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Plant pathogen resistance is mediated by recruitment of specific rhizosphere fungi

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Beneficial interactions between plants and rhizosphere microorganisms are key determinants of plant health with the potential to enhance the sustainability of agricultural practices. However, pinpointing the mechanisms that determine plant disease protection is often difficult due to the complexity of microbial and plant-microbe interactions and their links with the plant's own defense systems. Here, we found that the resistance level of different banana varieties was correlated with the plant's ability to stimulate specific fungal taxa in the rhizosphere that are able to inhibit the *Foc* TR4 pathogen. These fungal taxa included members of the genera *Trichoderma* and *Penicillium*, and their growth was stimulated by plant exudates such as shikimic acid, D-(-)-ribofuranose, and propylene glycol. Furthermore, amending soils with these metabolites enhanced the resistance of a susceptible variety to *Foc* TR4, with no effect observed for the resistant variety. In total, our findings suggest that the ability to recruit pathogen-suppressive fungal taxa may be an important component in determining the level of pathogen resistance exhibited by plant varieties. This perspective opens up new avenues for improving plant health, in which both plant and associated microbial properties are considered.

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INTRODUCTION

Plant pathogens represent a serious and growing concern with respect to global food security [1]. Plants, including important crops, have evolved intricate immune systems in which metabolic shifts can help reduce pathogen infection levels or mitigate the negative impacts of infection [2]. Plants can also modulate their susceptibility to pathogen attack by recruiting potentially beneficial microbes to their rhizospheres, via for instance the excretion of signaling compounds or microbial food sources such as salicylic acid [3, 4]. These beneficial microbes can impede the growth or spread of the pathogen, as well as induce the plant's systemic resistance system [5]. However, due to the complexity of interactions between the environment, plant traits, and plant-associated microbes, it often remains difficult to disentangle the mechanisms that differentiate between plant cultivation scenarios that are conducive or resistant to specific plant diseases.

The rhizosphere represents a zone where close interactions between plants and soil microorganisms impact plant performance and health [6], and the rhizosphere microbiome can help plants cope with a range of biotic and abiotic stresses, including pests and diseases, salinity, and drought [7–10]. Rhizosphere microbial community assembly is driven by a combination factors, including the features of the soil environment [11] and the species and genotypic composition of the aboveground vegetation [12, 13]. When threatened by soil-borne pathogens, plants can recruit specific microbial populations and alter the structure and function of their associated rhizosphere microbial community to help maintain plant health [14]. This microbiome engineering is achieved to a large degree by changing the composition of rhizosphere metabolites, which can vary greatly across plant species [15] and plant developmental stages [16]. Although changes in plant metabolic signaling in the rhizosphere have been linked to plant health, we still generally lack knowledge of how pathogen resistance across varieties of the same plant species is related to metabolic differences in the rhizosphere and the potential associated impacts on the rhizosphere microbiome.

Fusarium wilt disease, caused by *Fusarium oxysporum* f. sp *cubense* tropical Race 4 (*Foc* TR4), can have devastating effects on banana crops [17]. *Foc* TR4 is particularly difficult to combat because it produces resting spores (chlamydospores) that facilitate survival in soil in the absence of a host [18], and there is no known method for removing the pathogen from infected soils once plants have become infested [19]. Extensive efforts have therefore been dedicated to developing resistant varieties of banana, but complete disease resistance remains problematic [20]. Disease-resistant varieties are known for many crops, with the ultimate level of disease resistance being determined by both the plant genotype and the associated microbiome [21, 22]. However,

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it is not well known to what extent realized disease resistance is a product of the plant's own ability to resist pathogen attack or the plant's ability to recruit a functional microbiome via metabolic signaling that assists in pathogen suppression [23]. For the effective implementation of plant-resistant varieties and the delineation of proper disease management strategies, it is important to determine the mechanisms that help confer pathogen resistance to banana varieties, including plant attributes, the impact of the soil microbiome, and the combination of these aspects.

Although bacterial communities have often been shown to play a role in the suppression of soil-borne pathogens [21, 24, 25], recent evidence suggests that fungal communities can play a more important role in plant health by promoting plant nutrient uptake and enhancing plant pathogen suppression [26, 27]. Although soil fungal communities are increasingly being recognized for their role in disease suppression, the mechanisms involved in this function still remain poorly understood, especially as related to the in situ role of soil-borne fungi in the development of plant pathogen resistance. Furthermore, niche overlap is an important mechanism by which rhizosphere microbes can inhibit plant pathogens, making soil fungal communities a particularly interesting target of study with respect to *Foc* TR4 suppression in banana [28–30].

In this study, we sought to examine both plant and fungal community components correlated with pathogen suppression across different plant varieties, using banana resistance to *Foc* TR4 as a model system. To this end, we first examined the extent to which the fungal assemblages recruited by resistant varieties might be involved in the observed differences in resistance between banana varieties. We also compared the compositions of rhizosphere fungal communities across varieties with differing resistance capabilities to identify fungal taxa potentially involved in disease resistance. We then recovered isolates of some potential key fungal taxa to examine their ability to impact *Foc* TR4 invasion using a series of pot experiments. To further examine the mechanisms involved in the recruitment of key fungal microbes in the rhizosphere of resistant banana varieties, we analyzed the rhizosphere metabolites of three representative banana varieties (one highly resistant variety, one resistant variety, and one highly susceptible variety) and examined the impacts of specific variety-distinguishing metabolites on the growth of isolates of potential key fungal taxa as well as *Foc* TR4 via vitro assays. We also examined if microbial communities subjected to these metabolites could influence the level of resistance to *Foc* TR4 of susceptible and resistant banana varieties in vivo. Collectively, the aims of this study were to 1) identify fungal taxa associated with the disease-resistant abilities of different banana varieties to *Foc* TR4 and 2) examine potential interactions between rhizosphere metabolites and such recruited fungal taxa as a mechanism of pathogen inhibition. This work may provide crucial insights into the reliance of plant-resistant varieties on fungal recruitment to attain their resistant status.

RESULTS

Disease incidence and pathogen density

The relative abundance of the pathogen decreased along the increasing levels of resistance for the banana varieties examined in our study (Tukey's HSD test: $p < 0.05$; Fig. 1). Based upon resistance level, we grouped the ten varieties into three categories: highly susceptible varieties (50–100% disease incidence), containing "Zhangzhou no.8", "Baxi", and "Williams"; moderately resistant (20–50% disease incidence) and resistant (10–20% disease incidence) varieties, containing "Nongke no.1", "Dongjiao no.1", "Kangku no.1" and "Kangku no.5", respectively; and highly resistant varieties (0–10% disease incidence), containing "Haigong", "Zhongjiao no.9", and "Goldfinger". Overall, the highly

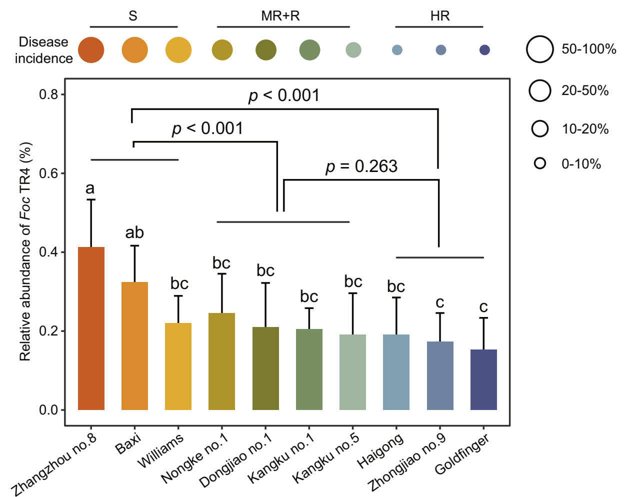

Fig. 1 Disease incidence and relative abundance of *Foc* TR4 in rhizosphere soils of different banana varieties. S represents highly susceptible banana varieties (50–00% disease incidence), containing "Zhangzhou no.8", "Baxi", and "Williams"; MR + R represents moderately resistant (20–50% disease incidence) and resistant (10–20% disease incidence) banana varieties, containing "Nongke no.1", "Dongjiao no.1", "Kangku no.1" and "Kangku no.5"; and HR represents highly resistant banana varieties (0–10% disease incidence), containing "Haigong", "Zhongjiao no.9", and "Goldfinger". Bars with different letters indicate significant differences as defined by one-way ANOVA with Tukey's HSD test ($p < 0.05$). The p values between group comparisons as defined by one-way ANOVA with Tukey's HSD test.

susceptible banana varieties showed the highest relative abundance of *Foc* TR4, followed by the moderately resistant and resistant varieties. The highly resistant banana varieties contained the lowest relative abundances of *Foc* TR4. Therefore, the stronger the resistance of the banana variety, the lower the relative abundance of *Foc* TR4 in the rhizosphere.

