Isolation and Characterization of Novel Biocontrol Agents for Controlling Tomato Bacterial Wilt

(トマト青枯病に対する新規生物防除エージェントの分離と特性解析)

2018

The United Graduate School of Agricultural Science,

Gifu University

Science of Biological Resources

(Gifu University)

Malek Khaled Mahmoud Marian

Isolation and Characterization of Novel Biocontrol Agents for Controlling Tomato Bacterial Wilt

(トマト青枯病に対する新規生物防除エージェントの分離と特性解析)

Malek Khaled Mahmoud Marian

Contents

General introduction

General introduction

1. Tomato plant

The commercial tomato belongs to a species most frequently referred to as *Lycopersicon esculentum* Miller. Other alternative names *Solanum lycopersicum* L., or *Lycopersicon lycopersicum* L. Karsten have appeared also in the literature. Tomato plant is a member of the *Solanaceae* (nightshade) family and belongs to the genus *Solanum*, which include also other economically important crops such as potato (*S. tuberosum* L.) and eggplant (*S. melongena* L.). Tomato is native to South America, especially Peru and Galapagos Islands, being first domesticated in Mexico. The Nahuatl (Aztec language) word *tomatl* gave rise to the Spanish word "tomate", from which the English word tomato derived. The fruit was thought to be poisonous, like its relative, the deadly nightshade. Its importance as a vegetable has occurred only in the 19th century (14).

Tomato plant is grown for their edible fruit which is often red in color. The plants typically grow to a 1–3 meters in height and have a weak stem that sprawls. It is a perennial in its native habitat, and cultivated as an annual crop (14). Five million hectares of tomatoes are estimated to be grown annually worldwide, producing >177 million tons, with China accounting for 32% followed by India (10.4%), United Stated of America (7.4%), and Turkey (7%). In terms of productivity, the Netherlands and Belgium were the most productive countries, with a nationwide average of 507 tons per hectare (56). In Japan, tomatoes are grown on a total area of 12 thousand hectares, with an annual production of 727 thousand tons. Usually it is cultivated in two periods, summer (July – November) and winter (December – June), with

Hokkaido prefecture accounting for 15.5% of the total production in the summer season, and Kumamoto prefecture accounting for 26.4% of the total production in the winter season (125).

2. *Ralstonia solanacearum* species complex

Following its discovery, *Ralstonia solanacearum* was first classified as a member of the genus "*Bacillus*" (188). The application of DNA-based methods eventually resulted in its transfer to the genus *Burkholderia* (227) and then to the genus *Ralstonia* (228). The species is classified into races and biovars (75) and recently into phylotypes (57), according to host range, biochemical, and molecular characteristics, respectively. More recently, *R. solanacearum* species complex was taxonomically organized into three genomic species: (i) *R. solanacearum*, including phylotype IIA and IIB; (ii) *R. pseudosolanacearum*, including phylotype I and III; and (iii) *R. syzygii*, including the former *R. solanacearum* phylotype IV and the clove pathogen *R. syzygii* (162, 178). The phylotypes are subdivided into sequevars based on sequence variation in the endoglucanase (*egl*) partial gene (57).

3. Bacterial wilt

Bacterial wilt was among the first diseases that Smith (188) proved to be caused by a bacterial pathogen. Bacterial wilt is caused by the gram negative β-proteobacteria soil-borne pathogens *R. solanacearum* (228), *R. pseudosolanacearum*, and *R. syzygii* subsp. *indonesiensis* (formerly classified as *R. solanacearum*) (178), and is the second most destructive bacterial disease of plants

worldwide (126). Together, these pathogens infect more than 200 plant species belonging to an over 50 different plant families, mostly *Solanaceae* and *Musaceae*. The disease affects the yield of many economically important solanaceous crops, such as tomato, potato, tobacco (*Nicotiana tabacum* L.), eggplant, and *Capsicum* species (75). Direct yield losses by bacterial wilt vary widely according to the host, cultivar, climate, soil type, cropping pattern, and strain. For instance, yield losses vary from 0 to 91% in tomato, 33 to 90% in potato, 10 to 30% in tobacco, 80 to 100% in banana, and up to 20% in groundnut (*Arachis hypogaea* L.) (53). To date, in Japan, more than 46 species belonging to an over 24 families have been reported to be hosts (212), and new hosts continue to be found (233).

