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RESEARCH

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The impact of microbial inoculation on resilience towards biotic stresses in plants under greenhouse cultivation

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Abstract

There is a lack of understanding on the collateral effects of microbial inoculations on culturable plants. The purpose of this study was to investigate if, and to which extent eventual changes in secondary metabolome and/ or microbiome compositions would correlate with increased resilience to biotic stressors, i.e. to *Fusarium oxysporum* for tomato and lettuce, and western flower thrips (WFT) for chrysanthemum plants. Tomato plants were individually treated with ten different microbial strains. Furthermore, four selected strains were also applied to lettuce and chrysanthemum plants. Inoculated plants of all three species were co-inoculated with the corresponding biotic stressor or remained mock inoculated. Plant colonization by applied microbial strains was established by cultivation or molecular detection from surface-sterilized roots and stems. Tomato and lettuce plant growth reduction, as a result of *F. oxysporum* colonization, was established by dry weight measurements. Damage in chrysanthemum plants was established by counting silver spots on leaves, as a result of WFT infestation. Secondary metabolome analysis was performed on leaves, and bacterial and fungal community analyses on roots of plants of the three species. Measured parameters in plants were compared between individual microbial treatments and the corresponding untreated control, separate for biotic stressor and mock-inoculated plants. In addition, measured parameters of biotic stressor and mock-inoculated plants, within each individual microbial and control treatment, were compared. All four selected strains colonized the three plant species while showing different prevalences for location in the plant and species type. Two strains, i.e. *Trichoderma viride* TV02 and *Pseudomonas putida* P9, evoked mitigating effects on damage caused by, respectively, *F. oxysporum* in lettuce, and WFT in chrysanthemum plants. Treatment with *Bacillus mycoides* strain 2003/84 in *F. oxysporum*-infected tomato plants tended to aggravate biotic stress caused by the pathogen. Impact of microbial inoculations on the secondary metabolomes of the three plant species was found for strain P9 in tomato and lettuce plants in the absence of the biotic stressor and for strains TV02 and *Isaria javanica* FE9901 in tomato plants inoculated with *F. oxysporum*. With the exception of strain P9, all applied strains impacted the tomato plant microbiome, either with or without *F. oxysporum*. The four selected strains also impacted the microbiomes of lettuce and chrysanthemum plants, with or without biotic stressor. In conclusion, microbial inoculations overall have strong effects on plant microbiome composition and modulations in leaf secondary metabolome and in root microbiome compositions are shown to be independent processes.

Keywords: plant microbiome, secondary metabolome, biotic stress, plant resilience, microbial inoculant, greenhouse production

Introduction

There is a lack of understanding on collateral effects of microbial inoculations on culturable plants (Litchman, 2010; Mawarda *et al*., 2020). Microbial products as disease and pest control agents (biocontrol agents) and as plant growth promoters (biofertilizers), and further denoted in the text as 'microbials', will become increasingly relevant in the transition towards more sustainable agricultural practices. Often, the active compound of microbials consists of single microbial strains originating from different habitats as wherein they are applied (Collinge *et al*., 2022; Koide, 2023). These microbial strains can, upon application, excessively exceed taxonomically related indigenous species in number, inside and near plants and this may have consequences for plant microbiome composition and functioning (Mallon *et al*., 2018). Microbial inoculation may impact plant resilience towards biotic and abiotic stresses and the effects are context dependent (Pangesti *et al*., 2013). Namely, the effects of microbials applied to plants will differ between plant species, production-impeding organisms, growth

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circumstances and applied agricultural practices. The many variables regularly occurring under agricultural circumstances determine the success of microbial products, sometimes with opposite effects. This explains the large variation in the efficacy of microbial products experienced in practice (Nkebiwe *et al*., 2024).

Ecological knowledge about microbials in different soil-plant ecosystems is scarcely available (Bünger *et al*., 2020). For that reason, it is so important to understand the modes of action of the active compounds of microbials under different growth conditions. Successful applications of microbials depend on timing, dose and location on, or near plants (Dimopoulou *et al*., 2021) and on the metabolic activity of the microbial strain under the applied circumstances (Köhl and Schlösser, 1989). Upon inoculation, microbial strains will be exposed to harmful ambient circumstances and to competition with indigenous microbes. Because plant-beneficial microbes often are avid root (Shoresh *et al*., 2010; Boamah *et al*., 2023) and endosphere colonizers (Dini Andreote *et al*., 2009; Hardoim *et al*., 2015; Baron and Rigobelo, 2021; Niu *et al*., 2022), some protection to harmful conditions and competition can be provided by the plant. Plant colonization by microbial strains is therefore considered as the limiting factor in the application of microbial products (Timmusk *et al*., 2023).

A holistic view on the interferences of microbials with plant host defences is currently lacking. Plants have, besides innate immunity, also an adaptable immune system (Bakker *et al*., 2013). The current state of knowledge is that the mechanisms behind immunity are largely unexplored in most of the cultured plants (Jacoby *et al*., 2021). Mutual interactions between plants and micro-organisms are often initiated by biochemical communication, leading to crosstalk between host plants and their associated microbes (Beneduzi *et al*., 2012; Pangesti *et al*., 2013; Reverchon *et al*., 2023). Compounds produced by plant-associated microbes can promote resilience towards different forms of biotic stress. For instance, coronatine, produced by *Pseudomonas syringae* pv. Tomato, induces resistance to Western Flower Thrips (WFT) (Chen *et al*., 2018). In this study, major shifts in secondary metabolite composition were observed in plants in concert with induction of systemic resistance upon separate treatments with *P. syringae* and coronatine. Furthermore, entomopathogenic fungi produce a wide spectrum of secondary compounds (Bojke *et al*., 2018; Baron and Rigobelo, 2021) that interfere with plant resistance against insects. The plant secondary metabolome can be considered as an important indicator for the plant immune status against biotic and abiotic stresses (De Vos *et al*., 2007; Jacoby *et al*., 2021). Changes in the secondary metabolome of plants thus may report on activation of defence pathways towards increased immunity against biotic stressors.

Plant host immunity and microbiome together are responsible for plant resilience towards many biotic and abiotic stresses and most likely they influence each other. Plant secondary metabolites shape microbiomes via recruitment of beneficial microbes from soil and the plant microbiome plays an important role in the protection of plants against invasive pathogens by competition and by induction of secondary compounds (Bakker *et al*., 2013; Jacoby *et al*., 2021; Park *et al*., 2023). Therefore, besides their intended attenuating effects on plant damage and diseases, microbial inoculants may interfere in microbial recruitment with consequences for the composition and functioning of microbiomes associated with plants (Litchman, 2010; Mallon *et al*., 2018; Mawarda *et al*., 2020). For that reason, deeper knowledge on the impact of microbials on resilience of cultured plants is needed to understand the extent to which they facilitate, or possibly, compromise plant resilience towards biotic and abiotic stresses.