To test the ability of rhizosphere microbiota recruited by different banana varieties to inhibit the invasion of *Foc* TR4, we conducted three microbial community transplantation experiments using three banana varieties with different levels of resistance to *Foc* TR4 (Zhongjiao no.9, a highly resistant banana variety; Kangku no.5, a resistant banana variety; Baxi, a highly susceptible banana variety). We found that the abilities of recruited rhizosphere communities from these three varieties to suppress *Foc* TR4 were consistent known resistance levels of the varieties, with the strength *Foc* TR4 suppression displaying the following order: highly resistant banana variety (Zhongjiao no.9) > resistant banana variety (Kangku no.5) > highly susceptible banana variety (Baxi) (Tukey's HSD test: $p < 0.05$; Fig. S1). Thus, banana varieties with a high disease suppression capacity appear to be able to recruit microbiota to the rhizosphere that impede the invasion of the pathogen.

Banana rhizosphere fungal diversity and community structure

Based upon Chao1 and Shannon index values, fungal rhizosphere microbial community diversity was found to be inversely correlated with the ability of the different banana varieties to resist Fusarium wilt disease (Tukey's HSD test: $p < 0.05$; Fig. S2). The highly resistant banana varieties (Haigong, Zhongjiao no.9, Goldfinger) showed lower levels of fungal diversity based upon Chao1 and Shannon index values as compared to fungal communities derived from highly susceptible (Zhangzhou no.8, Baxi, Williams) and moderately resistant and resistant banana varieties (Nongke no.1, Dongjiao no.1, Kangku no.1, Kangku no.5). The highest Chao1 and Shannon index values were observed for

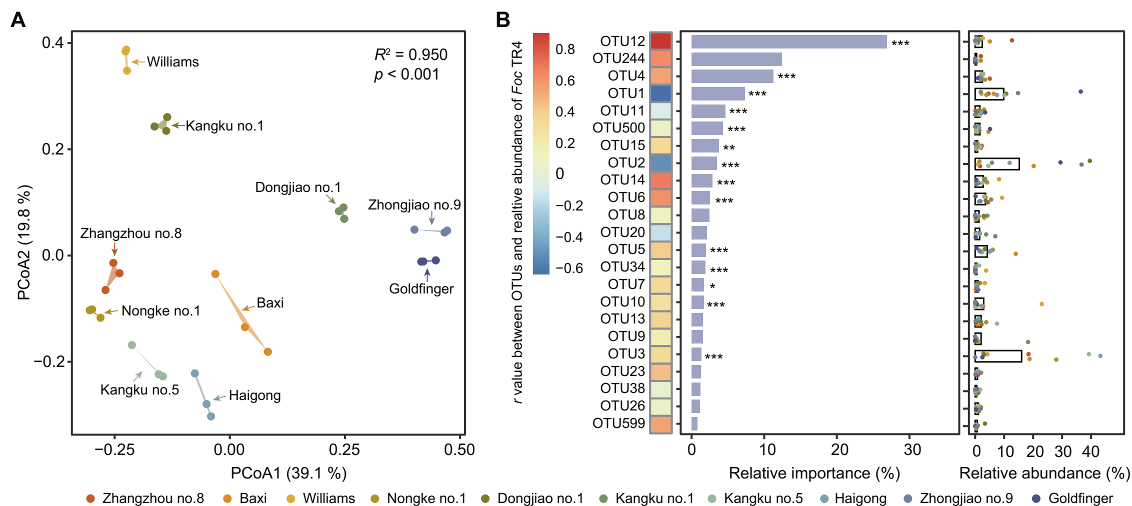

Fig. 2 Properties of the rhizosphere fungal communities associated different banana varieties. A Principal coordinate analysis (PCoA) of fungal communities based on Bray-Curtis distance of rhizosphere soils. **B** Linear models (LM) describing the relationships of microbial indicators with the relative abundance of *Foc* TR4. The relative importance refers to the contributory importance of selected microbial indicators for relative abundance of *Foc* TR4 in linear models. The bar plot indicates the average relative abundance of each OTU in the all samples of the ten varieties, which were calculated by dividing the total abundance in all samples by the number of samples. The points showing the average relative abundance of these OTUs in the rhizosphere soil of each variety, and the average relative abundances were calculated as the total abundance divided by the number of samples for each variety. The p value represents the significance of the predictor in the linear model, as determined by ANOVAs (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

the highly susceptible banana varieties (Zhangzhou no.8, Baxi, Williams). Principal coordinate analysis (PCoA) based on the Bray-Curtis distance revealed significant differences in fungal community composition across the categories of pathogen resistance (PERMANOVA, permutation = 999, $p < 0.001$; Fig. 2A). An overview and additional details of the fungal community sequencing data are given in Supplementary Materials (Figs. S3–5).

Fungal OTUs correlated with resistance to *Foc* TR4

To identify fungal taxa associated with disease resistance in banana varieties, we performed linear models and Spearman's rank correlation analysis. OTU12, OTU4, OTU1, OTU11, OTU500, OTU15, OTU2, OTU14, OTU6, OTU5, OTU34, OTU7, OTU10, and OTU3 were selected from our initial microbial indicators in the linear model, showing significant explanatory power for the relative abundance of *Foc* TR4 ($p < 0.05$; Fig. 2B). In addition, based on our Spearman's correlation analyses between the relative abundance of *Foc* TR4 and these OTUs, we found that OTU1 and OTU2 showed significantly negative relationships with the relative abundance of *Foc* TR4 ($p < 0.05$), with a high level of relative importance in determining the relative abundance of the pathogen (OTU1: 7.3%, OTU2: 3.5%; Fig. 2B). The relative abundances of OTU1 and OTU2 were highest in the highly resistant banana varieties, averaging 20% and 26% of the total fungal community, respectively, and lowest in the highly susceptible banana varieties (Tukey's HSD test: $p < 0.05$; Fig. S6). Based on these results, OTU1 and OTU2, assigned as *Trichoderma* sp. and *Penicillium* sp., respectively, were selected as potential key microbial taxa for resistant banana varieties.

Penicillium and *Trichoderma* isolates and their disease suppression abilities

A total of 74 fungal isolates were recovered from the rhizosphere soil of variety Zhongjiao no.9. Based upon ITS sequence analysis, this collection represented 9 families and included 17 *Penicillium* and 10 *Trichoderma* isolates. Among these, the isolates that were most similar to OTU1 and OTU2, based upon sequence analysis, were *Trichoderma*_72 (T72) and *Penicillium*_61 (P61), respectively (Fig. 3A).

The selected strains included T72, P61, and other *Trichoderma* (*Trichoderma*_19 and *Trichoderma*_22) and *Penicillium* (*Penicillium*_1 and *Penicillium*_91) strains that were phylogenetically distant from OTU1 and OTU2, as well as the negative control strains (*Alternaria*_15, *Aureobasidium*_59, *Humicola*_40, and *Davidiella*_6). We carried out a pot experiment to determine the ability of this extended range of strains to suppress the pathogen. The results showed that for three banana varieties, all selected *Trichoderma* strains (T72, *Trichoderma*_19, and *Trichoderma*_22), P61, and the multi-isolate treatments significantly induced *Foc* TR4 suppression, with the combination of T72 with P61 yielding the greatest level of *Foc* TR4 suppression. The negative control strains had no significant inhibition effects on the pathogen (t test: $p < 0.05$; Fig. 3B).

