Tomato seedlings were transplanted into plastic pots with five kg of homogenized dry soils at four-leaf stage. The soil moisture content was manipulated using five treatments with 40%, 50%, 60%, 70%, and 80% of maximum water holding capacity, and twelve replicate pots were used per treatment. This moisture range covered dry (40%) and flooded (80%) soils. Plants were acclimated in greenhouse conditions for three weeks before

pathogen inoculation and then grown in the same conditions until the end of the experiment (constant temperature of 30 °C ± 3 °C, relative air humidity of 80%, and 14 h of light and 10 h of dark daily cycle). Water content was kept constant in each treatment by adding sterile water to each pot during acclimatization period before the infection and until the end of the experiment after the infection~~accordingly~~. After three weeks of acclimatization, *R. solanacearum* pathogen strain QL-Rs1115 (a strong virulent reference strain) was inoculated to all pots using soil drenching method with resulting in final concentration of 5.0×10^6 CFU·g^{-1} soil (Wei et al., 2011). The same amount of water (10 mL) was used with all the pots, which led to only momentary increase in water holding capacity in some of the low moisture treatments during the drenching. The disease development was monitored on a daily basis and quantified as a disease index on a scale ranging from 0 to 4 where one whole number change corresponds to 25% increase in the proportion of wilted leaves per plant (Schandry, 2017).

2.4 Data analyses

2.4.1 Comparing differences in abiotic and biotic properties of healthy and diseased plant rhizosphere samples

All measured abiotic and biotic properties were normalized between the range 0 – 1 using min-max normalization before statistical analyses (Patro and Sahu, 2015). Nonparametric Wilcoxon rank sum test (Wilcoxon test) was used to compare differences between healthy and diseased plant rhizosphere soil samples (Cuzick, 1985). The microbial community composition was ordinated by principal coordinates analysis (PCoA) using Bray-Curtis distance and differences between healthy and diseased plant rhizosphere soil samples were compared using the nonparametric permutational multivariate analysis of variance

(PERMANOVA, $P < 0.05$, 999 permutations) using Adonis function in R vegan package (Dixon, 2003). Principal component analysis (PCA), based on the Euclidean distance of the range normalized values for overall abiotic and biotic properties, was used to visualize differences between healthy and diseased plants (FactoMineR R package (Lê et al., 2008); statistical significance tested using nonparametric PERMANOVA ($P < 0.05$) with 999 permutations using Adonis function in R vegan package (Dixon, 2003)).

2.4.2 Identifying key abiotic and biotic predictors for pathogen abundance and plant health

To identify key abiotic and biotic predictors for pathogen abundance, we build up a model using multiple linear regression function in R stats package (R Core Team, 2020) to predict *R. solanacearum* densities based on all measured soil properties. The relative importance of different predictors ~~were~~was estimated based on their significance for the model performance (% of R^2) using Anova (R Core Team, 2020) and relweights functions in R (Kabacoff, 2015). To understand the relationship between environmental variables and disease incidence, we used Random forest approach using randomForest package in R as follows (Cutler and Wiener, 2018). We first randomly selected 80% of the entire 139 sample dataset as a training set (n = 111) to generate a classification model for predicting plant health status (healthy vs. diseased) based on soil abiotic (moisture, pH, P, K, C, N) and biotic properties including Chao1, Shannon and Bray-Curtis metrices of the bacterial community in tomato rhizosphere soils. Tenfold cross-validation was performed 10 times using the rfcv function to select appropriate number of predictor properties whose importance and cross-validation curves were visualized by using the R ggplot2 package (Wickham et al., 2020). Remaining 20% of samples (n = 28) were used as a test set to predict plant health based on the abiotic and biotic rhizosphere soil properties.

2.4.3 Analysis of disease dynamics in a greenhouse experiment

The effect of soil moisture on disease dynamics was analysed based on temporal changes in disease index values using a logistic growth curve (Schandry, 2017). The disease dynamics curves were fitted individually for each plant using gcFitModel function in R grofit-package (Kahm et al., 2010). As described previously (Wei et al., 2015b), this fit could be divided into three variables describing different stages of disease development: 1) lag phase referred to as the delay time of disease symptom onset after inoculation of the pathogen (early infection stage); 2) disease rate referred to as the exponential increase of disease progression (exponential infection stage); 3) area under progression of the disease dynamics curve (AUDPC) referred to as the overall severity of wilt disease (late infection stage). Shapiro-Wilk and Bartlett's tests were used to test the normality and homogeneity of the fitted variables using the R stats-package. If the data matrix followed a normal distribution with homogeneous variances, ANOVA and post hoc Tukey's HSD tests were used to compare differences between different soil moisture groups ($P < 0.05$) using R multcomp-package (Hothorn et al., 2020). Otherwise, non-parametric Kruskal-Wallis and post hoc Dunn's tests were used for statistical analyses using R agricolae package (Mendiburu, 2020).

3 Results

3.1 Rhizosphere soil properties vary between healthy and diseased plants

We first compared the abiotic physiochemical and biotic rhizosphere soil properties (Table 1) of diseased and healthy tomato plants across six sampled provinces in China (Fig. 1a, CS: Changsha; NB: Ningbo; NC: Nanchang; NJ: Nanjing; NN: Nanning; WH: Wuhan). Pathogen densities were on average 15.79 times higher in diseased compared to healthy plants (P < 0.0001, Wilcoxon test, Supp. Fig. 1-2), and also the other rhizosphere soil properties differed between diseased and healthy plants (Fig. 1, Supp. Fig. 1-2). Specifically, diseased plants were characterized by 1.15 times higher soil moisture (P = 0.001), and 10.53 times higher total bacterial densities (P = 0.0002) compared to the healthy plants (Wilcoxon test; Fig 1b and Supp. Fig. 1-2). While other physiochemical soil properties, or microbial community diversity, did not differ between the diseased and healthy plants (P > 0.05; Wilcoxon test; Fig 1b, Supp. Fig. 1-2), PCoA analysis revealed that microbial community composition varied depending on the plant health status (R^2 = 0.10, P = 0.002) and between provinces (R^2 = 0.56, P = 0.001, PERMANOVA; Fig. 1c and Supp. Fig. 3). Moreover, differences in microbial community composition between the healthy and diseased plants were location-specific: significant differences were found in CS, NB, NJ and NN (P > 0.05) but not in NC or WH provinces (P < 0.05, PERMANOVA; Supp. Fig. 3). Moreover, when analysed together using PCA, abiotic physicochemical and biotic soil properties differed between provinces (R^2 = 0.62, P = 0.001), and between healthy and diseased plants within each province (R^2 = 0.09, P = 0.001, PERMANOVA; Fig. 1d and Supp. Fig. 4). Together, these results suggest that diseased and healthy plants were associated with distinct soil properties despite clear between-province variation in environmental conditions.

3.2 Soil moisture is the relatively most important factor distinguishing diseased and healthy plant samples

To compare the relative importance of different soil properties, we used correlation analysis and machine learning. We found statistically significant relationships between abiotic physicochemical and biotic parameters and *R. solanacearum* pathogen densities in case of all variables except for P and K availability ($P > 0.05$, Fig. 2a and Supp. Fig. 5). Specifically, pathogen densities correlated negatively with N availability ($R^2 = 0.24$, $P = 0.005$) and average Bray-Curtis dissimilarity (average Bray-Curtis distance of microbial community composition from other 139 samples, $R^2 = 0.28$, $P = 0.001$). In contrast, pathogen densities were positively associated with total bacterial densities ($R^2 = 0.60$, $P < 0.0001$), soil moisture ($R^2 = 0.55$, $P < 0.0001$), pH ($R^2 = 0.28$, $P = 0.001$), Shannon diversity ($R^2 = 0.20$, $P = 0.017$) and Chao1 richness ($R^2 = 0.21$, $P = 0.014$; Fig. 2a and Supp. Fig. 5). Of all predictor variables, soil moisture (relative weight = 40.36%), total bacterial density (relative weight = 22.77%) and soil pH (relative weight = 14.59%) were the most significant predictors of pathogen densities in the tomato rhizosphere (multiple regression model, AIC: 324.09; $F_{10,128} = 10.6$, $R^2 = 0.45$, $P < 0.0001$, Supp. Table 1).