A wide taxonomical variety of microbial species living in soils can be recruited by plants, often leading to beneficial interactions with the plant host. For instance, plant-growth-promoting bacteria like *Pseudomonas* and *Bacillus* strains living in soils can induce systemic resistance in plants in a manner that resembles pathogen-induced systemic acquired resistance (Beneduzi *et al*., 2012). Furthermore, entomopathogenic fungi like *Metarizium brunneum* and *Beauveria bassiana* strains can induce systemic resistance towards bacterial and fungal pathogens in tomato plants (Partida-Martínez and Heil, 2011 Gupta *et al*., 2022). Also hitherto uncultured microbes have been shown to interact with potato and leek plants, such as particular *Verrucomicrobium* subdivision 1 (*Luteolibacter*) strains (Da Rocha *et al*., 2010; Nunes da Rocha *et al*., 2013). It has been shown before that plant treatments with microbials modify plant microbiomes (Andreote *et al*., 2009; Berg *et al*., 2021; Park *et al*., 2023). Treatments of maize plants with two *Pseudomonas* strains (*P. putida* RA2 and *P. corrugata* IDV1) resulted in shifts in microbial composition of the rhizosphere (Kózdroj *et al*., 2004). Potato plants treated with another *P. putida* strain (P9) resulted in a shift in the microbial composition of the endosphere (Andreote *et al*., 2009). In principle, plant microbial inoculation can revert dysbiosis into a healthy state of microbiomes in cultivated plants, leading to enhanced resilience to biotic and abiotic stresses (Berg *et al*., 2021; Allsup *et al*., 2023).

The effects of microbial inoculations on plant resilience thus are difficult to predict. Untargeted metabolite and microbial community composition screening technologies can be helpful tools to assess impact effects of microbial inoculations on plant resilience (De Vos *et al*., 2007; Chen *et al*., 2018; Dimopoulou *et al*., 2021). Therefore, the impact of different microbial strains on secondary metabolome and microbiome compositions was investigated in three different plant species relevant for greenhouse food and ornamental flower production in the Netherlands, i.e. tomato, lettuce and chrysanthemum. Interaction between different microbial groups in the plant microbiome will occur, especially between bacteria and fungi (Hardoim *et al*., 2015; van Overbeek and Saikkonen, 2016), and therefore, these two groups will be targeted for plant-associated microbial community analyses. The purpose of this study was to investigate if, and to which extent eventual changes in secondary metabolome and/ or microbiome compositions would correlate with increased resilience towards an introduced biotic stressor typical for each plant species, comprehending specific *Fusarium oxysporum formae specialis* (*f.sp.*) strains for tomato and lettuce, and WFT for chrysanthemum plants. The root system as target for microbial community analysis was chosen because it is the prime location for colonization by soil-borne microbes. Secondary metabolome analysis was performed on leaves as the most distinct location from roots to determine whether eventual observed effects on plant resilience, caused by microbial treatments, are systemic or not. Selected microbial strains covered a wide taxonomical diversity, and also, strains differed in origin of isolation and antagonism towards plant pathogens or harmful insects.

Methods

MICROBIAL STRAINS

Microbial strains used for treatments of tomato, lettuce and chrysanthemum plants are shown in Table 1. Four fungal strains, *B. bassiana* BbRb, *M. brunneum* F52, *Isaria javanica* FE9901 and *Trichoderma viride* TD50 have been applied for control of WFT in chrysanthemum flower production. Strain BbRb originated from cabbage root fly (*Delia radicum*) and strains F52, FE9901 and TD50 from chrysanthemum plants. *Trichoderma viride* TV02 was isolated from the bark of a pine tree in Germany, and it was demonstrated to suppress germination of sclerotia of the plant-pathogenic fungus *Botrytis cinerea* under cold circumstances (Köhl and Schlösser, 1989). The bacterial strain *Serratia marcescens* A2 was isolated from the endosphere of *Arabidosis thaliana* plants and evaluated for registration as a biological control agent against mites and thrips under greenhouse conditions. *Pseudomonas fluorescens* C17 was isolated from the endosphere of a chrysanthemum

Table 1. Microbial strains used for treatments of tomato, lettuce and chrysanthemum plants.

Strain	Taxonomic identity	Origin
BbRb	*Beauveria bassiana* (F)	RDIPP, Romania
F52	*Metarhizium brunneum* (F)	(Bio 1020) Bayer
FE9901	*Isaria javanica* (F)	(PreFeRal) Biobest
TD50	*Trichoderma viride* (F)	RDIPP, Romenia
TV02	*Trichoderma viride* (F)	WUR: Bark of pine tree, Germany
A2	*Serratia marcescens* (B)	WUR: *Arabidopsis thaliana* endosphere
C17	*Pseudomonas fluorescens* (B)	WUR: Chrysanthemum plant, endosphere
P9	*Pseudomonas putida* (B)	WUR: Potato plant, endosphere
2003/84	*Bacillus mycoides* (B) (B)	WUR: Potato plant, endosphere
C20	*Verrucomicrobium* subdivision 1 (B)	WUR: Potato plant, rhizosphere soil
Nt90	*Fusarium oxysporum* f.sp. *lycopersici* race 1EU/2US (F)	Bejo seeds, Netherlands
P188	*Fusarium oxysporum* f.sp. *lactucae* PD015-4750888 (F)	Dutch food and consumer product safety authority

plant, but it was never tested for antagonism towards any plant pathogen in particular. *Pseudomonas putida* P9 was isolated from the endosphere of a surviving potato plant in a field infested with *Phytophthora infestans* and was shown to suppress *P. infestans* in some late-flowering potato cultivars. Its ecological behaviour as an endophyte in potato plants was described in Andreote *et al*. (2009). *Bacillus mycoides* 2003/84 was isolated from the endosphere of a field-grown potato plant and shown to antagonize the soil-borne plant pathogen *Rhizoctonia solani* AG3 *in vitro* (van Overbeek and van Elsas, 2008). *Verrucomicrobium* subdivision 1 (*Luteolibacter*) C20 was isolated from potato rhizosphere soil (Da Rocha *et al*., 2010) and shown to be ecologically competent in rhizosphere soils (Nunes da Rocha *et al*., 2013). Strain C20 has never been demonstrated to antagonize any plant pathogen in particular. *Fusarium oxysporum f. sp. lycopersici* race 1 strain EU/2US (Foxlyc) and *F. oxysporum f. sp. lactucae* race 4 strain PD015-4750888 (Foxlac) strains were provided for this study by, respectively, Bejo and the Dutch food and consumer product safety authority (NVWA). Western flower thrips (WFT), reared on flowering chrysanthemum plants cv. Miramar, were used in greenhouse studies with chrysanthemum plants by WUR Greenhouse horticulture.

All fungal strains were standard cultured for 2 weeks at 20°C on potato dextrose agar (PDA, Oxoid). For making spore suspensions, a volume of 25 ml sterile water with 0.1% Tween-80 was added to each plate and spores were harvested from mycelium grown on the agar surface by scraping with a sterilized spatula. Resulting suspensions were filtered through sterile Miracloth (Sigma Aldrich, St Louis, Mi) and spores in filtered suspensions were counted in a Bürker Türk counting chamber, after which densities were set at 10^7 spores ml^{-1} using sterile water. Bacterial strains were grown in sterile 50 ml Erlenmeyer flasks, either filled with 10 ml Trypton Soy Broth (TSB; ThermoFisher Scientific, Waltham, Ma) for strains A2, P9 and A17, or in 1/10 strength TSB for strain 2003/84. All strains were overnight cultured under rotation (120 RPM) at 25°C. Strain C20 colonies were allowed to grow out on R2A (Sigma Aldrich) for 7–10 days at 20°C, after which 10 ml sterilized water was added to each plate and cells were harvested by scraping colonies with a sterilized spatula. Bacterial cells from suspensions thus obtained were harvested by centrifugation for 5 min at 10,000 g and pellets were resuspended in sterile water, followed by washing with the same centrifugation and pellet resuspension step. Final bacterial cell densities were all set at 10^8 cells ml^{-1} with sterile water. Fungal spore and bacterial cell suspensions were all stored at 16°C for not longer than 24 h before plant treatment.