Composition of rhizosphere metabolites of Baxi, Kangku no.5, and Zhongjiao no.9 banana varieties

A total of 119 compounds were detected from 18 rhizosphere soil samples of the three examined banana varieties by GC-MS (Fig. S7). Principal component analysis (PCA) ordination showed that the rhizosphere metabolites of the three banana varieties were significantly distinct from each other (Adonis, permutation = 999, $p = 0.004$; Fig. S8), and the first two principal components explained 39.6% (24.8% and 14.8%, respectively) of the total variation. Many of the same metabolites were detected across the three banana varieties, but distinct differences in the relative abundances of some compounds were observed (Table S1). The relative abundances of five compounds in Zhongjiao no.9 were significantly higher as compared to Baxi, while the relative abundances of 17 compounds were significantly lower in the comparison of these two varieties (Fig. 4A). The five compounds enriched in Zhongjiao no.9 variety were nitrilotriethanol, shikimic acid, stearic acid, silane, and D-(-)-ribofuranose (Fig. 4B). When comparing Kangku no.5 with Baxi, 12 compounds were relatively higher in Kangku no.5, including (2-propenylthio)-acetic acid, other-C1, ketone-C, nonanoic acid, butylphosphonic acid, other-C2, acetamide, amine, N-ethyl-acetamide, heterocyclic-C, nitrilotriethanol, and propylene glycol (t test: $p < 0.05$; Fig. 4C, D).

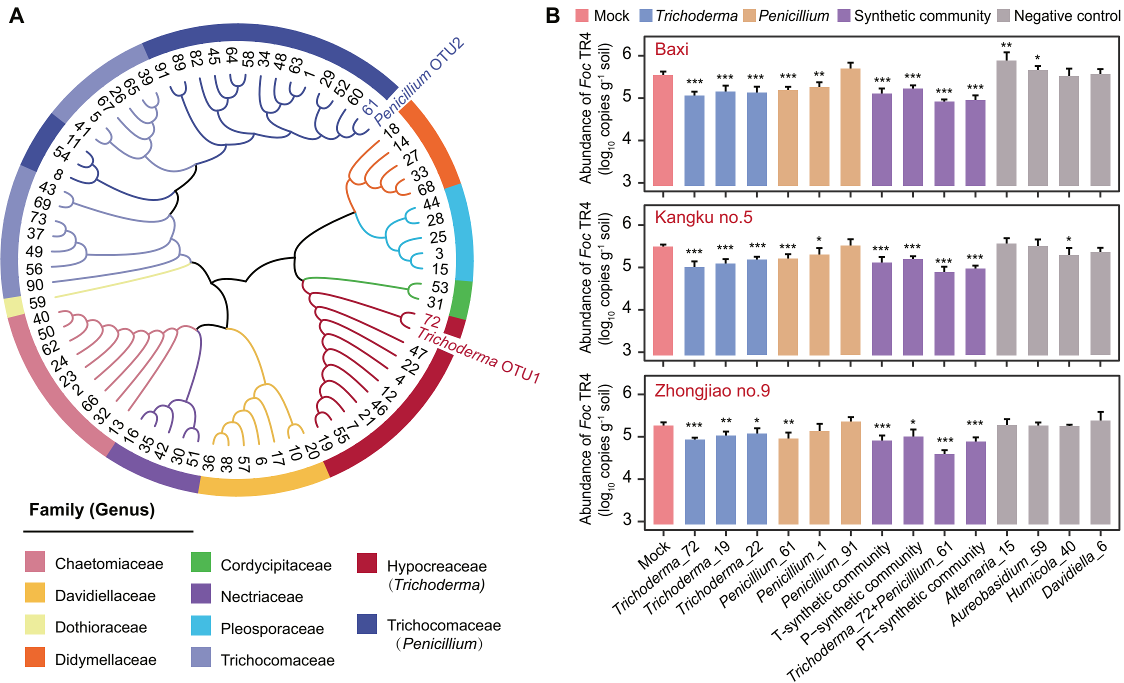

Fig. 3 Identification of fungal isolates and pathogen suppression effects of selected fungal isolates. A Cladogram showing the phylogenetic relationships around OTU1, OTU2, and 74 rhizosphere fungal isolates. Leaf labels indicated representative sequence IDs. The inner ring indicates the species-level taxonomy. **B** Abundance of *Foc* TR4 in rhizosphere soils of Baxi, Kangku no.5, and Zhongjiao no.9 varieties inoculated with the given fungal isolates. Baxi_ highly susceptible banana variety; Kangku no.5: resistant banana variety; Zhongjiao no.9: highly resistant banana variety. Asterisks indicate significant difference between the control and each isolate inoculation treatment in the corresponding variety as determined with a *t* test and resulting *p* values were corrected using false discovery rate (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

Interactions between variety-distinguishing metabolites and isolates from key fungal taxa

We conducted network analyses to explore potential relationships between metabolites whose concentrations differed across varieties and the corresponding differential enrichment of fungal OTUs in the rhizosphere. The results showed that the fungal OTUs that were relatively more abundant in the rhizosphere soil of Zhongjiao no.9 were positively correlated with the compounds nitrilotriethanol, shikimic acid, stearic acid, silane, and D-(-)-ribofuranose. In contrast, specific fungal taxa associated with the rhizosphere soil of Baxi were negatively correlated with these compounds. We also found a consistent pattern in the Kangku no.5 rhizosphere soil: fungal OTUs enriched in the Kangku no.5 rhizosphere were positively correlated with the compounds that increased significantly in this variety, while an opposite pattern of OTU enrichment was observed in the Baxi rhizosphere soil. This suggests that the observed differences of fungal communities in the rhizosphere of different banana varieties may be influenced by changes in the relative abundances of these metabolites (Fig. S9).

To examine potential interactions between differentially detected metabolites and putative key fungal taxa, four compounds (shikimic acid, D-(-)-ribofuranose, propylene glycol, and acetamide) were tested for their effects on the growth of isolates T72 and P61, as well as that of *Foc* TR4. The results of 96-well cell culture assays showed that high concentrations of shikimic acid had a significant inhibitory effect on T72 and P61, as well as *Foc* TR4. High concentrations of propylene glycol and acetamide also inhibited the growth T72 and P61. For the T72 isolate, the selected compounds generally had the most positive effect on growth in the concentration range of 0.1 to 0.5 mM. The largest growth effects for P61 were mostly seen at a concentration of 0.1 mM (Tukey's HSD test: $p < 0.05$; Fig. S10). In light of these results, 0.1 mM was selected for a subsequent shaking flask experiment. For *Foc* TR4, acetamide was shown to significantly promote growth, while the other three compounds had no clear effects.

We found that shikimic acid, D-(-)-ribofuranose, propylene glycol, and acetamide significantly increased the abundance of T72 and P61 using a shaking flask experiment (*t* test: $p < 0.05$; Fig. 5A). Shikimic acid also significantly promoted the growth of *Foc* TR4 (*t* test: $p < 0.05$; Fig. 5A), while D-(-)-ribofuranose, propylene glycol, and acetamide had no discernable effects. When T72 or P61 was co-cultured with *Foc* TR4, they inhibited the growth of the pathogen. The strongest effect was observed when *Foc* TR4 was grown together with a combination of T72 and P61. Compared with the control, the addition of shikimic acid, D-(-)-ribofuranose, and propylene glycol significantly enhanced the inhibitory effect of T72 or P61 on *Foc* TR4, while acetamide decreased the level of inhibition (*t* test: $p < 0.05$; Fig. 5B). Thus, these selected rhizosphere metabolites not only affected the growth of T72 and P61, but also impacted their interactive effects on the pathogen. Importantly, shikimic acid, D-(-)-ribofuranose, and propylene glycol appeared to enhance the pathogen inhibition abilities of T72 and P61.