Random forest modelling was further used to analyse associations between soil properties and plant health. By using all measured soil properties, we could predict bacterial wilt disease outcomes with 78.6% accuracy (AUC = 0.89; Supp. Fig. 6). To eliminate the obvious link between pathogen abundance and disease incidence, we re-ran the model without pathogen density data (Fig. 2b). The high predictability of the model was retained, and bacterial wilt disease outcomes could still be predicted with 75% accuracy (AUC = 0.75; Fig. 2c). Based on ten-fold cross-validation with 10 independent model simulations (inset of Fig. 2b), soil moisture was ranked as the most important individual predictor of plant health

followed by the total bacterial abundances (Fig. 2b). Together, these results suggest that abiotic and biotic soil properties can reliably predict bacterial wilt disease occurrence, with soil moisture being the relatively most important factor.

3.3 Variation in soil moisture can causally drive bacterial wilt disease occurrence

To directly test if soil moisture can drive variation in bacterial wilt disease incidence, we performed a greenhouse experiment where tomato plants were exposed to *R. solanacearum* type strain under different soil moisture treatments. We found that bacterial wilt disease dynamics differed depending on soil moisture content and the stage of infection (Fig. 3). On average, the highest disease incidence was observed in 60% followed by 70% soil moisture content treatments, while no differences were observed between the other treatments (Fig. 3a-b). Specifically, soil moisture effects were visible during the early stages of infection in terms of reduced lag-phase of disease onset ($F_{4,21}$ = 7.48, P < 0.0001, ANOVA; Fig. 3b) and as overall differences in area under disease progression curve (AUDPC, χ^2 = 13.73, P = 0.008, AUDPC panel), while soil moisture content had no effect on the disease rate during the exponential phase of infection (χ^2 = 4.07, P = 0.396, Kruskal-Wallis test; Fig. 3b). Together, these results demonstrate that soil moisture alone can causally drive bacterial wilt disease outcomes in otherwise homogenous tomato rhizosphere environments.

4 Discussion

Here we studied if plant-level variation in bacterial wilt disease occurrence could be explained by local abiotic and biotic soil properties across six provinces in China. Our sampling data shows that healthy and diseased plant rhizosphere soils were associated with distinct abiotic and biotic properties which could predict bacterial wilt disease occurrence with 75% accuracy. Soil moisture was identified as the most important predictor, and its causal role was tested directly in a greenhouse experiment with tomato. It was found that variation in soil moisture alone, could considerably change the disease dynamics resulting in different levels of disease incidence. Our results are in line with previous studies that have identified a tight link between moisture and plant diseases (Huber and Gillespie, 1992) with *Pseudomonas syringae* (Xin et al., 2016) and *M. oryzae* pathogens in the plant phyllosphere (Li et al., 2014) ~~and~~ by expand~~s~~ings this association to crops and other soil-borne bacterial diseases.

Moisture could affect plant pathogens in several ways (Aung et al., 2018) ranging from effects on pathogen survival, movement and growth in the soil (Smilanick and Mansour, 2007; Kearns, 2010) to effects on pathogen invasiveness (Li et al., 2014) or indirect effects on the activation of plant defences (Panchal et al., 2016; Velásquez et al., 2018). While the relationship with moisture has previously been observed with other soil-borne pathogenic fungi and bacteria, including *R. solanacearum* (Chairman et al., 1981; van Elsas et al., 2000; Islam and Toyama, 2004; Satou et al., 2006; Mondal et al., 2014; Jiang et al., 2018), we here show that moisture was the relatively most important factor predicting bacterial wilt occurrence across broad geographical scale spanning six Chinese provinces. Our sampling area covered various soil types, tomato cultivars and climate conditions. While clear geographical variation between locations was observed, the significance of soil moisture on plant health status was significant within each field. As a result, this difference could not be

explained by local climate or agricultural practises, such as use of certain tomato cultivars. In the future, it will be important to see if our findings can be extrapolated to other countries and agricultural areas experiencing recurrent *R. solanacearum* outbreaks.

In addition to identifying an important country-wide link with the soil moisture, we show that this association might not be an indirect consequence of *R. solanacearum* infection, which typically leads to increased soil moisture via reduced water uptake and transpiration in the infected plants (Jiang, 2016). Instead, by using controlled greenhouse experiment, we demonstrate that soil moisture alone can causally drive bacterial wilt disease outcomes in otherwise identical soil environmental conditions. Highest levels of disease incidence were observed at 60% and 70% maximum water holding capacity soil moisture treatments, and there are several potential reasons for this. First, it is possible that this moisture content level was optimal for the plant growth (Kramer, 1983) leading to more efficient root exudation (Larson and Funk, 2016) and improved growth and colonisation of the plant by the pathogen (van Elsas et al., 2000; Islam and Toyama, 2004). Moreover, non-optimal soil moisture levels have previously been shown to lead overexpression of plant resistance genes (Sinha et al., 2016; Jiang et al., 2018), which could have also affected the observed differences in disease occurrence, as reported before (Mondal et al., 2014). Alternatively, it is possible that certain moisture levels were directly beneficial to the pathogen, allowing more efficient growth, movement and colonisation of the plant (Beattie, 2011; Aung et al., 2018; Velásquez et al., 2018). Finally, soil moisture is known to affect the availability of oxygen (Mainiero and Kazda, 2005) and nutrients (Cavagnaro, 2016), which could have affected the *R. solanacearum* growth (Dalsing et al., 2015) or the strength of microbiome-mediated pathogen suppression (Chen et al., 2007; Brockett et al., 2012) in the rhizosphere. Further experiments are ~~hence~~ however needed to directly test these explanations ~~directly~~.

In addition to soil moisture, also some soil physiochemical properties, such as microbiome composition, pH and nitrogen availability, differed between healthy and diseased plants depending on the sampling sites. This is in line with previous findings showing a clear link between bacterial community composition and bacterial wilt disease outcomes (Wei et al., 2018, 2019), highlighting also the importance of microbial interactions for *R. solanacearum* infections (Wei et al., 2019; Wen et al., 2020; Lee et al., 2021). While differences in bacterial community richness and diversity of healthy and diseased plants were only significant in Nanjing, bacterial community composition was more consistently associated with plant health status indicative of its importance in predicting bacterial wilt disease occurrence (Wei et al., 2019). In the future, it would be interesting to test if the abundance and activity of certain *R. solanacearum*-suppressing bacteria, such Firmicutes and Actinobacteria (Lee et al., 2021), were positively or negatively affected by the soil moisture content. Furthermore, it has previously been shown that bacterial wilt disease is aggravated in acidic soils (Li et al., 2017a, 2017b; Wang et al., 2017) and by high nitrogen availability (~~Y. Gu et al., 2020~~Gu et al., 2020a), while high C, N, P and K availabilities have been linked ~~with~~ to healthy plant rhizosphere (Wang et al., 2017; Wei et al., 2018; Wu et al., 2020). However, we found that the physicochemical soil properties did not consistently differ between healthy and diseased plants ~~machine learning algorithm~~. It is also possible that some of the healthy plants were latently infected by *R. solanacearum*, and hence, did not show visible disease symptoms despite being infected (Hayward, 1991; Genin and Denny, 2012). While certain healthy plants overlapped in their soil properties with the diseased plants, we did not see clear clustering of healthy plant samples at the field level. This suggests that the proportion of latently infected plants was low, or that their microbiome properties were more similar to healthy plants. In the future, it would be interesting to test if our algorithm can predict

bacterial wilt disease occurrence using other unrelated datasets, and if its performance can be improved by taking latent infections into account. Furthermore, model predictions could potentially be improved by including temporally, locally and globally varying ~~some other~~ abiotic and biotic variables that vary temporally~~,~~ such as temperature, humidity and ~~humidity~~precipitation.~~ Furthermore,~~As it is likely that ~~certain~~ these environmental factors will have interactive effects, ~~which should~~their effects be further explored experimentally.