PLANT GROWTH AND TREATMENTS WITH MICROBIAL STRAINS AND BIOTIC STRESSORS

The outline of the study consisted of three separate experiments with the following plant species: tomato (*Solanum lycopersicum* cv Topspin), lettuce (*Lactuca sativa* cv Starley) and chrysanthemum (*Chrysanthemum morifolium* cv Baltica). Plants of these species were microbially treated with all ten (tomato) or with four selected microbial strains (lettuce and chrysanthemum), i.e. FE9901, TV02, P9 and 2003/83 (Table 1). Co-inoculations were performed with *F. oxysporum* pathogenic strains Foxlyc or Foxlac on, respectively, tomato and lettuce plants and with WFT on chrysanthemum plants. Details specific for each plant species are provided below.

Tomato plants, grown from seeds, were allowed to germinate in potting soil under greenhouse conditions for 16 h in light, at 27°C with an air humidity of 85%, and for 8 h in dark, at 20°C with an air humidity of 70%. After 3 days, plants were treated by application of 1 ml spore/ cell suspension or sterile water (as control) to the soil in close proximity to the plant stem base and all treatments were repeated 1 week later (microbial treatment). One week after the second treatment, roots remaining in soil were artificially wounded by random cutting with a sterilized scissor. Half of the plants were co-inoculated by application of 3 ml Foxlyc spore suspension in close proximity to the stem base (Foxlyc inoculation), whereas the other half were treated in the same way with 3 ml sterile water (mock inoculation). Both Foxlyc and mock-inoculated plants were further allowed to grow for 2 more weeks under the same conditions as described before. The experiment consisted of 220 plants, based on ten microbial and a control treatment, for each type of co-inoculation, i.e. Foxlyc and mock-inoculation, with ten replicate plants for each treatment per type of inoculation (experimental unit).

Lettuce plants, also grown from seeds, were placed in potting soil mixed with vermiculite and allowed to germinate under the same conditions as described for tomato plants. Three days after sowing, the young plants were treated with four microbial strains or sterile water, as control, and the same treatments were repeated 1 week later. One week after the last microbial treatment, roots were artificially wounded, as described before for tomato plants, and 3 ml Foxlac spore suspension (Foxlac inoculation) or sterile water (mock inoculation) was applied in close proximity to the stem base. Then, all Fox-lac and mock-inoculated plants were further allowed to grow out for 2 weeks under the same conditions as described before for tomato plants. The experiment consisted of 100 plants, based on five microbial and a control treatment and two types of

inoculation, i.e. Foxlac and mock-inoculation, with ten replicate plants per experimental unit.

Chrysanthemum plants were grown from non-rooted and auxin-treated cuttings. These cuttings were planted into pots, containing a combination of perlite and potting soil, and pots were placed inside insect cages and incubated under the same greenhouse conditions as described before for tomato and lettuce plants. After 1 week, plants were treated with the four microbial strains and water, as control, and all treatments were repeated 11 days later. After 4 weeks, plants were placed in the dark to induce flower formation and 1 week later, when flowers were formed, half of the plants of each treatment was inoculated with WFT by placing five female thrips on each plant, which was repeated 1 week later. The other half remained uninoculated and as such these plants were considered as 'mock-inoculated plants'. All plants were further incubated for 11 weeks under the same conditions until all WFT-treated plants were fully infested. The experiment consisted of 50 plants, based on five microbial and a control treatment and two types of inoculation, i.e. WFT and mock-inoculation, with five replicate plants per experimental unit.

PLANT DEVELOPMENT MEASUREMENTS AND MICROBIAL STRAIN RECOVERY

The effects of the applied biotic stressors on plants of the three species were recorded by measuring plant dry weight or leaf damage parameters. Plant development was determined by dry weight measurements on all Foxlyc, Foxlac and mock-inoculated tomato and lettuce plants. Therefore, entire shoots of plants, using five replicates per experimental unit, were incubated at 100°C for 24 h, after which dried plants were weighed. Damage on chrysanthemum leaves, as a result of WFT infestation, was measured by counting the number of silver spots per leaf. Therefore, silver spot numbers on four full-grown leaves per replicate plant were counted using five replicate plants per experimental unit. The number of WFT (nymphs and adults) was, in addition, determined on four flowers per replicate plant, initially stored in 70% ethanol prior to counting.

Recovery of applied microbial strains was performed on surface-sterilized roots and stem base samples (lowest 3 cm of the stem and further denoted as 'stem') of all mock-inoculated plants of the three plant species. For that purpose, roots were separated from stems of all plants, using a sterilized scalpel knife, and thoroughly washed in sterile tap water for removal of adhering soil particles. Washed roots were then split into two parts along the top-to-bottom axis. One part was directly processed for recovery of the microbial strains by cultivation (not for strain C20), whereas the other part was instantly frozen at −70°C for later DNA extraction. Both root and stem samples were subjected to surface-sterilization by incubation in sterile 1% sodium hypochlorite solution, for 2 min, followed by incubation in a sterile solution of 0.02% Tween-20 in water, for 2 min, after which ethanol and Tween-20 residues were removed by three times washing in sterile water (1 min per washing step). In addition, stem samples were incubated for 1 min in 96% ethanol, after which ethanol was burned off from the stem surface and thus flamed samples were immediately washed in sterile water for 1 min. Final wash water from surface-sterilized root and stem samples were checked for sterility by plating 100 µl subsamples in duplicate onto PDA and R2A.

PROCESSING OF ROOT AND STEM SAMPLES, AND MICROBIAL AND MOLECULAR IDENTIFICATION OF RECOVERED STRAINS

Applied strains were recovered from surface-sterilized root and stem samples by cultivation or molecular detection. Therefore, 1 g subsamples of sterilized root and stem samples were transferred to 2 ml sterile bead beat tubes containing 1 ml water and two sterile steel beads (3.2 mm in diameter) and samples were bead-beaten in four separate runs of 15 s at 8000 RPM in a Precellys Evolution Homogenizer (Bertin technologies, Montigny-le-Bretonneux, Fr), with 15 s intervals between runs. Two hundred microliter of the resulting homogenates were either plated onto PDA or R2A, for, respectively, treatments with fungal or bacterial inoculants. Subsamples from control roots and stems were plated onto both agar types. All agar plates were incubated for maximally 4 weeks at 20°C, after which the identities of the recovered strains were, in a first step, confirmed by making visual comparisons, in colour and morphology, with the original strains grown from stocks on corresponding agar types. Recovered colonies identified as strain 2003/84 were not further confirmed, because of its characteristic expansive colony growth, whereas the identities of all other recovered colonies were additionally confirmed using DNA-based methods. For that purpose, cell lysates were made first by boiling a loopful of colony material suspended in 100 µl sterile water in Eppendorf tubes, for 5 min. One microliter subsamples of these cell lysates were used as targets for species-specific PCRs, for strains TD50, TV02, BbRb, F52, FE9901 and A2 and BOX-PCR fingerprinting for strain C17. The identity of presumed strain P9 colonies was determined by a strain P9-specific TaqMan system. A minimum of three randomly selected colonies per sample was used for identification. References to all primers, probes and PCR conditions are shown in Table S1 in Supplementary Material. Cell lysates from strain P9 colonies were used as targets for the TaqMan assay in Takara master mix with ROX dye supplemented with 0.3 µM of each primer and 0.1 µM hydrolysis probe and amplified at a thermocycle regime of one cycle at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 55°C for 1 min.