Plant Pathogenic *Ralstonia* species are well adapted to grow and survive in the bulk soil for many years in the absence of susceptible host plants. When the pathogen encounters a susceptible host, it enters the root through wounded roots or natural openings such as lateral root emergence points, colonizes the root cortex and then invades the xylem vessels by degrading the cell wall and produces large amounts of exopolysaccharides that block water flow (42). The most frequent external symptoms of the infected plants are wilting, stunting and yellowing of the foliage. Other symptoms are leaves bent downward showing leaf epinasty, adventitious roots growing in the stems, and the observance of narrow dark stripes corresponding to the infected vascular bundles beneath the epidermis. The most frequent internal symptoms are progressive discoloration of the vascular tissue, mainly the xylem, at early stages of infection, and of portions of the pith and cortex, as disease develops, until complete necrosis. Slimy viscous ooze typically appears on transverse-sectioned stems at the points corresponding to the vascular bundles.

As a result, collapse and death of the plant take place because of the degradation of occluded xylem vessels and the destruction of surrounding tissues (10).

3. Management of bacterial wilt

The current management strategies used against bacterial wilt includes chemical controls, cultural practices, and biological control methods. These approaches have been investigated for decades, extensively reviewed by Elphinstone (53) and Yuliar et al. (238). In general, the control of bacterial wilt is very difficult owing to the viability, adaptability, and genetic diversity of the responsible pathogen (53). Many attempts are made to control this devastating disease. The following approaches have been taken.

3.1 Chemical control

The chemical control methods have not been always efficient in eradicating *R. solanacearum*, and due to the environmental concerns, chemical control is being discouraged (177). Chemicals such as soil fumigants (1,3-dichloropropene, chloropicrin, and dazomet), bactericides (streptomycin sulfate), and plant activators generating systemic resistance on different plants (acibenzolar-S-methyl, DL-3-aminobutyric acid, and validamycin A) have been used to control bacterial wilt (13, 74, 87, 115, 127, 128). In addition, biofumigation using volatile plant essential oils from thyme (*Thymus* spp.), palmarosa (*Cymbopogon martini* Roxb.), Indian mustard (*Brassica juncea* L. Czern), lemongrass (*C. citratus* L.), eucalyptus (*Eucalyptus*

globulus), bergamot (*Citrus aurantium* var. *bergamia* L.), and sweet orange (*Citrus sinensis* L.) oils have also been used to control bacterial wilt (7, 11, 91, 153).

3.2 Cultural practices

The growth of plant cultivars which are resistant to bacterial wilt has been accomplished in the past (59, 114). However, resistance to bacterial wilt in many crops has generally been negatively correlated with yield and quality. Moreover, public acceptance in Japan is needed prior to the commercial use of such genetically modified crops. Thus, the release of resistant cultivars is difficult (238).

Crop rotation and intercropping has also been used for controlling bacterial wilt. However, in addition to being a high labor intensive method, it has been indicated that crop rotation and intercropping, depending on the companion plant used might have little suppressive effective on bacterial wilt (134, 135). Crop rotation of susceptible tomato line with corn (*Zea mays* L.), okra (*Abelmoschus esculentus* L. Moench), cowpea (*Vigna unguiculata* L. Walp.), or partially resistant tomato line has been shown to delay onset and reduce disease severity of the bacterial wilt (2). Potato cultivation rotated with wheat (*Triticum aestivum* L.), sweet potato (*Ipomoea batatas* L. Lam), maize, millet (*Pennisetum glaucum* L. R.Br., carrots (*Daucus carota* subsp. *sativus* Hoffm. Schübl. & G. Martens), sorghum (*Sorghum bicolor* L. Moench), or phaseolus beans (*Phaseolus vulgaris* L.) reduced the incidence of wilt by 64 to 94% while the yield of potatoes was 1- to 3-fold higher than that of monocultured potatoes (95). Intercropping tomato with *Allium* plants, such as Chinese chive (*Allium tuberosum* Rottler ex Spreng) and garlic (*Allium sativum* L.), has also been reported to suppress bacterial wilt (106, 235).