5 Conclusions

We conclude that soil properties can be used as reliable predictors of bacterial wilt disease occurrence, with soil moisture being one of the most import single factors that consistently differed between healthy and diseased plants across all sampling locations. Moreover, while other soil properties played important roles, their effects were often sampling location-specific, indicative of their potential importance at the local scale. The causal role of soil moisture was directly validated in a greenhouse house experiments, which highlights the value of direct experimentation in separating causes from consequences in plant pathology studies. The obtained information will be helpful for developing predictive modelling to better understand the epidemiology of bacterial wilt disease outbreaks in spatially and temporally varying agricultural environments and should be validated in the future with unrelated datasets from other countries and agricultural areas. Finally, the importance of soil moisture suggest that relatively simple water management practises could potentially be effective way to control bacterial wilt disease occurrence.

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Conflict of Interest

The authors declare that there are no relevant conflicts of interest.

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Table 1: Differences in abiotic physicochemical and biotic soil properties between healthy and diseased plants

Factor	Name (Units)	Statistical method	Diseased vs healthy plants (*P*-values)*					
			CS	NB	NC	NJ	NN	WH
Moisture	Soil moisture content (%)	Wilcoxon test	<0.001	0.026	0.115	0.009	0.006	0.922
pH	Soil pH value	Wilcoxon test	0.312	0.729	0.025	0.016	0.954	0.431
Phosphorus	Available phosphorus ($mg \cdot kg^{-1}$)	Wilcoxon test	0.514	0.63	0.606	0.079	0.862	0.224
Potassium	Available potassium ($mg \cdot kg^{-1}$)	Wilcoxon test	0.114	0.319	0.001	0.928	0.012	0.699
Carbon	Water-soluble carbon ($mg \cdot kg^{-1}$)	Wilcoxon test	0.799	0.378	0.599	0.009	0.008	0.047
Nitrogen	Water-soluble nitrogen ($mg \cdot kg^{-1}$)	Wilcoxon test	0.887	0.143	0.028	0.211	0.419	0.401
Pathogen density	*R. solanacearum* density (log_{10} *fliC* gene copies g^{-1} soil)	Wilcoxon test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Bacterial density	Total bacterial density (log_{10} *16S rRNA* gene copies g^{-1} soil)	Wilcoxon test	0.002	0.04	0.645	0.005	0.014	0.081
Shannon	Shannon index for bacterial community diversity (OUT level)	Wilcoxon test	>0.999	0.198	0.519	0.002	0.291	0.133
Chao1	Chao1 index for bacterial community richness (OTU level)	Wilcoxon test	0.755	0.977	0.133	0.002	0.198	0.401
Bray-Curtis	Bray-Curtis dissimilarity index for bacterial community composition	PERMANOVA test	0.034	0.023	0.298	0.001	0.003	0.067

*Sampling locations are abbreviated as follows: CS = Changsha, NB = Ningbo, NC = Nanchang, NJ = Nanjing, NN = Nanning and WH = Wuhan. P-values less than 0.05 are shown in red colour. Details of the analysis are listed in Supplementary Figures 2 – 4.

Figure legends

Figure 1. Differences in abiotic physicochemical and biotic soil properties between healthy and diseased plants. (**a**) Map of China showing sampling locations and provinces (CS = Changsha, NB = Ningbo, NC = Nanchang, NJ = Nanjing, NN = Nanning and WH = Wuhan; numbers in parentheses show the number of samples included in each location). (**b**) Comparison of the normalised physicochemical (blue) and biotic (black) parameters between healthy (green) and diseased (red) tomato plant rhizosphere samples (ns denote for non-significant correlation ($P > 0.05$) and stars (**, ***, ****) denote significant correlation at levels $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively). Pathogen and total bacterial abundances are abbreviated as 'Pathogen' and 'Bacteria', respectively. (**c**) Comparison of microbial community composition (PCoA) between healthy and diseased tomato plant rhizosphere samples (*status*) at each sampling location (*site*). (**d**) Comparison of abiotic soil physicochemical properties and biotic soil properties (PCA) between healthy and diseased tomato plant rhizosphere samples (*status*) at each sampling location (*site*).

Figure 2. The relative importance of abiotic physicochemical and biotic soil properties in predicting bacterial wilt disease occurrence. (**a**) Correlation coefficients (ranging from negative (purple) to positive (cyan)) between *R. solanacearum* pathogen densities and abiotic physicochemical (blue) and biotic (black) soil properties across all tomato rhizosphere samples (ns denote for non-significant correlation ($P > 0.05$) and stars (**, ***, ****) denote significant correlation at levels $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively). (**b**) Relative importance rank of abiotic physicochemical (blue) and biotic (black) soil properties and ten-fold cross-validation of random forest model (inset in **b**) based on the training set (80% of randomly selected rhizosphere samples). Total bacterial abundances are abbreviated as

'Bacteria'. (**c**) Validation of random-forest model with a test set (20% of remaining samples) predicting plant disease outcomes based on soil properties: green and red filled cells denote for correct predictions and filled cells with white crosses denote for false predictions.

Figure 3. Causal validation of the role of soil moisture driving bacterial wilt disease dynamics in a greenhouse experiment. (**a**) Mean disease progression curves in different soil moisture treatments based on logistic curve fitting (left inset shows goodness-of-fit and significance for each treatment). (**b**) Comparison of disease dynamics between different treatments in terms of lag-phase before disease onset (early stage), disease rate (exponential stage) and area under progression of disease curve (AUDPC, late stage). Different small letters above violin plots denote for significant differences between treatment groups ($P < 0.05$).

Dear Dr. Jiang:
Manuscript ID SEL-2020-0105 entitled "The relative importance of soil moisture in predicting bacterial wilt disease occurrence" which you submitted to the Soil Ecology Letters, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

The reviewer(s) have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript.

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Sincerely,
Editorial Office, Soil Ecology Letters

Response to Editor: Thanks for handling our manuscript and your encouraging decision of 'minor revision'. We have carefully revised the manuscript following the constructive comments and suggestions from reviewers, and our point by point answers can be found below.

Reviewer(s)' Comments to Author:

Reviewer: 1

Comments to the Author
This paper deals with defining the soil properties to predict bacterial wilt occurrence in fields. Based on field evaluation of bacterial wilt occurrence in tomato, soil properties from various fields in China, microbiome/statistical analysis, and validation experiments in green house, authors propose that soil moisture is the most important predictor for bacterial wilt in field-growing tomato. Both biotic and abiotic factors differed among sites and between diseased and healthy plants, and this was the same in soil microbial composition. Among the soil properties, soil moisture was the most important predictor of pathogen density and disease outcome in fields. Contribution of soil moisture content to bacterial wilt was further validated in a greenhouse.

The main conclusion of this paper is acceptable based on the analysis and interpretation of the result, however, I found a couple of points to be clarified in detail in methods or to be discussed in discussion.

Response 1 to first reviewer: Thank you very much for your time to revise our manuscript. Please find our responses to your comments below.

1) Selection of diseased or healthy plants from fields: what was the exact criteria to differentiate the plants "diseased" or "healthy"? Was it solely based on wilting symptoms? If this is the case, authors should consider latent infection status of bacterial wilt in fields. Please discuss this.

Response 2 to first reviewer: Yes, the diseased and healthy plants were chosen solely based on visible wilting symptoms as described on lines 138-139. We fully agree that some of the healthy plants could have been latently infected by *Ralstonia* and now consider this possibility in the text. It is possible that some of the healthy plants were latently infected by *R. solanacearum*, and hence, did not show visible disease symptoms despite being infected. While certain healthy plants overlapped in their soil properties with the diseased plants, we did not see clear clustering of healthy plant samples at the field level. This suggests that the proportion of latently infected plants was low, or that their microbiome properties were more similar to healthy plants. This is now discussed on lines 394-399.

2) Methodology to validate disease severity depending on soil moisture; the Ralstonia challenge to tomato plants was done by a soil drenching method which will change the soil moisture content. Authors described a way to maintain the constant soil moisture before pathogen inoculation. But with soil drenching inoculation, how did they control the soil moisture? Did authors only focus on the water content during acclimatization period?