Effects of the exogenous metabolites on the key fungal taxa and the susceptibility of different varieties to *Foc* TR4

For the soil incubation experiment, natural soil was conditioned by amendments of shikimic acid, D-(-)-ribofuranose, and propylene glycol at two application concentrations (0.1 mM and 1 mM), and the abundances of *Trichoderma* and *Penicillium* were tracked in soil in response to the addition of these three metabolites. Compared with the control, shikimic acid significantly increased the abundance of *Trichoderma* and *Penicillium* at the higher concentration (1 mM) (*t* test: $p < 0.05$; Fig. S11). In addition, slight stimulation by exogenous D-(-)-ribofuranose (0.1 mM and 1 mM) and propylene glycol (0.1 mM) was observed for *Trichoderma* and *Penicillium*, as compared to the control

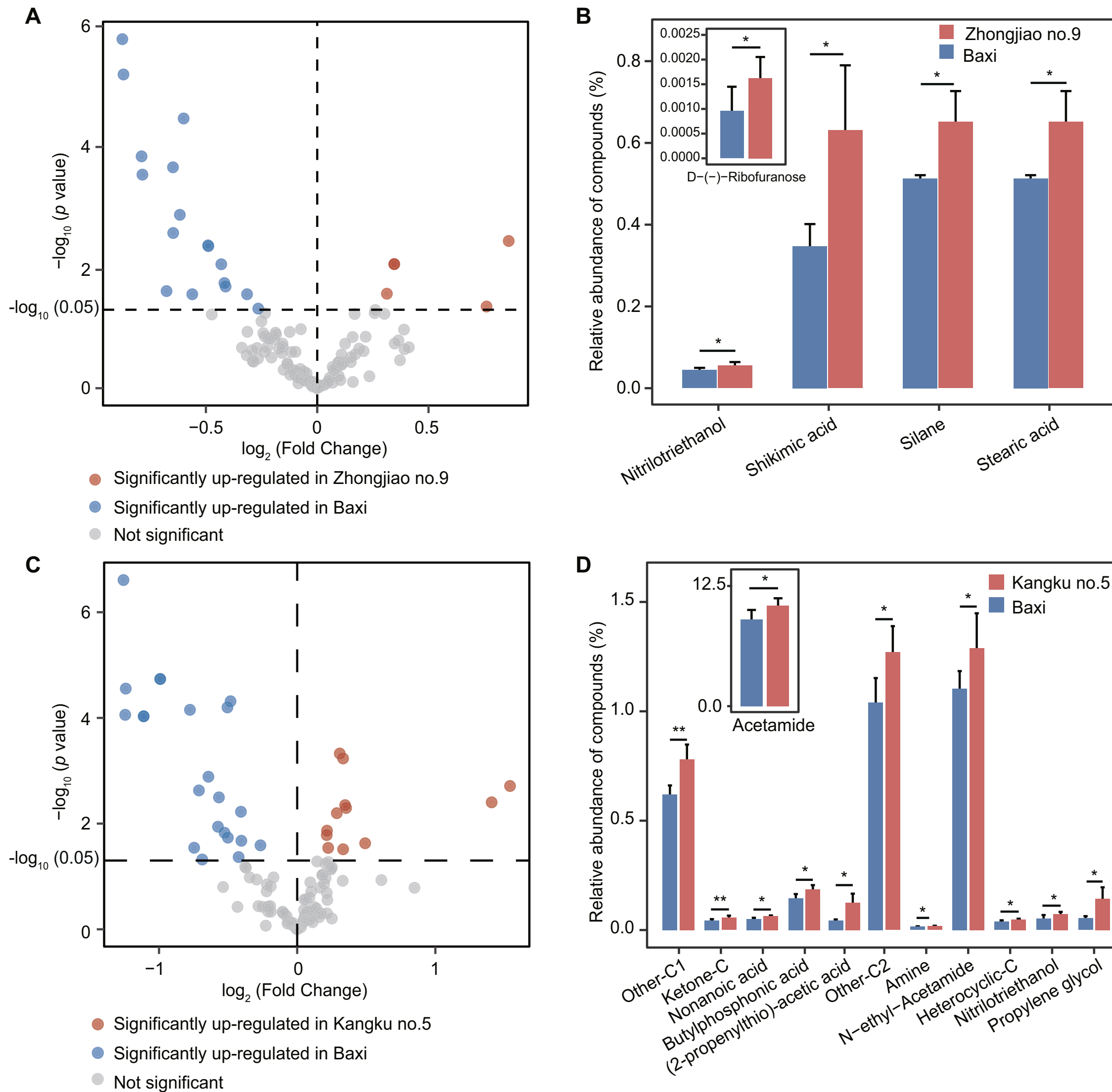

Fig. 4 Metabolite analysis of rhizosphere soils of different banana varieties. A Volcano plots of metabolites in Baxi and Zhongjiao no.9 rhizosphere soils. **B** The relative abundance of compounds enriched in Zhongjiao no.9 rhizosphere soil as compared to Baxi. **C** Volcano Plots of metabolites in Baxi and Kangku no.5 rhizosphere soils. **D** The relative abundance of compounds enriched in Kangku no.5 rhizosphere soil as compared to Baxi. Baxi_ highly susceptible banana variety; Kangku no.5: resistant banana variety; Zhongjiao no.9: highly resistant banana variety. The grey line on the x axis is used to determine whether the metabolites in Zhongjiao no.9 or Kangku no.5 varieties have increased or decreased compared to the Baxi variety, with lower on the left and higher on the right. The gray line on the y axis was determined by the value of $-\log_{10}$ (0.05), which refers to the $\log_{10}$ value of $p = 0.05$. Dots above the line indicate a significant difference, and dots below indicate no significant difference. Red, blue and gray dots represent significantly up-regulated, down-regulated and not significant compounds compared with Baxi, respectively. Asterisks indicate significant differences between treatments as determined with a *t* test, and resulting *p* values were corrected using false discovery rate (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Bars represent the average of six replicates and error bars show standard deviations.

(*t* test: $p > 0.05$; Fig. S11). These results suggest that the supplementation of exogenous metabolites to soil had a stimulatory effect on potentially disease-suppressive fungal taxa, although the effects were expectedly smaller than those observed using in vitro assays.

We further examined the effect of exogenous supplementation of these three metabolites on the susceptibility of different varieties to *Foc* TR4 using a pot-based experiment. We again observed that there were significant differences in the abundance of the pathogen across the three varieties (Baxi, Kangku no.5, and Zhongjiao no.9) in the unamended control (Tukey's HSD test: $p < 0.05$; Fig. 6A), with the addition of exogenous metabolites reducing the difference in pathogen abundances in the rhizospheres of varieties with different levels of pathogen resistance (*t* test: $p > 0.05$; Fig. 6B). Specifically, in the highly resistant variety Zhongjiao no.9, the difference in pathogen abundance in the presence of exogenous metabolites compared to the control was marginal (*t* test: $p > 0.05$; Fig. 6B). In the resistant variety Kangku no.5, the abundance of the pathogen was significantly reduced after exogenous supplementation of propylene glycol, as compared to the control (*t* test: $p < 0.05$; Fig. 6B). For the susceptible variety Baxi, the abundances of *Foc* TR4 were lower after the addition of the three metabolites, compared to the control (*t* test: $p < 0.05$; Fig. 6B). Shikimic acid and D-(-)-ribofuranose had more significant inhibitory effects on pathogen density at the higher concentration (1 mM), while propylene glycol showed a stronger inhibitory effect on the pathogen at the lower concentration (0.1 mM).