Previous studies evaluated the potential of soil amendments with rock dust, urea, mineral ash, calcium oxide, and fertilizers such as calcium and silicon on the incidence of bacterial wilt (13, 17, 113, 230). However, some of these components have been shown to be not effective. In addition, their biocontrol effect can be site-specific, and the generation of several toxic substances may also affect their potential use.

Cultural practices through commercially grafted seedlings (grafting resistant rootstock with susceptible scion) restrict pathogen multiplication and movement in the rootstock, thereby suppressing the infection and wilting in the scion, and through an anaerobic reductive soil disinfestation (RSD) method reduces the pathogen population in the soil and is widely adopted in Japan (137). However, grafting is expensive, requires more labor, and result in the production of fruits of inferior quality (taste, color, and sugar contents) (110). Furthermore, new virulent races of the pathogen might overcome the resistance, resulting in colonization and migration of the pathogen into susceptible scions and causing wilt symptoms (141). Moreover, for the RSD method, achieving sufficient disinfection in the deep soil layers where the pathogen might localize is difficult (137).

3.3 Biological control

Great interest in the biological control method has increased over the past decade due to concerns from the excessive use of chemicals (218). The advantages of using biological control agents (BCAs) is that; they are effective, safe, potentially self-sustaining, spread on their own after initial establishment, reduced input of non-renewable resources, and long-term disease suppression in an environmentally

friendly manner (136, 219). Among the natural soil microorganisms, researchers mainly focused on identifying bacteria with an *in vitro* antibacterial activity against RSSC, as potential BCAs for controlling bacterial wilt. Despite the large body of literature describing their use as BCAs in the past decade (Table. 1), there are only few commercialized biocontrol products available in the world, mostly in China, such as a wettable powder of *Bacillus subtilis* (Cohn) Y1336, a water suspension of *Pseudomonas fluorescens* (Migula), a mixture of wettable powder and granule of *Paenibacillus polymyxa* (Ash, Priest and Collins) (195). In Japan, rhizospheric *P. fluorescens* isolates were previously commercialized as a biocontrol product against bacterial wilt (Cell Nae Genki, Taki Chemical, Kakogawa, Japan); however this product was abolished and no longer exists, therefore it is necessary to develop a new biocontrol product against bacterial wilt.

4. Mechanisms employed by bacteria in the biocontrol of bacterial wilt

4.1 Competition for nutrients and niche

The root surface and the surrounding rhizosphere are significant carbon sinks. Photosynthate allocation to this zone can be as high as 40%. Thus, along root surfaces there are various suitable nutrient rich niches attracting a great diversity of microorganisms, including phytopathogens. Competition for these nutrients and niches is a fundamental mechanism by which biocontrol bacteria protect plants from phytopathogens (39). Recently, Huang et al. (82) showed that the bacterium *Chryseobacterium nankingense* sp. WR21 effectively suppresses *R. solanacearum* via intensive root exudates competition, particularly four amino acids (i.e.,

Asparagine, Glutamine, Histidine, and Leucine), against *R. solanacearum*. This effect might aid in the colonization of this isolate, thereby effectively suppressing tomato bacterial wilt. Additionally, Wu et al. (221) showed that the competitive ability of biocontrol bacteria *Bacillus amyloliquefaciens* SQYUV162 to use the tomato root exudate citric acid directly affected not only the population density of *R. solanacearum* but also its pathogenicity, thus efficiently suppressing the incidence of bacterial wilt.

Competition for niches has been also suggested to be one of the mechanism by which biocontrol bacteria suppress bacterial wilt. For instance, McLaughlin and Sequeira (131) suggested that the avirulent *R. solanacearum* strain B82 confer protection against bacterial wilt disease by competitive exclusion of the pathogen, particularly, in the crown region of the vascular system. Moreover, Etchebar et al. (54) suggested that competition for space in the xylem vessels is one of the possible explanations for the protective ability of a HrcVÀ mutant strain of *R. solanacearum* against subsequent invasion by the wild *R. solanacearum* strain. Through tagging the biocontrol bacteria *B. amyloliquefaciens* T-5 and *R. solanacearum* with different fluorescent protein markers, Tan et al. (204) revealed that the root colonization of pathogen was almost completely suppressed in the presence of biocontrol strain T-5-GFP when both soil and plant seedlings were treated with T-5-GFP, suggesting that the biocontrol bacteria may prevent the pathogen infection through competitive exclusion.