Response 2 to first reviewer: We agree that soil drenching infections will increase the soil moisture content levels. However, this effect was kept consistent to all moisture treatments and limited to soil drenching application period (one day): soil moisture contents were otherwise carefully manipulated between treatments before and after pathogen inoculation. We have now described this in the manuscript on lines 233-239.

3) Some of the references in text were not indicated with the proper citation format. Authors need to double check this.

Response 2 to first reviewer: We have now corrected the format of referencing throughout the manuscript (on lines 81, 93, 95 and 391 and in the 'References' list).

Reviewer: 2

Comments to the Author

Considering the poor understanding of the relative importance of different factors, or their combinations, for plant disease, this study is timing to show a clear link between soil properties, pathogen density and plant health, by analysing 139 tomato rhizosphere soils samples isolated from six provinces in China. The authors found disease outcomes were positively associated with soil moisture that determined bacterial community, especially abundance of some bacterial groups. The exquisite experimental design together with advanced algorithm allows quantify the relative importance of soil properties in predicting bacterial wilt disease occurrence. The study certainly has sufficient novelty and updated our knowledge of what edaphic variables promote pathogenic Ralstonia solanacearum thus caused occurrence of bacterial wilt. I like the overall story and don't have much criticism with the research. Before considering acceptance, I have a few concerns as below.

Response 1 to second reviewer: We thank reviewer for positive comments. Please, find our detailed responses to your comments below.

1. The valid experiment was conducted to test the causality though, I have one major question about the collected 139 samples based on which "water" was attributed to the biggest predictor to wilt. As water content depend on the weather of the sampling day (rain or not), the local climatic information, i.e. average precipitation per year or during growth, thus, might be better used as variable.

Response 2 to second reviewer: This is a very good point, and we fully agree that local weather data could potentially be very useful for predicting bacterial wilt disease incidence

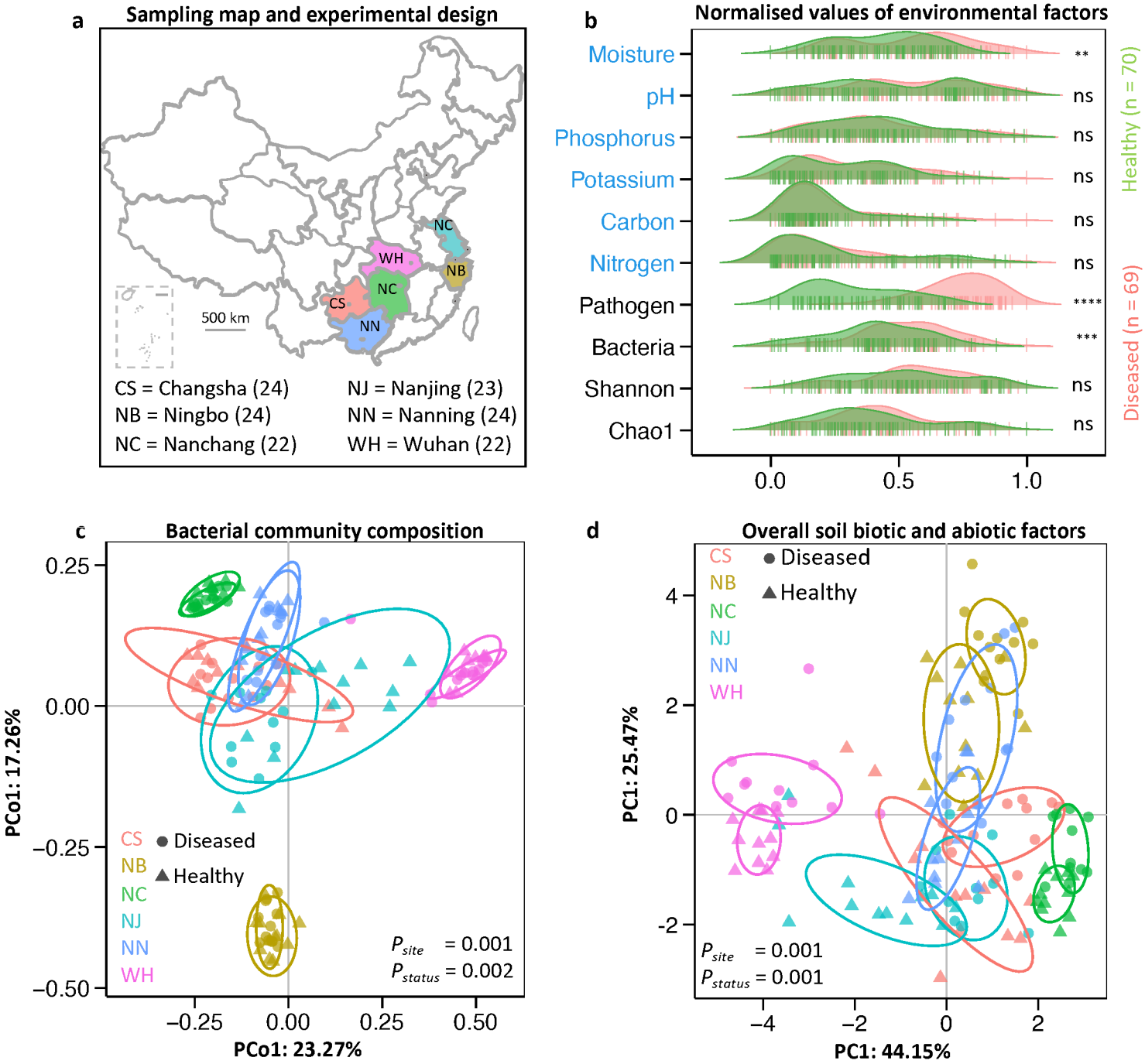
across wider geographical areas. This would be especially useful in the face of global climate change. We now briefly discuss about this on lines 402-404.

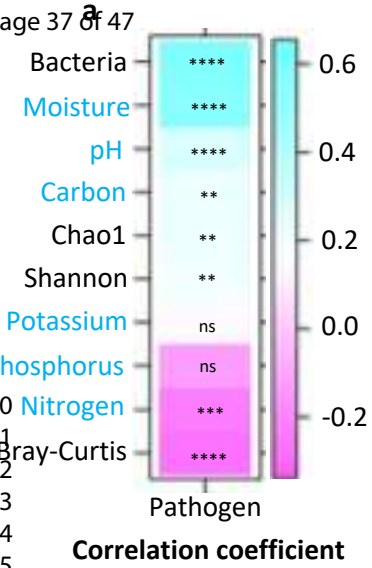
2. Line 68, maybe move this sentence to the end of introduction

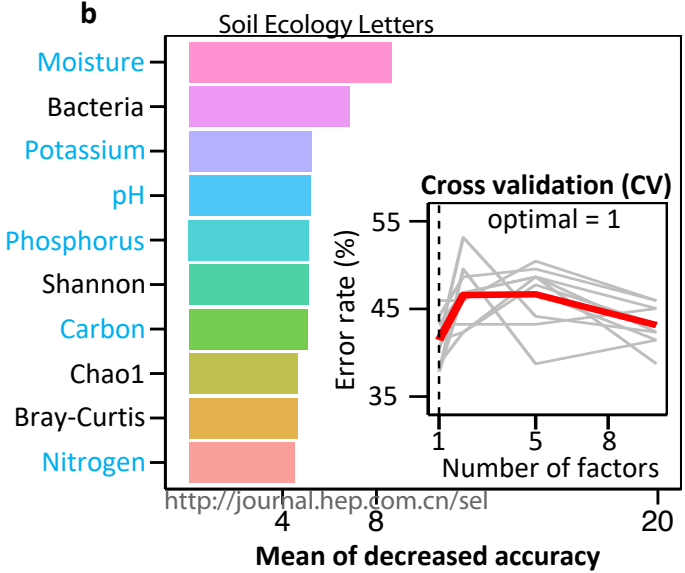
Response 3 to second reviewer: Thank you for your suggestion. We would prefer to give readers an overview of our research question already at the beginning of the introduction. We believe this is helpful for communicating our research questions clearly and setting everything in the relevant context.