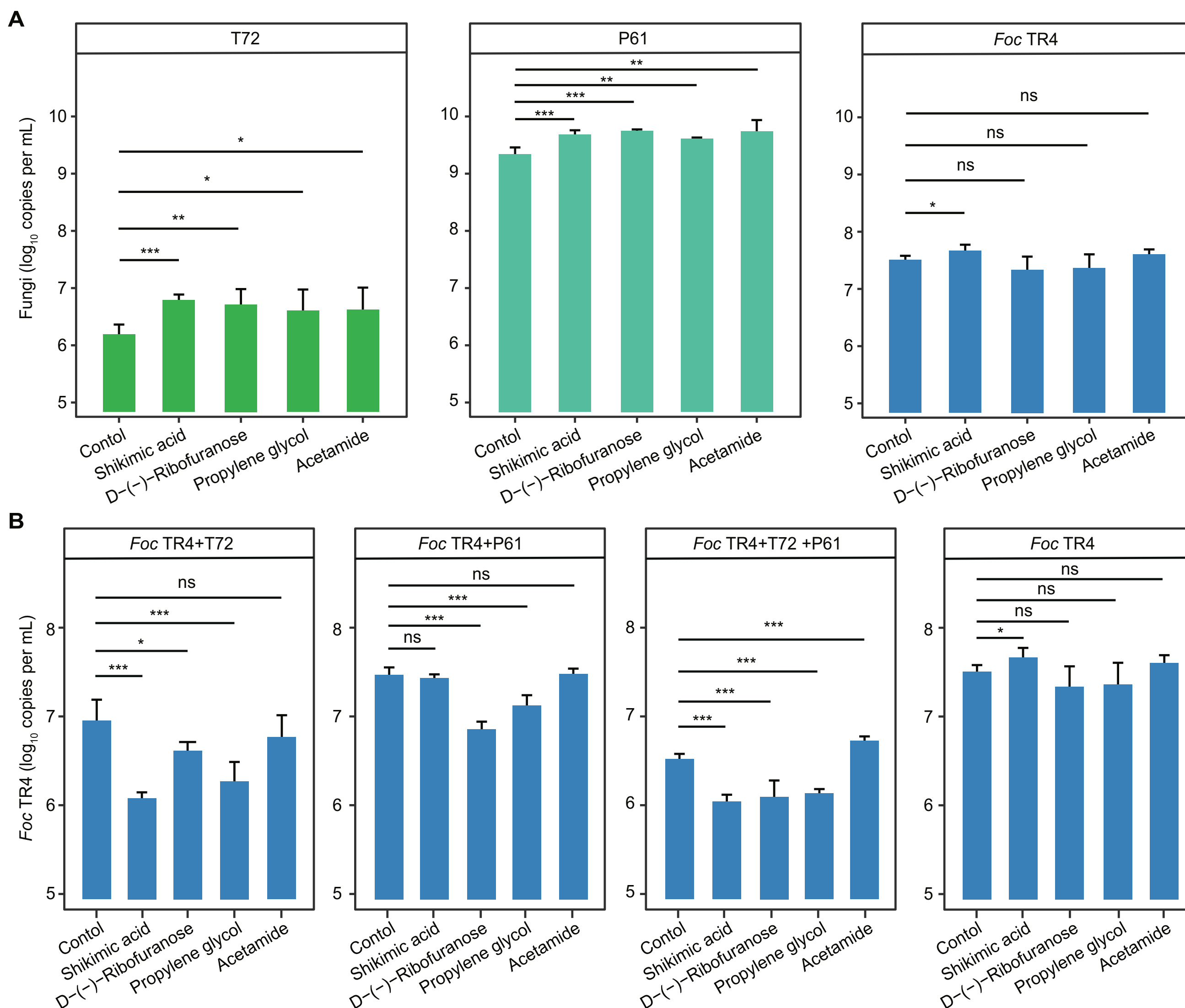

Fig. 5 Effects of pure metabolites on the growth of putative key fungal taxa in vitro. A Abundance of T72, P61, and *Foc* TR4 in the treatments with different compounds added during separate shaking culture, respectively. **B** Abundance of *Foc* TR4 in the treatments with different compounds added during the shaking culture alone or in combination. Asterisks indicate significant differences between control and other treatments by the *t* test and resulting *p* values were corrected using false discovery rate (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), ns indicates that there was no significant difference between the two treatments. Bars represent the average of six replicates, and error bars show standard deviations.

DISCUSSION

In this study, we sought to examine the extent to which resistant plant varieties might achieve their disease resistance via recruitment of specific microbial taxa that help to assist in pathogen inhibition. We specifically focused on the development and impact of fungal communities in our study, which have recently been implicated as important determinants of plant disease resistance and health [26, 27]. Furthermore, fungal isolates, especially of the genus *Trichoderma*, have been studied intensively for their biocontrol potential against fungal plant pathogens. However, relatively little is yet known about the in situ role of soil-borne fungi in the development of plant pathogen resistance. We first examined fungal communities in the rhizosphere of ten banana varieties to identify fungal taxa that were correlated with the level of disease resistance observed. We also explored potential interactions involved in disease suppression by combining metabolomics and cultivation-dependent approaches. We demonstrate that disease-resistant phenotypes of banana varieties are related to differential secretion of specific root exudates that recruit beneficial *Trichoderma* and *Penicillium* populations able to inhibit the pathogen.

We showed that resistant banana varieties are associated with a lower relative abundance of pathogens, which strongly determines the health of the plant [31]. Rhizosphere fungal community structure also varied across the ten banana varieties, which is similar to previous findings [21, 24]. These results suggest that fungal recruitment in the rhizosphere may represent an important component of plant disease resistance. We observed that the rhizosphere fungal community composition of susceptible varieties (e.g. Baxi and Williams) responded differently, suggesting that additional resistance mechanisms such as plant immune systems exist in these varieties that may account for their different disease susceptibility phenotypes [32, 33]. Furthermore, we found that specific fungal taxa, namely *Trichoderma* spp. and *Penicillium* spp., were important determinants of disease suppressive plant phenotypes. These taxa have previously been identified for their roles in the control of fungal plant pathogens [34, 35]. We also observed a negative correlation between the level of plant

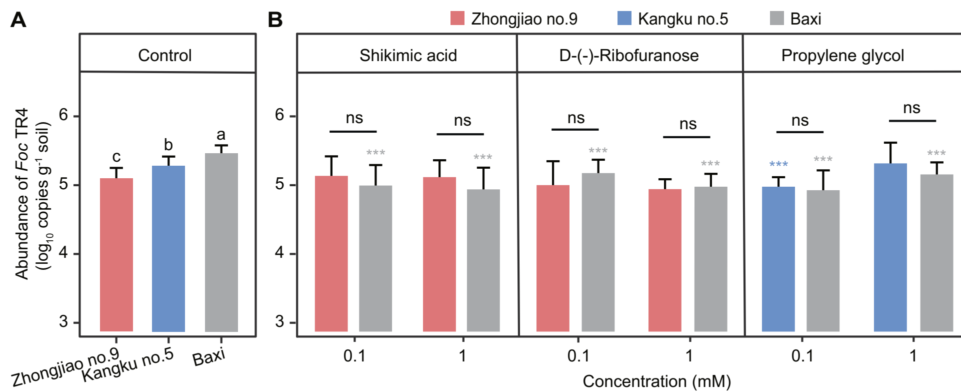

Fig. 6 Effects of the exogenous metabolites on the susceptibility of different varieties to *Foc* TR4. A Abundance of *Foc* TR4 in rhizosphere soils of Baxi, Kangku no.5, and Zhongjiao no.9 treated with sterile water. **B** Abundance of *Foc* TR4 in rhizosphere soils of Baxi, Kangku no.5, and Zhongjiao no.9 treated with shikimic acid, D-(-)-ribofuranose, and propylene glycol. Baxi_ highly susceptible banana variety; Kangku no.5: resistant banana variety; Zhongjiao no.9: highly resistant banana variety. Bars with different letters indicate significant differences as defined by one-way ANOVA with Tukey's HSD test ($p < 0.05$). Asterisks indicate significant differences between control and other treatments in the corresponding variety by the *t* test and resulting *p* values were corrected using false discovery rate (***$p < 0.001$). Ns indicates that there was no significant difference between the two varieties with exogenous supplementation of metabolites by the *t* test and resulting *p* values were corrected using false discovery rate.

resistance and alpha diversity of the rhizosphere fungal community. Numerous studies have examined the relationship between soil-borne microbial diversity, including fungal diversity, and soil functions such as resistance to pathogen invasion [36, 37]. In our experiment, it appears that the strong recruitment of specific fungal taxa, such as OTUs 1 and 2, leads to lower evenness in the rhizosphere communities of more resistant varieties, resulting in lower measures of alpha diversity. Similar decreases in fungal alpha diversity were previously found to be associated with increased suppression of soil-borne plant diseases [38].