Table 1. Recent examples of the use of bacteria as potential biocontrol agents against bacterial wilt reported in the past decade.

Potential BCAs	Host plant	Proposed mechanism(s)	References
Bacillus amyloliquefaciens Bg-C31	Capsicum	Production of antimicrobial protein	78
B. amyloliquefaciens BZ6-1	Peanut (*Arachis hypogaea* L.)	Production of antimicrobial compounds (surfactin and fengycin)	214
B. amyloliquefaciens QL-5 and QL-18 + organic fertilizer	Tomato (*Solanum lycopersicum* L.)	Antibiosis	216
B. amyloliquefaciens HR62 + organic fertilizer (BIO62)	Tomato	Production of antibacterial compounds (Macrolactin A and 7-O-malonyl macrolactin A)	81
B. amyloliquefaciens CM-2 and T-5	Tomato	Antibiosis. Induced systemic resistance (ISR). Competition for niches	203, 204
B. amyloliquefaciens S13-3	Tomato	Antibiosis.	229
B. amyloliquefaciens WF02	Tomato	Antibiosis. Siderophore production. Biofilm formation. ISR	79
B. amyloliquefaciens SQRT3	Tomato	Antibiosis. Production of siderophore and protease. Biofilm formation. ISR	37
B. amyloliquefaciens JK6	Tomato	Production of antimicrobial compounds (surfactin, fengycin, and *YndJ* protein). Production of siderophore, indole acetic acid (IAA) and protease. Biofilm formation	223
B. amyloliquefaciens SQYUV 162	Tomato	Competition for nutrients	221
B. amyloliquefaciens S20 + organic fertilizer	Eggplant (*Solanum melongena* L.)	Production of antimicrobial compound (iturin A)	33
B. amyloliquefaciens + organic fertilizer (BIO23) and *B. subtilis* + organic fertilizer (BIO36)	Potato (*Solanum tuberosum* L.)	Antibiosis	44
B. amyloliquefaciens SQR-7 and SQR-101 *and Bacillus methylotrophicus* SQR-29	Tobacco (*Nicotiana tabacum* L.)	Antibiosis. Production of siderophore and indole acetic acid	236
Bacillus thuringiensis	Tomato	ISR	85
B. thuringiensis UFV-56 and *Bacillus cereus*	Eucalyptus	Production of siderophore and volatiles	180

UFV-62		compounds	
B. cereus BC1AW and *Pseudomonas putida* PP3WT	Tomato	Antibiosis. Production of siderophore, protease, and quorum sensing inhibiting molecules. ISR	105
Bacillus subtilis Lu144	Mulberry (*Morus alba* L.)	Antibiosis. Competition	92
Bacillus sp. (RCh6) and *Pseudomonas mallei* (RBG4)	Eggplant	Antibiosis. Siderophore production	167
Bacillus vallismortis ExTN-1	Tomato	ISR	209
Bacillus velezensis Y6 and F7	Tomato	Production of antimicrobial compounds (surfactin, iturin, and fengycin)	28
B. subtilis PFMRI, *Pseudomonas fuorescens* PF20, and *Paenibacillus macerans* BS-DFS	Potato	Antibiosis	8
B. subtilis 1JN2, *Myroides odoratimimus* 3YW8, *B. amyloliquefaciens* 5YN8, and *Stenotrophomonas maltophilia* 2JW6.	Ginger (*Zingiber officinale* Roscoe)	Antibiosis. Production of siderophore and protease	232
B. subtilis and *P. fluorescens*	*Coleus forskohli*	Antibiosis	185
Pseudomonas brassicacearum J12	Tomato	Production of antimicrobial compound (2,4diacetylphloroglucinol). Production of siderophore and protease	242
Pseudomonas monteilii + *Glomus fasciculatum*	*C. forskohli*	Antibiosis	186
P. fluorescens EB69	Eggplant	Antibiosis. Siderophore production	166
Pseudomonas putida A1+organic fertilizer	Tomato	Antibiosis. Biofilm formation	194
Pseudomonas aeruginosa NXHG29	Tobacco	Antibiosis	123
Streptomyces virginiae E36 and Y30	Tomato	Siderophore production	202
Streptomyces mycarofaciens SS-2-243 and *Streptomyces philanthi* RL-1-178	Chili pepper (*Capsicum annuum* L.)	Antibiosis	26
Brevibacillus brevis L-25 + *Streptomyces roche* L-9 + organic fertilizer	Tobacco	Antibiosis	117
Acinetobacter sp. Xa6 and *Enterobacter* sp. Xy3	Tomato	Rhizocompetence and root colonization	225