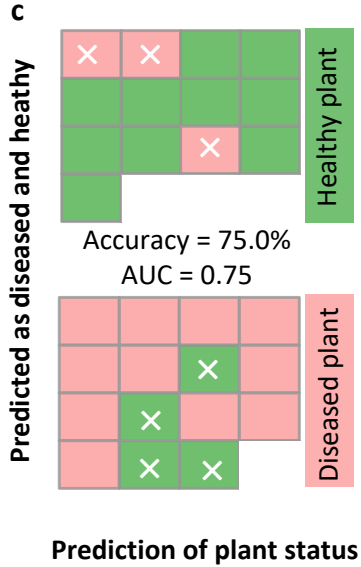
3. Fig. 1c and d, any chance to give the legend of abiotic variables, I might miss it.

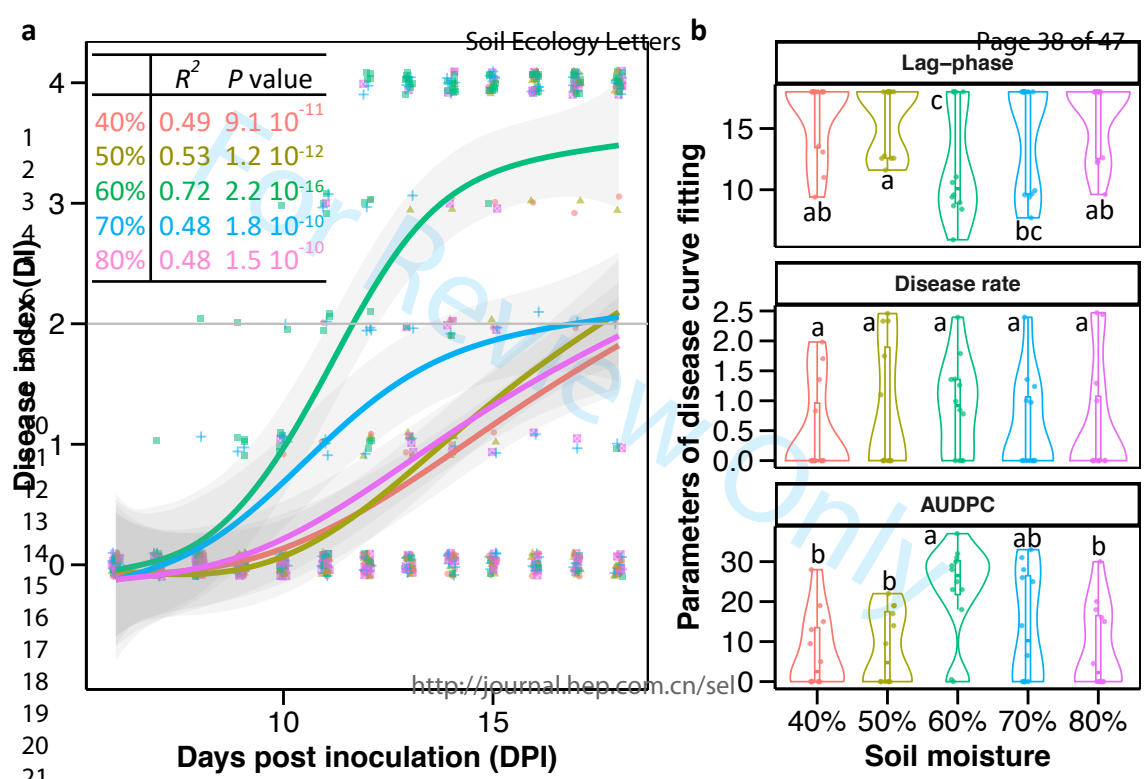
Response 4 to second reviewer: Panels C and D describe overall differences in bacterial community composition and abiotic soil properties using multivariate analysis (Principal component analysis, *i.e.*, PCA). Individual samples are further separated along with the health status of the plant (healthy vs. diseased) and field of isolation in both panels. As a result, each observation (individual dots) represents an overall value based on multiple variables.

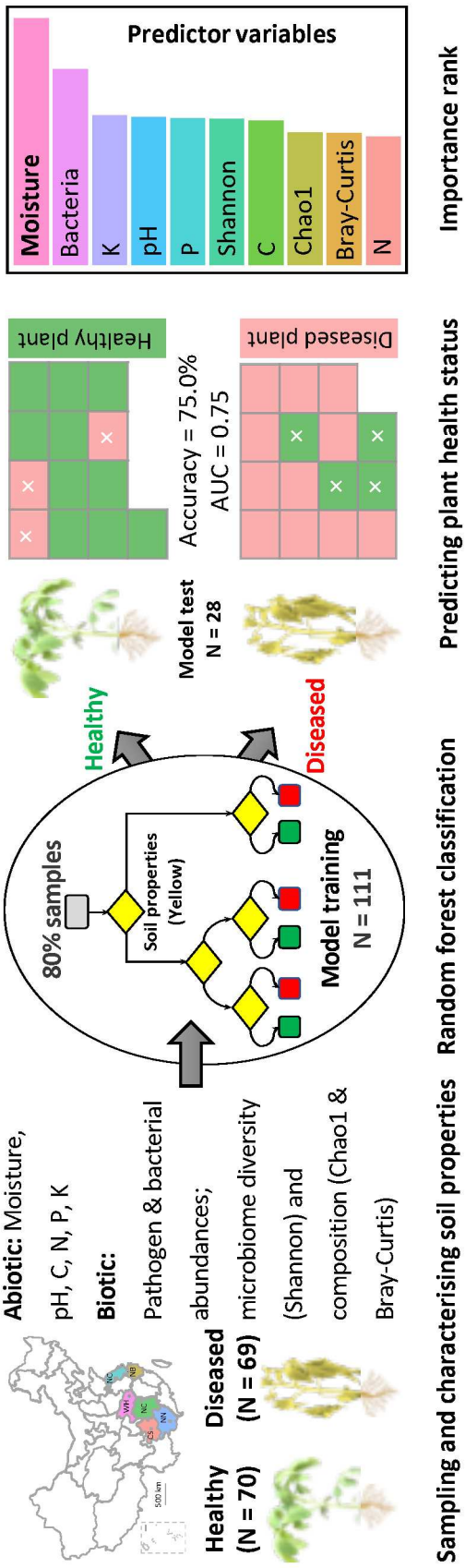

a
Sampling map and experimental design
NC
WH
NB
NC
CS
NN
500 km
CS = Changsha (24)
NJ = Nanjing (23)
NB = Ningbo (24)
NN = Nanning (24)
NC = Nanchang (22)
WH = Wuhan (22)
b
Normalised values of environmental factors
Moisture
pH
Phosphorus
Potassium
Carbon
Nitrogen
Pathogen
Bacteria
Shannon
Chao1
**
ns
ns
ns
ns
ns
****
***
ns
ns
0.0
0.5
1.0
Healthy (n = 70)
Diseased (n = 69)
c
Bacterial community composition
0.25
0.00
−0.25
−0.50
PCo1: 17.26%
CS
NB
NC
NJ
NN
WH
Diseased
Healthy
P_{site} = 0.001
P_{status} = 0.002
−0.25
0.00
0.25
0.50
PCo1: 23.27%
d
Overall soil biotic and abiotic factors
CS
NB
NC
NJ
NN
WH
Diseased
Healthy
4
2
0
-2
PC1: 25.47%
P_{site} = 0.001
P_{status} = 0.001
−4
−2
0
2
PC1: 44.15%










Predictor variables
Moisture
Bacteria
K
pH
P
Shannon
C
Chao1
Bray-Curtis
N
Importance rank
Healthy plant
Diseased plant
Accuracy = 75.0%
AUC = 0.75
Model test
N = 28
Predicting plant health status
Healthy
Diseased
80% samples
Soil properties
(Yellow)
Model training
N = 111
Random forest classification
Abiotic: Moisture,
pH, C, N, P, K
Biotic:
Pathogen & bacterial
abundances;
microbiome diversity
(Shannon) and
composition (Chao1 &
Bray-Curtis)
Diseased
(N = 69)
Healthy
(N = 70)
Sampling and characterising soil properties