We also found a large degree of variation in the relative abundances of different rhizosphere metabolites across banana varieties, a result that is line with a previous examination of rhizosphere metabolites across different rice accessions [39]. The composition and abundance of plant exudates has been demonstrated to be closely linked with rhizosphere microbial community assembly and function [40]. We found that the concentrations of specific metabolites differed significantly between resistant and susceptible varieties, suggesting differential exudate production as a mechanism for recruiting functionally different fungal communities across varieties. This mechanism was further explored via correlation-based network analyses, which indicated positive associations between heightened levels of certain metabolic compounds and specific fungal taxa in resistant varieties. The strong positive correlation between metabolites and *Trichoderma* OTU1 and *Penicillium* OTU2 further suggested that these taxa may play an important role in conferring pathogen resistance. Both *Trichoderma* and *Penicillium* have been shown to be able to inhibit pathogenic fungal growth [41, 42], with competition for nutrients being one of the important mechanisms involved [43].

We further substantiated the role of *Trichoderma* strain T72 and *Penicillium* strain P61, which corresponded to *Trichoderma* OTU1 and *Penicillium* OTU2, in the suppression of the pathogen using a combination of in vivo and in vitro experiments. Both strains inhibited pathogen proliferation, and their combination was most effective at controlling the pathogen, which is in line with the proposition that synergistic microbial interactions may be important determinants of soil functionality [44]. Moreover, we found that shikimic acid, D-(-)-ribofuranose, and propylene glycol enhanced the inhibitory effects of T72 or P61 against the pathogen in vitro. Furthermore, soil amendments with these metabolites in the absence of the plant lead to increased suppression of the pathogen, demonstrating that the stimulation of disease-suppressive fungal taxa acts through direct fungal interactions. Indeed, our results also showed that the presence of shikimic acid, D-(-)-ribofuranose, and propylene glycol further enhance the pathogen inhibition capabilities of the co-inoculation of T72 and P61. Previous studies have also revealed that the beneficial effects of functional microbes are modulated by plant genotype [45] and plant-derived compounds [46]. Our findings also showed that the exogenous amendment of metabolites affects the susceptibility of susceptible and resistant varieties to *Foc* TR4, and these metabolites enhanced the resistance of the susceptible variety, but had no effect for the resistant variety. Recently, similar types of metabolite amendments were reported to cause varying levels of morbidity [47]. Future studies are required to disentangle the mechanisms of molecular communication between plants and rhizosphere microbes, and how their combined effects are mediated by metabolites to promote disease suppression.

CONCLUSIONS

We demonstrate that an important component of observed disease resistance may be attributed to the plant's ability to recruit a disease-suppressive fungal community in the rhizosphere. More resistant plant varieties are able to enhance the abundance of particular beneficial fungi by secreting higher abundances of specific metabolites. The actions of these fungal recruits reduce the colonization capacity of the pathogen in the rhizosphere, ultimately protecting plants from pathogen infection and maintaining plant health (Fig. 7). Our data support the hypothesis that plant resistant varieties are resistant at least to some extent due to their ability to recruit pathogen-inhibitory microbes to their rhizospheres. This represents a new target for future breeding programs to improve disease resistance in which breeding is directed toward the ability to recruit pathogen inhibition to the rhizosphere rather than just breeding for direct plant resistance traits.

MATERIALS AND METHODS

Field site description and sampling

Soil samples were collected from fields cultivating ten different banana varieties with a range of resistance to *Foc* TR4 within Dongguan County (113°69′ E, 23°01′ N), Guangdong Province, China. The climate of this area is subtropical monsoon, with an annual mean total precipitation of

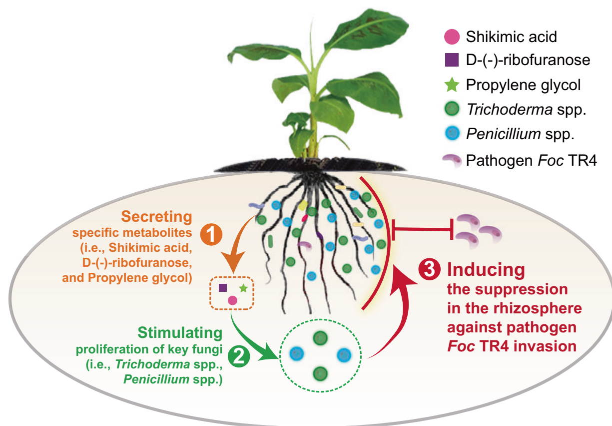

Fig. 7 Conceptual model for the interaction mechanisms among plant, pathogen and beneficial fungal key taxa derived by rhizosphere metabolites. In the rhizosphere of resistant banana variety, high content of specific metabolites can result in a compositional shift of the rhizosphere microbiota by enrichment of *Trichoderma* and *Penicillium*, which reduce the colonization of pathogen in the rhizosphere and protect plants from pathogen infection. *Foc* TR4: *Fusarium oxysporum* f. sp *cubense* tropical Race 4.

1800 mm. The average annual temperature is approximately 22 °C. The soil has been classified as paddy soil with a pH of 4.6, electrical conductivity of 133 µs/cm, available phosphorus of 80 mg/kg, and available potassium of 178 mg/kg. All of these fields are subject to typical banana cultivation practices. Bulk soil and rhizosphere soil samples were collected before the banana harvest in 2018. In brief, three healthy banana trees in each subplot were randomly selected following the S-type sampling method, and three soil cores near the drip line for each tree were collected using a 25 mm soil auger to a depth of 20 cm [48]. The soil cores collected from each plot were thoroughly mixed into a single composite soil sample, and about 300 g soil was retained by the quartering method for each plot. After removing plant residues by sifting through a 2 mm sieve and mixing the sample [49], a part of soil sample was naturally air-dried in the laboratory for soil chemical properties analysis, and the remaining sample material was stored at −80 °C for DNA extraction. Roots of banana trees were collected at the same time as bulk soil sampling, and rhizosphere soil was recovered as previously described [50], and stored at −80 °C prior to DNA extraction. In total, 60 soil samples (ten banana varieties × three replicates × two positions (bulk and rhizosphere)) were collected. The banana varieties used in this study and their resistance levels to *Foc* TR4 are given in Table S2.

DNA extraction and high-throughput sequencing

Soil (0.5 g) of each sample was used for DNA extraction using the PowerSoil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The concentration and quality of the extracted DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The ITS1 region of ITS was amplified from soil genomic DNA using the primers ITS1F and ITS2, in accordance with previously described protocols [51]. Details of the primers are provided in Table S3 [52, 53]. Paired-end amplicon sequencing was performed on a MiSeq PE 250 platform (Illumina) at Personal Biotechnology Co., Ltd (Shanghai, China).

Bioinformatic analyses

According to unique barcodes and primer sequences, raw sequences were split and then the adaptor and primer sequences were removed using QIIME [54] and FLASH [55]. Forward and reverse sequences of each sample were merged after discarding low-quality sequences. The retained sequences were then grouped into operational taxonomic units (OTUs) at a 97% similarity level using UPARSE [56], and taxonomic assignment was performed using the Ribosomal Database Project (RDP) against the UNITE Fungal ITS database with the online version of the RDP classifier [57]. All raw sequence data have been deposited in the NCBI Sequence Read Archive database under the accession number PRJNA830664.

The OTU table and the species annotation table were combined for subsequent microbial community analysis. To eliminate the result bias caused by uneven sequencing depth between samples, the samples were normalized according to the rarefaction (Fig. S12) at the minimum sample sequencing depth (the sequence number was 29,142 reads), and all further analyses were performed based on the normalized sequence data [58]. Firstly, the relative abundance of a given taxonomic group per sample was calculated as the percentage of the number of sequences affiliated with that group to the total number of sequences. Chao1 richness and Shannon index diversity, analyzed in MOTHUR [59], were used to estimate alpha diversity of rhizosphere fungal community. Principal coordinate analysis (PCoA, based on Bray-Curtis dissimilarity matrix) was performed to compare the differences in fungal community structure across all samples, and the significant differences among treatments were tested using permutational analysis of variance (PERMANOVA, with transformed data by Bray-Curtis, permutation = 999) with the "vegan" package [60].