DNA EXTRACTION FROM MOLECULAR DETECTION OF *LUTEOLIBACTER* AND *F. OXYSPORUM SENSU LATO* IN ROOTS AND STEMS

DNA was extracted from surface-sterilized and frozen root (50 mg) and stem samples (200 mg) of tomato, lettuce and chrysanthemum roots and stems, using the Qiagen DNeasy® Powersoil Kit. This kit was originally designed for soil DNA extraction; however, for this study, it was adapted for DNA extraction from root and stem samples. DNA extraction steps were performed as prescribed by the manufacturer with the exception of a preceding bead beating step of two runs at 8000 RPM for 15 s. The DNA concentration in all extracts was determined using the Quant-iT Picogreen dsDNA Assay kit (ThermoFischer) on a Tecan Infinite M200 pro plate reader and normalized to two ng μl^{-1} by dilution with elution buffer. DNA extracts were stored at −20°C for later downstream qPCR and PCR processing steps.

Strain C20-specific DNA sequences were quantified by TaqMan in tomato root and stem DNA extracts of five randomly selected samples per treatment using a *Verrucomicrobium* subdivision 1 (*Luteolibacter*)-specific primer and hydrolysis probe set (Supplementary Material: Table S1). *Fusarium oxysporum sensu lato*-specific DNA sequences were quantified in all tomato and lettuce root and stem DNA extracts by TaqMan using a *F. oxysporum s.l.*-specific primer and hydrolysis probe set (Supplementary Material: Table S1, Supplementary Material). Strain C20 and *F. oxysporum s.l.*-specific TaqMan assays were performed on 4 ng root or stem DNA, as targets, under the same thermocycle conditions as described before for identity confirmation of strain P9 colonies, with the exception that primer and hydrolysis probe concentrations were higher, i.e. 0.6 µM for each primer and 0.2 µM for the hydrolysis probe.

SECONDARY METABOLITE ANALYSIS OF LEAF SAMPLES

Untargeted analysis of plant secondary metabolites was performed by liquid chromatography coupled to mass spectrometry (LC-MS) on freeze-dried leaf samples of all three plant species. Upon harvest, leaves were instantly frozen in liquid N_2, according to the procedure described in de Vos *et al*. (2007). Mass peak alignments

were performed in MetAlign, in accordance to de Vos *et al*. (2007), whereas unbiased mass signal assembly into compounds and expression into relative abundances across all samples was performed in MSClust (Tikunov *et al*., 2012). Reduced data matrices for each plant species were used for further multivariate analyses.

BACTERIAL AND FUNGAL AMPLICON SEQUENCING FROM ROOT DNA

Microbial community analysis was performed by amplicon sequencing using bacterial and fungal-specific PCRs on all root DNA extracts from the three plant species. For bacterial amplification an equimolar mix of six frameshifting forward, and six frameshifting reverse primers, respectively, EMP515F and EMP806R (Cregger *et al*., 2018) was used, targeting the bacterial 16S rRNA V4 region. For fungal amplification, the primers ITS1F (Gardes and Bruns, 1993) and fITS7R (Ihrmark *et al*., 2012), directing the fungal ITS1 region, were used. All primers were elongated with standard Illumina adapter sequences, synthetized by Integrated DNA Technologies (BVBA, Leuven, Be). Polymerase chain reactions were performed in volumes of 50 µl using the Q5 High-Fidelity DNA Polymerase kit (New England Biolabs) that further included 0.5 µM of each forward and reverse primer, or of mixtures of primers in case of bacterial amplification, 200 µM dNTP, 2 mM of each PCR amplification blocker, mPNA and pPNA (PNA Bio Inc, Ca), for suppression of bacterial amplification from mitochondria and plastids (Lundberg *et al*., 2013), and 20 ng target DNA. Amplification reactions of all samples, including a DNA mixture from all ten microbial strains as positive, and sterile nuclease-free water as negative control, were performed in duplicate in separate runs on a Veriti Thermo Cycler (Thermo Fisher Scientific, Ma). For sequencing library preparation, duplicate samples were pooled to equimolar solutions and sequenced with 2 × 250 cycles for bacterial, and 2 × 300 cycles for fungal libraries, all according to standard Illumina MiSeq protocols (Illumina, Ca). Resulting bacterial amplicon sequence variants (ASVs) that remained unassigned at the taxonomical Kingdom level, or that were classified as 'Eukaryotes', 'mitochondria', 'chloroplast', or 'unidentified' were excluded from the bacterial ASV table, whereas for fungal ASVs, assignments to the Kingdom of 'Fungi' were maintained in the fungal ASV table. Bacterial and fungal ASVs of the samples were, separate for each experimental unit, rarefied to the lowest sequencing depth. Shannon diversity and community composition parameters were calculated using the R package phyloseq (McMurdie and Holmes, 2013) and vegan (R package vegan version 2.2-1), based on ASV abundances per sample.

DATA ANALYSES AND STATISTICS

For all three plant species, consistent pairwise comparisons were made between control and individual microbial treatments, separate for the two co-inoculation types (biotic stressor and mock-inoculation) and between the two co-inoculation types within the same microbial or control treatment. The following experimental parameters were included: *Luteolibacter* Ct values in tomato, and *F. oxysporum s.l.* Ct values in tomato and lettuce plants; dry weights of tomato and lettuce plants; silver spot per leaf and thrips numbers in chrysanthemum plants; secondary metabolite composition in leaves; Shannon diversity and composition of the bacterial and fungal communities in roots of all three plant species. Therefore, two-way ANOVA was performed on all Ct, plant dry weight, silver spot, thrips number and Shannon diversity values and in case of significance ($P \leq 0.05$), LSD values for each factor (treatment, co-inoculation and interaction between treatment and co-inoculation) were calculated for post-hoc analysis. Significance of differences between values was always confirmed by an additional two-tailed Students t-test on restricted datasets. Permutational multivariate analysis of variance (PERMANOVA) was performed on Bray-Curtis distance matrices derived from secondary metabolic compound and bacterial and fungal ASV tables, after which pairwise comparisons were made.