Supplementary files

The relative importance of soil moisture in predicting bacterial wilt disease occurrence

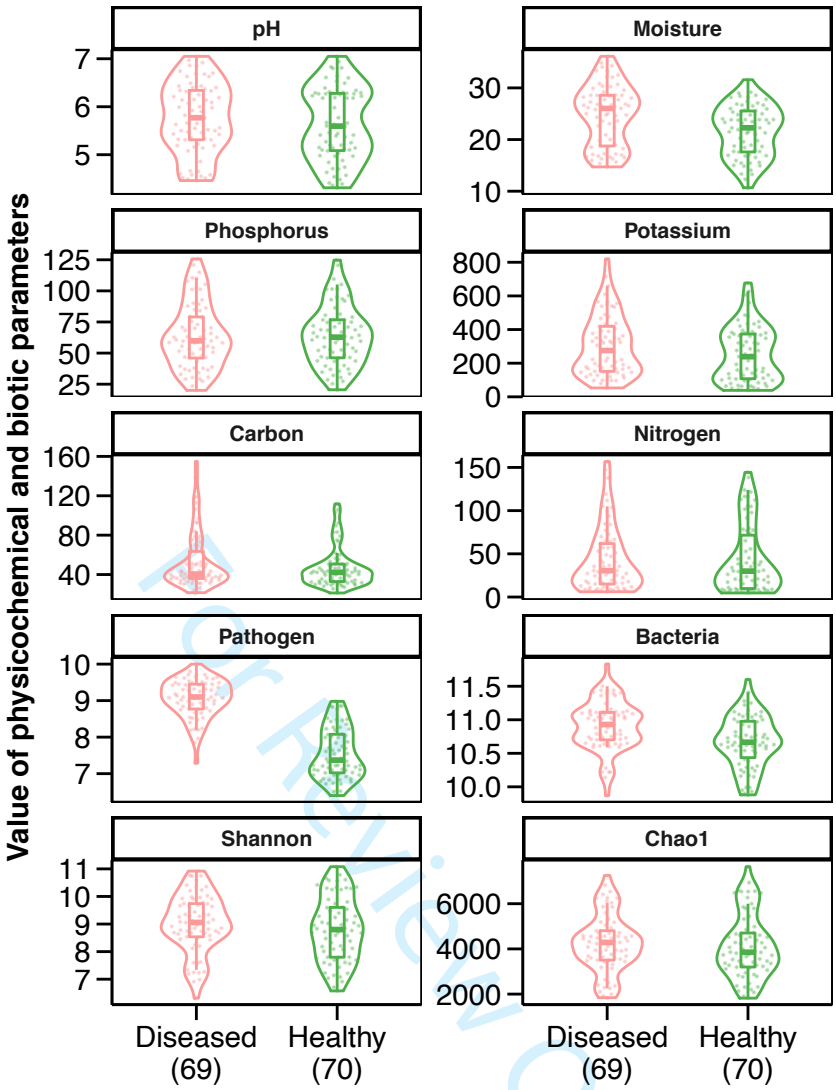
Gaofei Jiang, Ningqi Wang, Yaoyu Zhang, Zhen Wang, Yuling Zhang, Jiabao Yu, Yong Zhang, Zhong Wei, Yangchun Xu, Stefan Geisen, Ville-Petri Friman, Qirong Shen

The supplementary information contains six files. The table file includes statistical information about multiple regression analysis, and figure files provide further detail on the variation of physicochemical properties and bacterial communities in tomato rhizosphere and how it was linked to pathogen density and plant healthy in tomato rhizosphere microbiomes.

Supplementary Table 1. ANOVA table summarizing the relative importance of abiotic physicochemical and biotic soil properties in predicting pathogen densities in tomato rhizosphere samples.

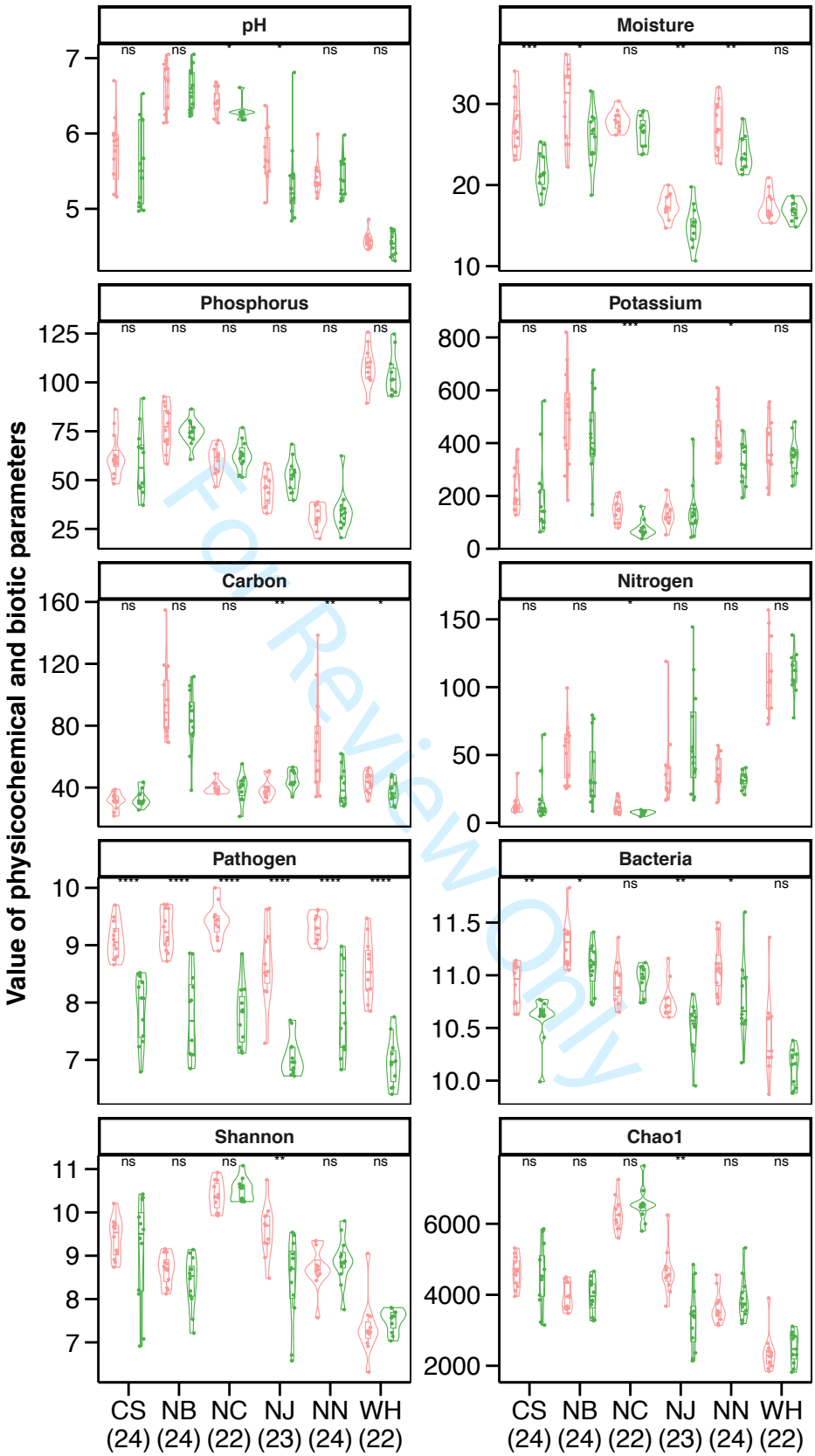
Predictor variable	Df	Sum Square	Mean Square	*F*-value	*P*-value	Relative weight
pH	1	10.29	10.29	18.68	<0.0001↓	14.59%
Moisture	1	28.45	28.45	51.66	<0.0001↑	40.36%
Phosphorus	1	0.14	0.14	0.25	0.6170↑	0.20%
Potassium	1	0.14	0.14	0.26	0.6141↑	0.20%
Carbon	1	0.07	0.07	0.12	0.7310↓	0.09%
Nitrogen	1	0.03	0.03	0.05	0.8271↓	0.04%
Bacterial abundance	1	16.05	16.05	29.15	<0.0001↑	22.77%
Shannon	1	1.78	1.78	3.24	0.0744↑	2.53%
Chao1	1	1.33	1.33	2.42	0.1226↓	1.89%
Bray-Curtis	1	0.11	0.11	0.21	0.6499↑	0.16%
Residuals	128	70.50	0.55			
Model Summary			AIC: 324.09; $F_{10,128} = 10.6$, $R^2 = 0.45$, $P < 0.0001$			

Note: The significant effects ($P < 0.05$) are shown in red colour and the 'up' and 'down' arrows denote for positive and negative effects, respectively, based on multiple regression model.