Quantitative real-time PCR analyses

The abundances of total fungi and *Foc* TR4 in rhizosphere soil samples were quantified by quantitative real-time PCR (qPCR), following previously reported protocols [61]. In brief, abundances of fungi and *Foc* TR4 were determined via ITS1f/5.8 s and FocSc-1/FocSc-2 targets, using the SYBR Green assay on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) [62, 63] (Table S3). Standard curves were prepared by using 10-fold serial dilutions ranging from 10^8 to 10^2 of plasmids containing the 18 S rRNA gene from *Saccharomyces cerevisiae* and the ITS gene from the *Foc* TR4 strain [64]. The qPCR amplifications were carried out in 96-well plates with a total volume of 20 µl for each reaction, containing 1 µl target DNA, 10 µl SYBR Green premix Ex Taq (2×), 1 µl of each primer and 7 µl distilled water. The thermal cycling conditions were 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s and 65 °C for 34 s. Three replicates were performed for each assay, and the results were expressed as $\log_{10}$ values for the target copy number per gram soil.

Effects of rhizosphere microbiota of different banana varieties on resistance to *Foc* TR4 invasion

Three banana varieties with different resistance levels to *Foc* TR4 (Zhongjiao no.9, a highly resistant banana variety; Kangku no.5, a resistant banana variety; Baxi, a highly susceptible banana variety) were selected to examine the extent to which the rhizosphere microbiome impacts the level of observed resistance to *Foc* TR4 invasion. Sterile banana tissue culture seedlings were cultivated in Erlenmeyer flasks and watered with sterilized modified strength Hoagland nutrition. One month later, the three banana varieties were transplanted into natural soil for an additional month to allow for the recruitment of rhizosphere microbiota. The pots were constructed using polypropylene pots filled with 1 kg dry soil and hydrated with sterile water, and cultivated in the greenhouse (daytime: 16 hours and 30 °C, night: 8 hours and 28 °C, 80% relative humidity) with periodic randomization throughout the experiment. The resulting materials were subsequently used in three experiments.

Greenhouse experiment 1: Effects of different varieties on the invasion of Foc *TR4*. A total of six treatments including three banana varieties from natural soil inoculated with or without *Foc* TR4 were established. The two-month-old banana plants described above were inoculated with *Foc* TR4 at $1{\times}10^4$ spores/g soil, using a *Foc* TR4 spore suspension or sterile water as a control. For each treatment, there were three replicates with six seedlings per replicate. Moisture and temperature were regularly adjusted to maintain optimum plant growth conditions. One month later, banana plants were harvested, and *Foc* TR4 densities in the soil were determined by plating a dilution series on Komada's medium [65]. The degree of *Foc* TR4 invasion was calculated using the following formula: (F-I)/F×100%, where F was the abundance of *Foc* TR4 in the rhizosphere soils after inoculating, and I was the abundance of *Foc* TR4 in the rhizosphere soils without inoculating *Foc* TR4.

Greenhouse experiment 2: Effects of rhizosphere microbiota of different varieties on the invasion success of Foc *TR4*. A total of six experimental treatments were established, which included the same three banana varieties either cultivated in natural soil or grown to a similar size as sterile banana tissue culture seedlings in hydroponic culture. Banana plants were uprooted from soil or taken from hydroponic cultures and transplanted into pots with 600 g sterilized dry soil, which had been prepared by Co75 γ-ray irradiation at Nanjing Xiyue Technology Co., Ltd, Nanjing, China. For

transplantation from soil, banana plants were carefully taken out of the pots (natural soil) so as to retain most of the rhizosphere soil. All treatments were inoculated with *Foc* TR4 a spore suspension at 1×10^4 spores/g soil. The same method of dilution counting was used to determine the density of *Foc* TR4 one month after transplantation. There were three replicates per treatment with six seedlings per replicate. The cultivation conditions were as described above. The inhibition rate of *Foc* TR4 invasion was calculated using the following formula: (NR-R)/NR×100%, where NR was the abundance of *Foc* TR4 in the rhizosphere soils derived from seedlings from hydroponics, and R was the abundance of *Foc* TR4 in the rhizosphere soils derived from seedlings from natural soil.

Greenhouse experiment 3: Effects of extracted rhizosphere microbiota of different banana varieties on resistance to Foc *TR4 invasion*. Rhizosphere soil was recovered from two-month-old seedlings after the one-month microbiome recruitment stage. These soils were then used to prepare soil suspensions, which were then used to inoculate jars containing 400 g sterilized soil, with the soil moisture content adjusted to 25% of field capacity. After one month of incubation in the dark at 28 °C, the soils were mixed, and a 20 g sample of each soil was taken and inoculated with a *Foc* TR4 spore suspension (final density of 1×10^4 spores/g soil). At the same time, the control treatment was established without *Foc* TR4 (sterilized water). This created a total of six experimental treatments: soils derived from three different banana varieties, each either with or without *Foc* TR4 inoculation. After one week, the density of *Foc* TR4 was determined using the method described above. The experiment consisted of six replications per treatment and was repeated three times. The relative level of *Foc* TR4 invasion was calculated by the following formula: (*Foc* TR4 -H_2O)/*Foc* TR4 ×100%, where *Foc* TR4 is the abundance of *Foc* TR4 in the soils of inoculating *Foc* TR4, and H_2O is the abundance of *Foc* TR4 in the soils of inoculating water. A schematic representation of this experiment is given in Fig. S13.

Metabolite collection and GC-MS analysis

In order to study the differences of rhizosphere metabolites across banana varieties with different levels of disease resistance, we selected three representative varieties: Zhongjiao no.9, Kangku no.5, and Baxi. These three varieties were chosen based upon three criteria: (1) they represent a range of resistance levels, allowing us to compare low, medium and highly resistant varieties; (2) there is a sizeable amount of genetic variation across these varieties; and (3) these varieties are very popular and widely accepted by farmers across China. Experimental soil was collected from the field in the above-mentioned field experiment site, and this soil was subsequently mixed and passed through a 2-mm sieve. Sterile banana seedlings were grown in polypropylene pots (20 × 18 × 13 cm) filled with 2 kg dry soil and hydrated with sterile water every three days. Each variety was grown in three independent pots, with experimental conditions identical to those used in the greenhouse experiments described above. After three months, roots were gently removed from the soil together with the tightly adhering soil, and then the soil attached to the root surface was carefully collected [66]. The samples were put into liquid nitrogen for five minutes and then stored at −80 °C. These frozen soil samples were subjected to GC-MS non-target metabolomics analysis, performed by BIOTREE Technology Co. Ltd. (Wuhan, China). Raw peak analyses were performed as reported elsewhere [67].

For the differences among groups, relative abundances were used to standardize the metabolite profiles. Principal components analysis (PCA, based on covariance matrix) was performed to explore the differences of rhizosphere metabolites. PERMANOVA was conducted to evaluate the differences in metabolites across varieties using the R vegan package [60]. Fold change of each compound in the highly resistant banana variety (Zhongjiao no.9) or the resistant banana variety (Kangku no.5) relative to that of the highly susceptible banana variety (Baxi) was calculated using the following formula: A/B, where A is the relative abundance of a given compound in Zhongjiao no.9 or Kangku no.5, and B represents the relative abundance of that compound in Baxi. Volcano Plots were used to plot the metabolites enriched by the different banana varieties.

Fungal strain isolation and assays of pathogen suppression

Fungal strains from the rhizosphere soil of the highly resistant variety Zhongjiao no.9, described above for Zhongjiao no.9 rhizosphere metabolite determination, were isolated by cultivation using Rose Bengal Agar (RBA) (Hopebio Company, Qingdao, China) and Potato Dextrose Agar (PDA) media (Hopebio Company, Qingdao, China) [28]. Plates were incubated at 28 °C for five days prior to selection of single colonies. A total of 74 such single colonies were chosen for further purification and subsequently identified according to previously described protocols [68]. Hierarchical clustering was performed on the phylogenetic tree generated for the sequences obtained from recovered fungal isolates together with OTUs that were most responsive to resistant banana varieties (OTU1 and OTU2), using the MEGA7 software package.