Results

PLANT COLONIZATION BY MICROBIAL STRAINS

With the exception of strain C20, all strains administered to the experimental plants were recovered by cultivation from surface-sterilized roots and stems. No colony growth was observed upon plating of final wash water samples, indicating that the surface sterilization procedure was effective for all plants of the three species. Nine strains endophytically colonized tomato, and all four selected strains, i.e. TV02, FE9901, P9 and 2003/84, also lettuce and chrysanthemum plants. It was anticipated that strain C20 colonies would be overgrown by colonies of copiotrophic microbes present in plant samples because of its low growth rate (Da Rocha *et al*., 2010). However, this strain was detectable by TaqMan in tomato roots because of significantly (Students t-test, $P < 0.001$) lower average strain *Luteolibacter* Ct values measured in strain C20-treated (29.1) than in non-treated root samples (36.0). Average strain *Luteolibacter* Ct values in treated and non-treated stem samples were below detection (Ct values > 39) in both cases, indicating that strain C20 only colonized roots and not stems of tomato plants. Expectedly, the applied sterilization procedure will not lead to full removal of DNA from dead cells; and therefore, it cannot be excluded that strain C20 DNA was co-amplified from the root surface.

The applied microbial strains showed differences in localization in three different plant species. For example, CFUs or target DNA of strains TV02, F52 and C20 were not recovered from stems, whereas the other seven strains were, from at least one of the five replicate samples (Table 2). Furthermore, strain BbRb CFUs were not recovered from tomato roots, whereas CFUs or target DNA of the other nine strains were, from at least two of the five replicate samples. Colony forming units of strains TV02, FE9901, P9 and 2003/84 were all recovered from lettuce roots and strains FE9901, P9 and 2003/84 also recovered from stems. Furthermore, CFUs of the selected four strains were all recovered from chrysanthemum stems and roots.

Strains FE9901, P9 and 2003/84 were present in roots and stems of the three plant species, although among samples there was variation in prevalence, i.e. the fraction of positive identifications to the total number of replicate plants. Prevalences were, across roots and stems, higher in tomato than in lettuce and chrysanthemum plants, indicating higher preference for tomato than for the other two plant species. Localization of strain TV02 in the plant substantially differed per species, because this strain was not recovered from stems of tomato and lettuce, but it was from chrysanthemum plants. Furthermore, this strain showed, across roots and stems, a higher prevalence in chrysanthemum than in tomato and lettuce plants, indicating a higher preference for chrysanthemum than for the other two plant species.

EFFECTS OF MICROBIAL STRAINS AND BIOTIC STRESSORS ON PLANT DRY WEIGHT AND DAMAGE

The effects of different microbial treatments on biotic stress symptoms caused by Foxlyc or Foxlac in, respectively, tomato and lettuce, and by WFT in chrysanthemum plants were assessed.

Microbial treatments resulted in variation in dry weight among all Foxlyc and mock-inoculated tomato plants (Fig. 1). There were overall effects of microbial treatment and type of co-inoculation on all tomato plants, as determined by ANOVA, whereas the interaction between both factors was not significant (Supplementary Material: Table S2). Infection of tomato plants with Foxlyc, calculated over all 11 microbial and control treatments by Students t-test, resulted in a significantly ($P < 0.001$) lower average dry weight value (0.86 g) compared to that of corresponding mock-inoculated plants (1.05 g). The overall lower dry weight measured in Foxlyc compared to mock-inoculated plants coincided with the significantly (Student t-test; $P < 0.001$) lower *F. oxysporum s.l.* Ct values of Foxlyc (31.7) compared to mock-inoculated plants (38.7), demonstrating that

Table 2. Microbial inoculants recovered from surface-sterilized roots and stems of tomato, lettuce and chrysanthemum plants.

	Number of samples scored positive for the introduced strain (n = 5)					
	Tomato		Lettuce		Chrysanthemum	
Strain	**stem**	**root**	**stem**	**root**	**stem**	**root**
Fox lyc/lac	BD	5*	BD	5*	NA	NA
TV02	0	3	0	2	4	5
FE9901	3	5	1	2	1	4
P9	5	5	3	3	3	2
2003/84	5	3	2	1	3	1
TD50	1	5				
BbRb	3	0				
F52	0	2				
A2	3	3				
C17	1	3				
C20	0*	5*				

*Absence/ presence determined via qPCR.
BD: below detection; NA: not applicable.

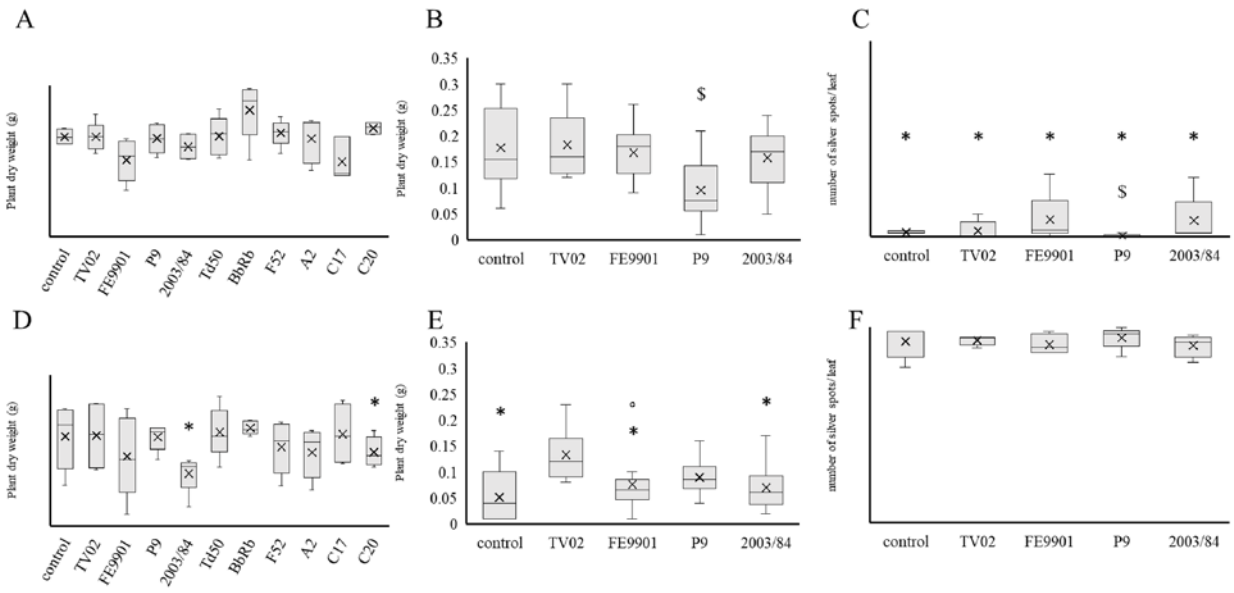

Fig. 1. Effects of microbial treatments on tomato (A, D) and lettuce (B, E) plant dry weights and on the number of silver spots on chrysanthemum leaves (C, F) in mock (A, B, C) and biotic stressor (D, E, F, respectively, Foxlyc, Foxlac and WFT) inoculated plants. $ significantly lower value for microbial treatment than for corresponding control. * significantly lower value for biotic stressor, than for mock-inoculated plants.

plant infection with Foxlyc led to an overall reduction in tomato plant growth. Individual comparisons in average tomato plant dry weight values between microbial and control treatments, separate for Foxlyc and mock-inoculated plants, never resulted in significant differences, as determined by Students t-test (Supplementary Material: Table S2). However, treatment with strain 2003/84 in Foxlyc-infected tomato plants resulted in a near-significant (for P values between 0.05 and 0.075; Table S2, Supplementary Material) reduction in plant dry weight, when compared to the corresponding control. When comparing Foxlyc and mock-inoculated plants within the same microbial treatment, it revealed that treatments with strains 2003/84 and C20 resulted in significantly lower dry weights in Foxlyc than in mock-inoculated plants.