Supplementary Figure 1. Differences in abiotic physicochemical and biotic soil properties between diseased and healthy plants.

All data is pooled over sampling locations and the numbers in the parentheses denote the sample size in each group. The 'ns' denotes for non-significant difference ($P > 0.05$) and stars (*, **, *** and ****) show significant differences at levels $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively. Pathogen and total bacterial abundances are abbreviated as 'Pathogen' and 'Bacteria', respectively. Each violin plot shows the distribution of 69 and 70 rhizosphere soil samples in diseased and healthy plants, respectively.



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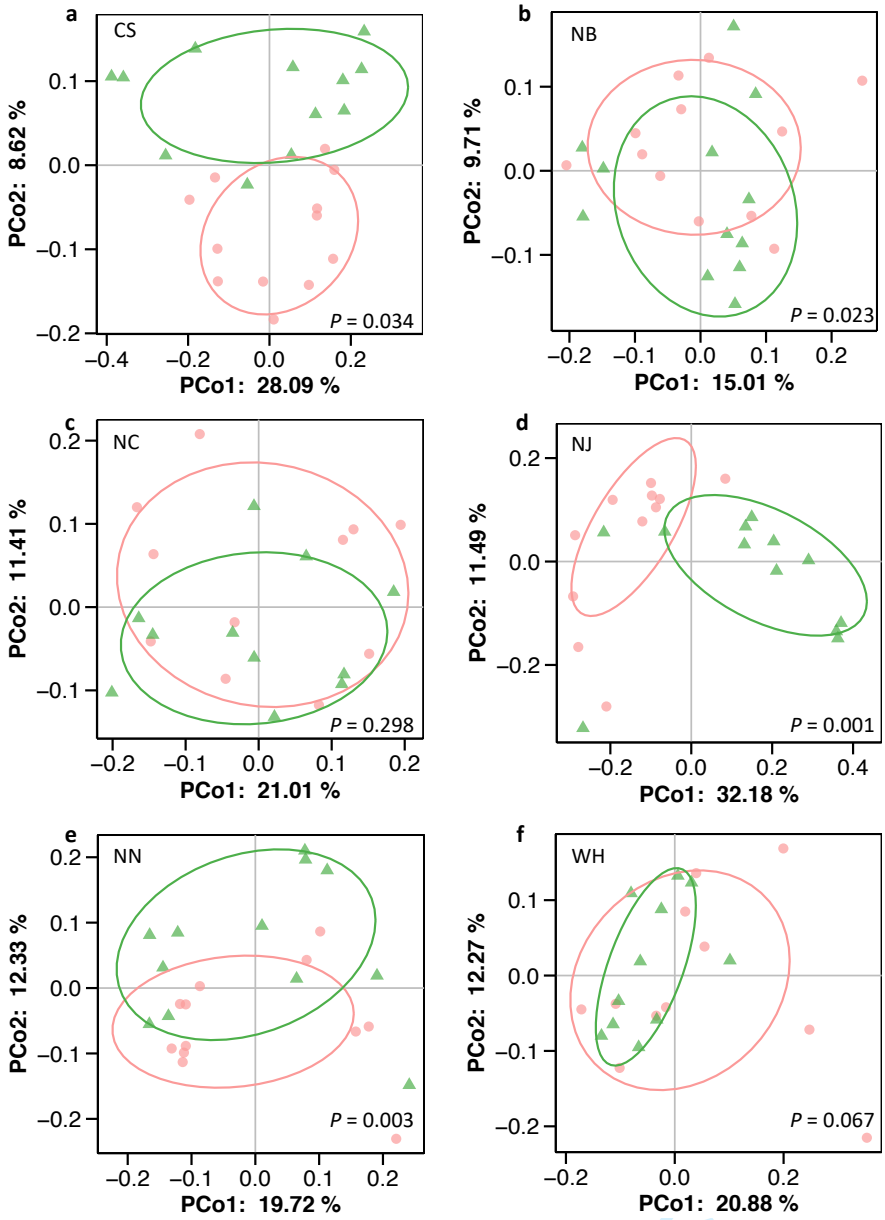
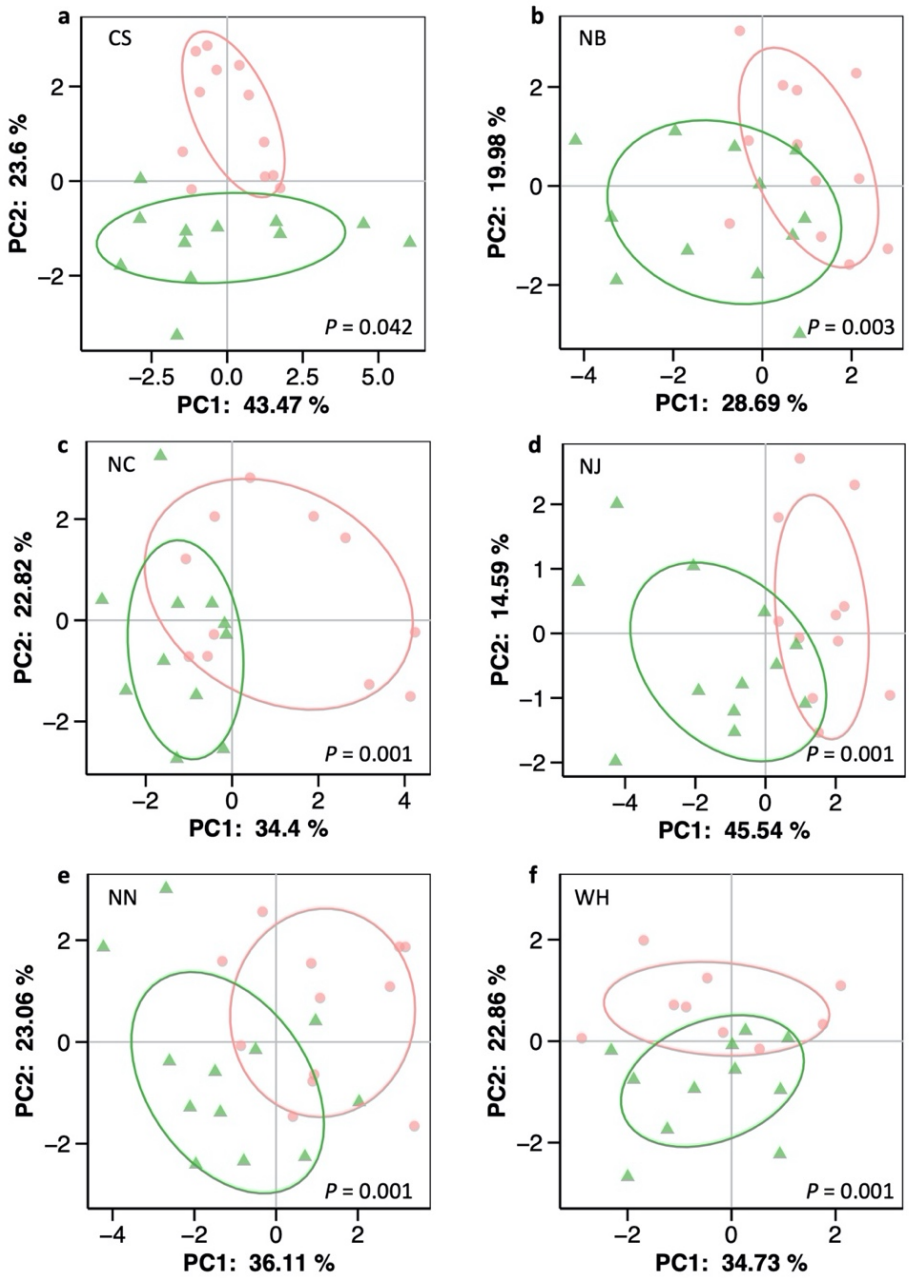
Supplementary Figure 2. Differences in abiotic physicochemical and biotic soil properties of diseased and healthy plants in different sampling locations (provinces). Numbers in the parentheses show sample size in each group. The 'ns' denotes for non-significant difference ($P > 0.05$) and stars (*, **, *** and ****) show significant differences at levels $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively. Pathogen and total bacterial abundances are abbreviated as 'Pathogen' and 'Bacteria', respectively. Each violin plot shows the distribution of rhizosphere soils in each province. Sampling locations are abbreviated as follows: CS = Changsha, NB = Ningbo, NC = Nanchang, NJ = Nanjing, NN = Nanning and WH = Wuhan.