Flavobacterium johnsoniae WR4 and *Chryseobacterium* sp. WR21	Tomato	Antibiosis and competition for nutrients	80, 82
Serratia sp. XY21	Tomato	Rhizocompetence and antagonistic activity.	226
Ralstonia pickettii QL-A6	Tomato	Competition	217
Paenibacillus sp. Pb28, *P. putida* Pp17, and *P. fluorescens* Pf11	Potato	Antibiosis. Production of siderophore and protease	97

4.2 Siderophore-mediated competition for iron

In general, the availability of soluble iron in the soil is extremely low (10^{-17} M), whereas a minimum concentration of 10^{-6} M is commonly needed for microorganisms to grow (143). Most microorganisms produce siderophores, which are low-molecular-weight metabolites with a high affinity for Fe^{3+}, under conditions of low iron availability (18). These siderophores chelate Fe^{3+} from the environment and transport it into the microbial cells (142). Earlier studies have indicated that siderophore production may contribute to disease suppression of bacterial wilt, possibly by limiting iron availability to *R. solanacearum* (138, 168). In addition, it was suggested that the siderophore pseudobactin, produced by some fluorescent *Pseudomonas* spp., is one of the determinants responsible of triggering induced systemic resistance in *Eucalyptus urophylla* against bacterial wilt (169).

4.3 Antibiosis

The basis of antibiosis as a mechanism employed by biocontrol bacteria has become increasingly better understood over the past two decades (70, 159, 146, 218). Antibiotics encompass a chemically heterogeneous group of organic, low-molecular weight compounds produced as secondary metabolites by microorganisms. At low concentrations, antibiotics are deleterious to the growth or metabolic activities of other microorganisms (60). A variety of antibiotics such as 2,4-diacetylphloroglucinol, surfactin, iturin A, and fengycin, produced by different biocontrol bacteria have been identified to be involve in the suppression of bacterial wilt (33, 214, 223, 242). Recently, Huang et al. (81) identified two types of

polyketides Macrolactin A and 7-O-malonyl macrolactin A produced by the *B. amyloliquefaciens* HR62 which could inhibit *R. solanacearum*. The production of these polyketides was pursued as another possible mechanism behind the biocontrol of bacterial wilt by HR62. Moreover, Hu et al. (78) indicated that the antagonistic action responsible for the inhibition of *R. solanacearum* was due to an antimicrobial peptide LCI produced by the endophytic bacterium *B. amyloliquefaciens* Bg-C31.

4.4 Lytic enzymes

Many bacteria produce and release enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, hemicellulose, and DNA. Expression and secretion of enzymes such as protease and polygalacturonase by different biocontrol bacteria can sometimes result in the direct suppression of plant pathogens or aid in their colonization ability (150). For example, Elhalag et al. (52) reported that the biocontrol activity of *Stenotrophomonas maltophilia* against bacterial wilt was due of the direct antagonism against *R. solanacearum*, which depends on the production of the enzyme protease. Moreover, the strong competitive ability of rhizobacteria to utilize pectin by producing extracellular pectinases may play a significant role in their rhizoplane competence, possibly resulting in suppressing the multiplication of *R. solanacearum* (86, 183). Cell wall-degrading enzymes such as pectinases also play some role in triggering defense mechanisms in plants, probably by releasing cell wall fragments (e.g., oligogalacturonides), which can act as elicitors of host defense responses (141).