Treatments with the four selected microbial strains resulted in variation in dry weight among all Foxlac and mock-inoculated lettuce plants (Fig. 1). The effects of microbial treatment, type of co-inoculation and interaction between both factors on lettuce plants, as determined by ANOVA, were all significant (Supplementary Material: Table S2). Inoculation of lettuce plants with Foxlac, under all five microbial and control treatments, resulted in a significantly (Student t-test; $P < 0.001$) lower average dry weight value (0.084 g) than that of mock-inoculated plants (0.156 g). The difference in average plant dry weights between Foxlac and mock-inoculated plants coincided with a significant (Student t-test; $P < 0.001$) lower average *F. oxysporum s.l.* Ct value (35.0) in roots of Foxlac, than of mock-inoculated plants (40.0), demonstrating that plant infection with Foxlac led to an overall reduction in lettuce plant growth. When comparing average lettuce plant dry weight values between control and the four microbial treatments separately in the mock-inoculated lettuce plants, it revealed that only the treatment with strain P9 resulted in a significantly lower dry weight (0.096 g)

compared to control (0.177 g) (Supplementary Material: Table S2). In Foxlac-inoculated lettuce plants, average plant dry weights under all four microbial treatments were higher than control, and difference was significant for the treatment with strain TV02 and near significant with strain P9. When comparing Foxlac and mock-inoculated plants within the same microbial and control treatment, it revealed that treatments with strains FE9901 and 2003/84 and control resulted in significantly lower average dry weight values in Foxlac, than in mock-inoculated plants.

The four selected microbial treatments resulted in variation in average silver spot per leaf numbers among all WFT and mock-inoculated chrysanthemum plants (Fig. 1). The effect of type of co-inoculation on chrysanthemum plants was significant, but not of microbial treatment or the interaction between both factors (Supplementary Material: Table S2). Overall, the effect of WFT inoculation of chrysanthemum plants resulted in a significant (Students t-test; $P < 0.001$) higher average number of silver spots per leaf (0.93) than in mock-inoculated plants (0.05). Higher leaf damage coincided with the observed significantly (Students t-test; $P < 0.001$) higher thrips numbers in flowers of WFT-inoculated (on average 42.4 nymphs and 135.2 adults) than of mock-inoculated chrysanthemum plants (on average 1.8 nymphs and 1.2 adults), demonstrating that higher WFT numbers in flowers correlated with higher silver spot damage in leaves. Only the treatment with strain P9 in mock-inoculated chrysanthemum plants resulted in significant lower leaf damage (0.004 silver spots per leaf) compared to the corresponding control (0.023 silver spots per leaf) (Supplementary Material: Table S2). In WFT-inoculated plants, there were no significant differences in average silver spot per leaf numbers for all microbial treatments, including treatment with strain P9, in comparison with control. Comparisons between average silver spot per leaf numbers between WFT and mock-inoculated plants, separate for each microbial and control treatment, revealed significant differences in all cases.

EFFECTS OF MICROBIAL STRAINS AND BIOTIC STRESSORS ON LEAF SECONDARY METABOLOME COMPOSITION IN PLANTS

The effects of microbial treatment in the presence and absence of a biotic stressor on the secondary metabolite composition (secondary metabolome) of the leaves of three plant species was investigated. Overall significant effects of microbial treatment, type of co-inoculation and the interaction between both factors were present on the secondary metabolome of tomato leaves (Table 3, Supplementary Material: Table S3). In comparison to respective controls, the effects of microbial treatments on the secondary metabolome were significant for strain P9 in mock, and for strains TV02 and FE9901in Foxlyc-inoculated tomato leaves. The overall effects of microbial treatment and type of co-inoculation on the secondary metabolome of lettuce leaves were significant, but not of the interaction between both factors. The effect of strain P9 treatment on the secondary metabolome of mock-inoculated lettuce leaves was significant in comparison to control. In chrysanthemum leaves, the overall effect of type of co-inoculation on the secondary metabolome was significant, but not of microbial treatment or the interaction between both factors. There were no significant effects of individual microbial treatments, compared to corresponding controls, in mock or WFT-inoculated chrysanthemum leaves.

EFFECTS OF MICROBIAL STRAINS AND BIOTIC STRESSORS ON THE BACTERIAL AND FUNGAL SHANNON DIVERSITY INDICES OF ROOTS

The impact of microbial treatments, either or not in combination with a biotic stressor, on Shannon diversity of microbial communities on, and inside roots of all three plant species was investigated.

In tomato roots, no overall significant effects of microbial treatment on bacterial and fungal diversities were present, whereas there was a significant effect of type of co-inoculation on fungal, but not on bacterial diversity, as calculated by ANOVA (Supplementary Material: Table S3). Furthermore, the interaction between both factors was significant for bacterial, but not for fungal diversity. Pairwise comparisons revealed significantly lower average bacterial diversity values for the treatments with strains A2 (diversity value of 4.95) and TD50 (4.99), compared to control (5.46), in roots of Foxlyc-inoculated, and for the treatment with strain C17 (4.60), compared to control (5.25), in roots of mock-inoculated plants (Table 3, Supplementary Material: Table S3). Treatment with strain TD50 resulted in a significantly higher average fungal diversity value (3.33) compared to control (2.52) in roots of mock-inoculated tomato plants.

In lettuce roots, there was a significant effect of type of co-inoculation on bacterial, but not on fungal diversity (Supplementary Material: Table S3). No significant effects of microbial treatment or the

Table 3. Impact effects of microbial treatments on secondary metabolome and on bacterial and fungal diversities and community compositions in three plant species in presence and absence of biotic stressors.

	Tomato		Lettuce		Chrysanthemum	
Microbial treatment	**mock**	**Foxlyc***	**mock**	**Foxlac***	**mock**	**WFT***
TV02	**Bβ**	**M, Bβ**	***Bβ*, Fβ**	**Bβ, Fβ**	*Bβ*	**Bβ**
FE9901	*Bβ*	**M, Bβ**	**Bβ, Fβ**	**Bβ, Fβ**	**Bβ**	
P9	**M**		**M, Bβ**	***M*, Bβ, Fβ**	**Bβ, Fβ**	**Bα,** *Fβ*
2003/84	**Bβ,** *Fβ*	***M*, Bβ**	**Bβ**	***M*, Bβ, Fβ**	**Bβ**	*Bβ*
TD50	**Bβ, Fα, Fβ**	**Bα,** *Bβ*				
BbRb	**Bβ**	***M*, Bβ**				
F52	**Bβ, Fβ**	**Bβ**				
A2	**Bβ, Fβ**	**Bα, Bβ**				
C17	**Bα, Bβ, Fβ**	*Bβ*				
C20	**Bβ**	**Bβ**				

*Foxlyc: *Fusarium oxysporum f.sp. lycopersici*; Foxlac: *Fusarium oxysporum f.sp. lactucae*; WFT: Western Flower thrips.
M: secondary metabolome; B: bacteria; F: Fungi; α: Shannon diversity; β: community composition; bold significant to control at $P \leq 0.05$; italic, near significant at $0.05 < P \leq 0.075$.
Significance of differences between average values are shown in Supplementary Material: Table S3.

interaction between both factors on bacterial and fungal diversities were found. Furthermore, there were no significant differences in average bacterial and fungal diversity values between individual microbial and corresponding control treatments in roots of Foxlac and mock-inoculated lettuce plants. Also, there were no significant differences in average diversity values between the two types of co-inoculation within each microbial and control treatment (Table 3, Supplementary Material: Table S3).