For the pot experiment, two fungal strains, T72 and P61 were chosen for plant-based disease inhibition assays based upon their close affiliations with *Trichoderma* OTU1 and *Penicillium* OTU2, respectively. For sake of comparison, other strains from *Trichoderma* (*Trichoderma*_19 and *Trichoderma*_22) and *Penicillium* (*Penicillium*_1 and *Penicillium*_91) with distant genetic distances to the key OTU1 and OTU2, as well as four strains (*Alternaria*_15, *Aureobasidium*_59, *Humicola*_40, and *Davidiella*_6, which were not negatively correlated with the relative abundance of pathogen) were selected as negative controls. Banana seedlings (at the growth stage of six true leaves) were grown in pot contained 500 g of sterilized soil. Plant seedlings of the varieties Zhongjiao no.9, Kangku no.5, and Baxi were used for the experiment. Ten days after planting, the seedlings were inoculated with potential biocontrol agents. Single isolate and microbial consortia suspensions were inoculated into pots with an initial density of 1×10^4 spores/g soil as describe above, and sterile water inoculation pots were used as a control. Each replicate contained ten pots with six replicates for each treatment. Pots were randomly placed in trays and watered with modified strength Hoagland solution. After ten days, a *Foc* TR4 spore suspension (final density of 1×10^4 spores/g of soil) was inoculated into the pots. The density of *Foc* TR4 was quantified by qPCR after an additional month of inoculation. Growth conditions were as described in the greenhouse experiments.

Effects of different pure metabolites on the growth of T72, P61, and *Foc* TR4

Based on the results from metabolite analysis, four compounds were chosen to examine the impacts of individual plant metabolites on the growth of T72, P61, and *Foc* TR4. All compounds were prepared at a concentration of 20 mM, filtered through a 0.22-µm sterile aqueous phase filtration membrane, and diluted to 8 mM, 4 mM, 2 mM, 0.4 mM, 0.2 mM, and 0.04 mM with sterile water, respectively. 150 µl of 10% Potato Dextrose Broth (PDB) liquid medium was added into 96-well cell culture plate (CoStar Clear) with 50 µl of the above six concentrations of each metabolite, and the spore suspensions of T72, P61, and *Foc* TR4 (1×10^4 spores/mL) were individually added into the microwells, respectively. The final concentrations of each metabolite in the wells were 5 mM, 2 mM, 1 mM, 0.5 mM, 0.1 mM, 0.05 mM, and 0.01 mM. Sterile water was used as a control for all experiments. 96-well cell culture plates were placed in a constant temperature incubator at 25 °C, and the fungal abundances were characterized by measuring absorbance at 750 nm every 24 h with a microplate analyzer [69]. Each treatment consisted of 12 microwells as a replicate, and the experiment was carried out four times to assure the accuracy of the results.

In order to explore the effects of different compounds on the growth of T72, P61, and *Foc* TR4, 20 mL 1/10 Potato Dextrose Broth liquid medium supplemented with different compounds to a final concentration of 0.1 mM was used to culture T72, P61, and *Foc* TR4 individually (final density of 1×10^4 spores/mL of each). To explore the influence of T72 and P61 on *Foc* TR4 growth in the presence of the different metabolites, either T72 or P61 or a combination of the two (final density of 1×10^4 spores/mL in total) was grown in the above-mentioned medium supplemented with each of the metabolite compounds, together with *Foc* TR4 (inoculated to at a density of 1×10^4 spores/mL). All the above Erlenmeyer flasks were cultured on an orbital shaker at 170 rpm and 25 °C for five days, after which the density of *Foc* TR4 was determined by qPCR as described above. Fig. S14 provides a schematic representation of this experiment.

Soil incubation with the addition of shikimic acid, D-(-)-ribofuranose, and propylene glycol

Based on the results from the effect of pure metabolites on the growth of key microbes, shikimic acid, D-(-)-ribofuranose, and propylene glycol were chosen for subsequent soil-based experiments. To test whether stimulation of disease-suppressive fungal taxa acts through direct fungal interactions, an additional treatment of soil with pathogens and metabolites without the host plant was conducted. 100 g of natural dry soil from the field was

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placed in a sterilized plastic bottle (V = 440 mL). Two concentrations, 0.1 mM and 1 mM of shikimic acid, D-(-)-ribofuranose, and propylene glycol standard, were added to the soil, respectively. Soils amended with sterilized water were established as controls. At the same time, all treatments were inoculated with *Foc* TR4, at a dose of 1×10^4 spores/g soil. Each treatment was comprised of six replicate bottles, and all bottles were randomly arranged during the incubation. Bottles were incubated at 28 °C for 30 days, and soil moisture content was regularly adjusted to maintain 25% of field capacity. After 30 days, 0.5 g soil samples were collected for DNA extraction.

Quantitative real-time PCR amplifications were used to determine the abundances of total fungi, *Trichoderma*, and *Penicillium* in the soil as described above. Abundances of total fungi, *Trichoderma*, and *Penicillium* were quantified with primers ITS1f/5.8 s [62], uTf/uTr [70], and Bt_Pbrev_F3/Bt_Pbrev_R4 [71], respectively (Table S3). The results of *Trichoderma* and *Penicillium* were expressed as relative quantifications to total fungi as previously described [72].

Effects of the microbial communities treated with exogenous shikimic acid, D-(-)-ribofuranose, and propylene glycol on disease suppression of different varieties

We carried out plant-based pathogen inhibition assays using shikimic acid, D-(-)-ribofuranose, and propylene glycol to test whether exogenous supplementation of metabolites affects the susceptibility of susceptible and resistant varieties to *Foc* TR4 grown in natural soil. Banana tissue culture seedlings (at the growth stage of six true leaves) were transferred to pots filled with 1.5 kg natural soil from the field. Plant seedlings of the varieties Zhongjiao no.9, Kangku no.5, and Baxi were used for the experiment. Two solutions containing equal proportions of shikimic acid, D-(-)-ribofuranose, and propylene glycol were added into the soils at the time of seedling transplantation at final total concentrations of either 0.1 mM and 1 mM. For each variety, there were two treatments and one control that was amended with sterile water. Each treatment contained six pots, and all pots were randomly arranged during the incubation. Twenty days after planting, the seedlings were inoculated with *Foc* TR4 spore suspension (final density of 1×10^4 spores/g of soil as describe above). After one month, the plants were harvested and the density of *Foc* TR4 was quantified by qPCR.

Statistical analyses

All statistical analyses were conducted using the IBM SPSS 20.0 (IBM Corporation, New York, USA) and R software programs (Version 3.5.0). Statistically significant differences in abundance of *Foc* TR4 among the varieties were determined by one-way analysis of variance (ANOVA) tests, along with the use of Tukey's HSD test for multiple comparisons. Unpaired *t* tests were performed for significance analysis of two groups, and *p* values were adjusted by the false discovery rate test. All statistical tests performed in this study were considered significant at $p < 0.05$. In addition, Spearman correlation analyses, calculated in the "corrplot" package, were used to examine correlations between the relative abundances of OTUs and the relative abundance of *Foc* TR4. To distinguish OTUs that were correlated with levels of disease suppression, a stepwise model selection was used to select the model with the best explanatory power (step function in R) with Akaike information criteria (AIC). Linear regression analyses between the relative abundance of *Foc* TR4 and the selected microbial indicators were then conducted using the "basicTrendline" package in R. The relative importance for each of the predictors in the model was determined using the "relaimpo" package in R. In order to examine potential associations between metabolites and microbes, network analyses was performed using the online interface Molecular Ecological Network Analysis Pipeline (MENAP) (http://ieg2.ou.edu/MENA) [73]. The fungal OTUs and metabolites were merged into an abundance table and Spearman correlation coefficients were calculated for network construction. Before network construction, the appropriate similarity threshold (St) was determined by random matrix theory. Cytoscape software was used for network visualization [74].

DATA AVAILABILITY

Raw amplicon sequencing data was deposited at the National Center for Biotechnology Information (NCBI) under the accession number PRJNA830664.

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AUTHOR CONTRIBUTIONS

SL, CT, ZS, CL and RL conceived experiments and discussed results. DX and OS performed field. SL, CT, LZ, ZW and JW designed assays and performed pot experiments. SL and CT analyzed data. SL wrote the manuscript with input from ZS, CL, CT, RL, GAK, WX and QS. All authors read and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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