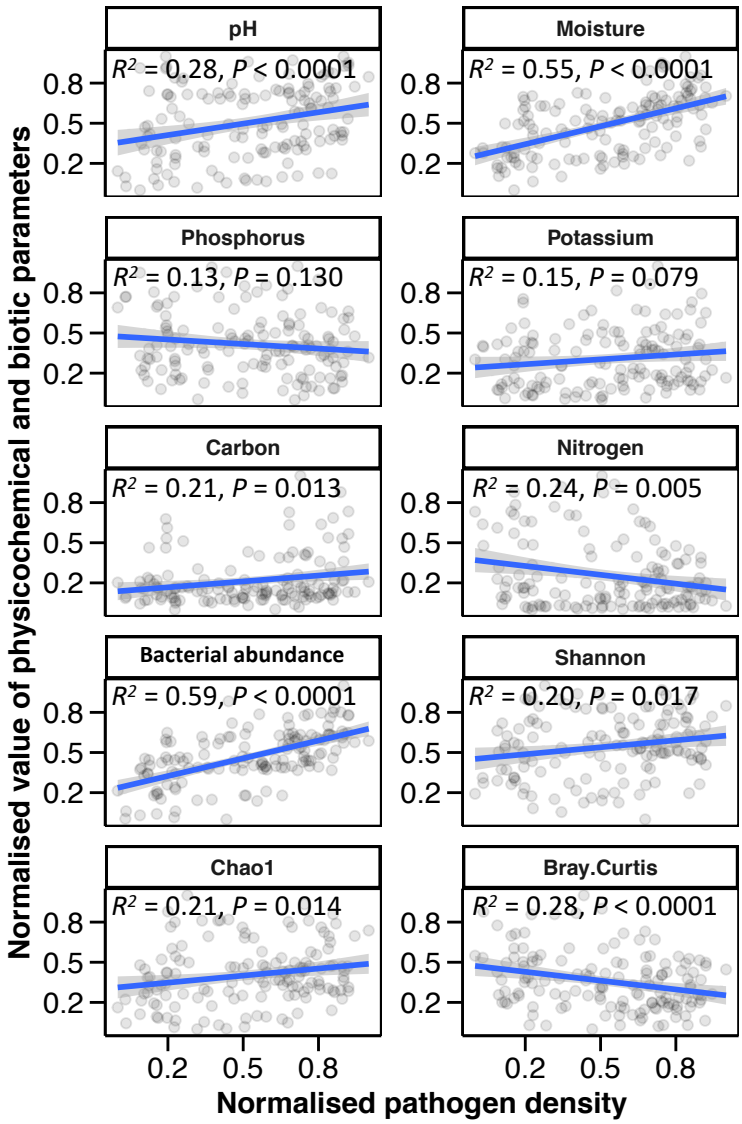
Supplementary Figure 3. Differences in bacterial community composition (PCoA) between healthy (green) and diseased (red) rhizosphere soil samples in different sampling locations (a-f).

PERMANOVA was used to identify microbial composition difference between the rhizosphere soil of diseased and healthy plants in each location based on Bray–Curtis distance matrices. *P*-values are indicated in each panel. Green triangles and red circles denote healthy and diseased plants, respectively. Sampling locations are abbreviated as follows: CS = Changsha, NB = Ningbo, NC = Nanchang, NJ = Nanjing, NN = Nanning and WH = Wuhan.



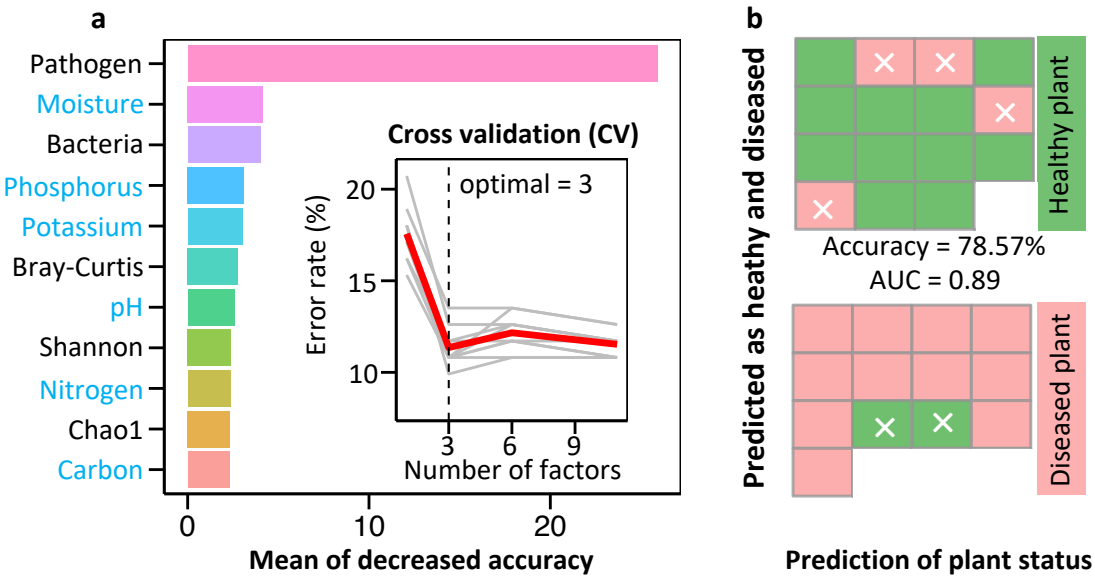
Supplementary Figure 4. Differences in physicochemical and biotic parameters (PCA) between healthy (green) and diseased (red) rhizosphere soil samples in different sampling locations (a-f).

PERMANOVA was used to for identify the environmental difference between the rhizosphere soil of diseased and healthy plants in each location based on Euclidean distance matrices. *P*-values are indicated in each panel. Green triangles and red circles denote healthy and diseased plants, respectively. Sampling locations are abbreviated as follows: CS = Changsha, NB = Ningbo, NC = Nanchang, NJ = Nanjing, NN = Nanning and WH = Wuhan.



Supplementary Figure 5. Linear correlations between the normalised pathogen densities and abiotic physicochemical and biotic soil properties (averaged over heathy and diseased plant samples).

Blue lines indices the linear fitting of curves, while R^2 indicates the Spearman correlation coefficient of the linear regression and P-values the significance of each correlation.



Supplementary Figure 6. Comparing the relative importance of all soil parameters in predicting plant health status.

(**a**) Relative importance rank of overall abiotic physicochemical (blue) and biotic (black) soil properties and ten-fold cross-validation of random forest model (inset in **a**) based on the training set (80% of randomly selected rhizosphere samples). Pathogen and total bacterial abundances are abbreviated as 'Pathogen' and 'Bacteria', respectively. (**b**) Validation of random-forest model with a test set (20% of remaining samples) predicting plant disease outcomes based on soil properties: green and red filled cells denote for correct predictions, while filled cells with white crosses denote for false predictions. The overall model gained an average accuracy (78.6%) in classifying plant status, with 84.6% accuracy for diseased and 73.3% accuracy for health plants (AUC = 0.89).