In chrysanthemum roots, there was a significant effect of type of co-inoculation on fungal, but not on bacterial diversity (Supplementary Material: Table S3). No effects of microbial treatment or the interaction between the two factors on bacterial and fungal diversities were found. The treatment with strain P9 resulted in a significantly higher bacterial diversity value (5.29; P = 0.009) compared to control (5.12) in roots of WFT-inoculated chrysanthemum plants. There were no significant differences in average diversity values between the two types of co-inoculation within each microbial and control treatment (Table 3, Supplementary Material: Table S3).

EFFECTS OF MICROBIAL STRAINS AND BIOTIC STRESSORS ON THE BACTERIAL AND FUNGAL COMMUNITY COMPOSITION OF ROOTS

The impact of microbial treatments, either or not in combination with a biotic stressor, on root-associated microbial communities of all three plants species was investigated.

The effects of microbial treatment, type of co-inoculation and the interaction between both factors on the bacterial community composition of tomato roots were all significant as determined by PERMANOVA (Supplementary Material: Table S3). In comparison to control, the difference in root bacterial community compositions of Foxlyc-inoculated tomato plants under nine microbial treatments were all significant, or near significant, but not under strain P9 treatment (Table 3). For roots of mock-inoculated plants, it was the same, namely, in comparison to control, differences in bacterial community composition under treatments with nine different microbials were all significant or near significant, but not with strain P9. There were significant effects of microbial treatment and type of co-inoculation on the fungal community composition of tomato roots, but the interaction between both factors was not significant. Differences in fungal community composition in roots of mock-inoculated plants were significant for treatments with strains TD50, F52, A2 and C17 in comparison to control. In roots of Foxlyc-inoculated tomato plants, differences in fungal community composition between microbial treatments and control were never significant.

The effects of microbial treatment, type of co-inoculation and the interaction between both factors on bacterial and fungal community compositions of lettuce roots were all significant (Supplementary Material: Table S3). Differences in root bacterial community composition between individual microbial treatments and corresponding controls in Foxlac, and mock-inoculated plants were all significant or near significant (Table 3, Supplementary Material: Table S3). In comparison to corresponding controls, differences in root fungal community composition were significant for treatments with all four microbial strains in Foxlac, and for treatments with strains TV02 and FE9901 in mock-inoculated plants.

The effect of microbial treatment on the bacterial community composition in chrysanthemum roots was significant, but not of type of co-inoculation and the interaction between the two factors (Supplementary Material: Table S3). In comparison to corresponding controls, differences in root bacterial community composition were significant for treatment with strain TV02 in WFT, and for treatments with strains FE9901, P9 and TV02 in mock-inoculated plants (Table 3, Supplementary Material: Table S3). The effects of the same factors on the root fungal community composition were never significant. Difference in root fungal community composition was significant between the treatment with strain P9 and control in mock-inoculated plants, whereas no further significant differences in root fungal community composition between individual microbial treatments compared to corresponding controls were present in WFT and mock-inoculated plants.

Discussion

The overall aim of this study was to establish eventual correlations between microbial inoculations, shifts in secondary metabolomes and microbiomes, and resilience to biotic stressors in plants commonly cultivated in Dutch greenhouses. Most studies on relationships between plant secondary metabolomes and microbiomes and resilience to biotic stressors were performed on Arabidopsis and not on agronomically relevant plants (Jacoby *et al*., 2021). Tomato, lettuce and chrysanthemum plants, used as objects in our study, are relevant crops in greenhouse production in the Netherlands and *F. oxysporum* and thrips are the major production-impeding organisms for these crops. Upon bans of chemical pesticides, there is a quest for broad spectrum alternatives among microbials and the motive for this study was to evaluate the effectiveness of different microbial strains in a wider variety of crops in the presence of their specific biotic stressors.

Based on endophytic colonization, the four selected strains showed differential preferences for the three plant species; i.e. strain TV02 showed higher preference for chrysanthemum, and strains FE9901, P9 and 2003/84 for tomato plants. The observed preferences for the different plant species cannot always be explained by the origin of isolation. Namely, strain FE9901 originated from chrysanthemum plants whereas strain TV02 did not. Strains 2003/84 and P9 originated from potato plants and, as may be expected from the close taxonomical relationship with tomato plants, these strains indeed showed higher preference for tomato, than for lettuce and chrysanthemum plants. Although with some variation in prevalence, strain P9 colonized roots and stems of all three plant species. Strain P9 previously has been described as a competent endophyte in potato plants (Andreote *et al*., 2009); therefore, this strain can now be described as a generic competent endophyte for different plant species.

Shifts in secondary metabolome composition of leaves did not always coincide with shifts in microbiome composition of roots upon microbial treatments of the three plant species in the absence of a biotic stressor. Application of strain P9 via soil to tomato plants resulted in a significant shift in the secondary metabolome composition of leaves, indicating that plants defence mechanisms are primed by strain P9 in the same way as described for some other *Pseudomonas* species (Bakker *et al*., 2013). However, priming by strain P9 in tomato plants did not coincide with any measurable shift in bacterial or fungal community composition in the roots. This to the contrary to applications with the other nine strains to soil near tomato plants that all led to measurable shifts in root bacterial, and sometimes also in root fungal community compositions, but never in shifts in the leaf secondary metabolome composition. In lettuce plants, the effect of strain P9 treatment was somewhat different from that in tomato plants in the sense that a measurable shift in the leaf secondary metabolome was accompanied by a measurable modification in the root bacterial community composition. The effect of strain P9 application to soil near chrysanthemum plants was again different from that in the other two plant species as root bacterial and fungal community compositions significantly shifted, but not the composition of the leaf secondary metabolome. It is a remarkable fact that secondary metabolomes are shifted by strain P9 in plant species from two distinct taxonomical families, namely in tomato (*Solanaceae*) and lettuce (*Asteraceae*) plants, whereas apparently not in chrysanthemum plants, that belongs to the same taxonomical family as lettuce. This illustrates the complexity of the impact of microbial inoculations with individual strains on physiology and microbial interactions in different plant species.

Complexity even increases when microbial treatments were combined with the inoculation of a biotic stressor to the three plant species.

In Foxlyc-inoculated tomato plants, significant shifts in the leaf secondary metabolome composition were induced by strains TV02 and FE9901. Strain 2003/84 induced a shift in the leaf metabolome composition of Foxlac-inoculated lettuce plants. Root microbiome composition sometimes differed between biotic stressor and mock-inoculated plants under the same treatment as was the case with strain FE9901 in chrysanthemum plants. Treatments with strain P9 in Foxlyc-inoculated tomato plants and with strain FE9901 in WFT-inoculated chrysanthemum plants were the only exceptions where no measurable shifts were observed, both in leaf secondary metabolome and in root microbiome compositions. The consensus for the majority of the microbial treatments was clear, namely, that microbial treatments led to measurable shifts in the root bacterial and occasionally also in fungal community composition of all three plant species, either in the absence or in the presence of a biotic stressor.

That the composition of the root bacterial, and to a lesser extent that of the root fungal community, can be affected by microbial inoculation is an observation that has been found before (Mallon *et al*., 2018; Lee Díaz *et al*., 2024). On the reason why bacteria in roots are more prone to microbials applied to plants than fungi can only be speculated upon. Even inoculation with strain C20, belonging to a phylotype of which the impact on plants was hitherto unknown, resulted in a significant shift in the bacterial fraction of the microbiome. Most likely, bacteria rather than fungi in, and near roots are more sensitive for the common traits of invasive microbes such as high growth rate, efficient resource utilization and competitive capacity (Litchman 2010). Typically, taxa lower in abundance in rhizosphere microbiomes are more often affected by microbial inoculants than the ones higher in abundance leading to more drastic changes in microbial composition than in species diversity (Hu *et al*., 2021), which was also an observation made in our study. Unsuccessful niche occupation by invasive microbes, in this particular study, an *Escherichia coli* O157:H7 strain alien to the plant-soil environment, could leave a microbial community legacy behind enabling a more successful establishment upon proceeding invasive events (Mallon *et al*., 2018; Liu *et al*., 2022). Microbial treatments in our study were always repeated, which may have reinforced the impact of microbial treatments on root bacterial communities in the three plant species.

Shifts in root microbiomes, as observed in our study, were not always accompanied by shifts in leaf secondary metabolomes. Discrepancy in observation between our study and that of Lee Díaz *et al*. (2024) may be explained by the fact that locality for secondary metabolome and microbiome analyses was the same, namely in roots, in the study of Lee Díaz *et al*. (2024) and different, namely in leaves and roots, in ours. The difference in sampling location in the plant for endophytic colonization, in roots and stem, and secondary metabolome analysis, in leaves, could in principle differentiate local from systemic induction of immunity. The location in the plants, where the biotic stressor was active, was different for chrysanthemum than for tomato and lettuce plants, because WFT will cause most of the damage in leaves and flowers, and *F. oxysporum* in roots and stems of the infected plants. This will have implications for the interaction between the applied microbial strain and biotic stressor in plants.

Direct interactions between microbial strains and *F. oxysporum* can be expected in the roots and stems, but for interaction with WFT, systemic colonization or induction of adaptable immunity in chrysanthemum plants is needed. The observed attenuation of the effects of Foxlac infection in lettuce plants by strain TV02 can be explained by local interaction between the microbial inoculant and the pathogen, because both Foxlac and strain TV02 only colonized the roots and not the stems of lettuce plants. Strain P9 tended to attenuate effects of Foxlac infection in lettuce plants and because this strain also internally colonized lettuce roots, it may have directly competed with Foxlac in lettuce roots. On the other hand, the observed shift in secondary metabolome composition would indicate that strain P9 primed a defence mechanism in lettuce plants. Priming has a fitness cost for the host plant (Pangesti *et al*., 2013), which may explain the observed initial reduction in lettuce plant growth upon inoculation with strain P9. However, endophytic colonization may lead to resource competition with the host plant (Partida-Martínez and Heil, 2011; Hardoim *et al*., 2015) and measured growth retardation in lettuce plants can also be the result of colonization by strain P9.

The observed reduction in silver spot damage caused by WFT in strain P9-treated chrysanthemum plants cannot be explained by activation of a defence pathway, because no shift in chrysanthemum leaf secondary metabolome composition was observed. Direct interaction between strain P9 cells and WFT might be an option, because strain P9 also internally colonized chrysanthemum stems and could, in principle, reach the leaves via the transport vessels. Strain P9 evoked a significant shift in the fungal community composition of mock-inoculated chrysanthemum plants and this would indicate an indirect effect of strain P9 on WFT in these plants. However, no significant reduction in thrips numbers in all strain P9-treated plants was observed, and this makes the exact mode of interaction between strain P9 and WFT in chrysanthemum plants unclear. Resistance pathways distinct from classical induced systemic resistance can be expected in cultivated plants, as demonstrated before with a non-pathogenic *Fusarium* strain in tomato plants (Constantin *et al*., 2019), and this might also be the case for strain P9 in chrysanthemum plants.

In summary, none of the treatments with the four selected strains resulted in increased resilience towards the applied biotic stressor in all three plant species, although of all applied strains, P9 offered some protection to Foxlac in lettuce and to WFT in chrysanthemum plants. Treatment of strain 2003/84 in Foxlyc-infected tomato plants tended to lead to aggravation of pathogenicity of the applied biotic stressor. For this strain, it would imply that there is synergism in disease occurrence between the microbial inoculant and the applied pathogen as described before for pathogen-pathogen interactions in plants (Lamichhane and Venturi, 2015). Microbiome modifications resulting from microbial treatments thus may not always result in beneficial effects to plants as stated before in Berg *et al*., (2021) and Park *et al*., (2023). Microbial treatments may lead to deteriorating effects on plants making them more vulnerable to biotic stressors and this is a critical note towards wider applications of microbials without further knowledge on their modes of action in plants. This study made clear that modulations in leaf secondary metabolome and in root microbiome compositions, as results of microbial treatments, must be considered as independent processes. Increased or decreased resilience towards applied biotic stressors could therefore not directly be linked to changes in microbiome or secondary metabolome composition. Effects of microbial treatments on plants are context dependent (Pangesti *et al*., 2013), making outcomes of microbial treatments on plants from different species and under attack by different biotic stressors hard to predict. Based on our results, we can state that, in general, plant treatments with microbial strains will lead to changes in root microbial community composition with undetermined phenotypic effects. Phenotypic effects of microbial inoculant-induced shifts in root microbiome composition need further attention in research and could, in principle, offer new opportunities for microbiome-based solutions in integrated crop management practices (French *et al*., 2021; Collinge *et al*., 2022).

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ETHICS STATEMENT

The authors confirm that the research meets any required ethical guidelines, including adherence to the legal requirements of the study country.

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AUTHOR CONTRIBUTIONS

LO contributed in conceptualization, formal analysis, funding acquisition, supervision, project administration, writing, reviewing and editing; LvH performed conceptualization, formal analysis, funding, reviewing and editing; PB contributed in conceptualization, formal analysis, funding, reviewing and editing;, EN performed formal analysis, investigating, methodology, data curation; SA performed formal analysis, investigating, methodology; and MD contributed in conceptualization, formal analysis, data curation, visualization, reviewing and editing.

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DATA AVAILABILITY

Raw data were generated at Wageningen Plant Research (WUR). Derived data supporting the findings of this study are available from the first author LvO on request.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the online version of this article.

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