4.5 Induced systemic resistance

Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance; in both SAR and ISR, plant defenses are preconditioned by prior infection or treatment that results in resistance (or tolerance) against subsequent challenge by a pathogen (213). ISR as a mechanism by which non-pathogenic rhizobacteria suppress plant diseases has been widely investigated during the last two decades (19, 100, 130, 159). Many studies have reported the ability of biocontrol bacteria to induce ISR against *R. solanacearum* in tomato plant (72, 94, 200). ISR is generally associated with a physiological state in which plant can react more efficiently to a pathogen attack; that is the priming of the plant defense mechanism (40). Primed defense reactions include an earlier oxidative burst and stronger upregulation of defense-related genes (4). Earlier studies have discussed the importance of host defense priming in the suppression of bacterial wilt by the treatment with biocontrol bacteria (4, 148).

5. Methods of improving the efficacy of biocontrol agents

The biocontrol effect exhibited by single BCAs can sometimes be low and/or last for only a short period of time, thus requiring uneconomically high inoculum densities or frequent applications in the field. These points were considered the most important disadvantages of BCAs in controlling bacterial wilt (238). Several sophisticated methods such as the combination of biocontrol bacteria with chemical pesticide (154), organic amendments (117), and other bacteria in a mixture (88) have been proven to be an effective way to overcome some of the drawbacks from the application of single BCAs against bacterial wilt. For example, the combined

application (foliar and/or soil drenching) of *P. fluorescens* Pf2 and the plant activator acibenzolar-S-methyl achieved higher biocontrol effect compared with their application individually against tomato bacterial wilt (1). The mechanism behind the improved effect might be due to the enhanced expression of several tomato defense-related enzymes. Moreover, a previous study reported that the combination of endophytic bacteria *Bacillus* sp. or *Serratia marcescens* with the susceptible tomato cultivar Santa Clara could reduce bacterial wilt for up to 35%, however the disease reduction was improved for up to 65% when they were applied with the resistant cultivar Yoshimatsu (22). Furthermore, Nion (145) have demonstrated that the suppressive effects against tomato bacterial wilt were enhanced by the combinations of the biocontrol bacteria *Burkholderia nodosa* G5.2.rif1 with lysine and sucrose. And the addition of these nutrients also improved the root colonization of this isolate. Recently, there has been increasing interest among researchers in using the combination of BCAs to exploit potential synergistic effects on plant health (reviewed by; 181, 224). Many previous studies reported that the combined application of multiple microbes may enhance the biocontrol efficacy and reliability against bacterial wilt on tomato, (88, 90), tobacco (117, 237), bell pepper (*Capsicum annuum* L.) (116), and *Coleus* (*Coleus forskohlii* Briq.) (186) plants. Additionally, the combination may also lead to a broad-spectrum protection against multiple pathogens (46, 89, 170) and may improve the growth, yield and quality of different crops such as tomato, rice (*Oryza sativa*), and potato (122, 179, 192).

6. Aims and outline of the thesis

The main goal of this thesis is to develop a practical biocontrol strategy for controlling tomato bacterial wilt. In an attempt to achieve the main objective, experiments described in the following chapters were conducted:

In chapter one, bacteria were isolated from the rhizosphere soil of tomato, Chinese chive, and Welsh onion. Rhizobacterial isolates were then screened for their antibacterial activity against *Ralstonia pseudosolanacearum* using the agar well diffusion assay. All isolates exhibiting antibacterial activity were identified based on the partial sequencing of the 16S rRNA gene. Antibacterial isolates were evaluated for their biocontrol effect against bacterial wilt using a tomato seedling bioassay. Isolates affiliated to the bacterial genera *Ralstonia* and *Mitsuaria* were assessed for their biocontrol effect in a series of pot experiments. Among the isolates, TCR112 (identified as non-pathogenic *Ralstonia* sp.) and TWR114 (identified as *Mitsuaria* sp.) showed consistent disease suppression in pot experiments, thus were selected for further evaluation under field conditions. We monitored the population dynamics of *R. pseudosolanacearum* in the rhizosphere and aboveground stem of TCR112- and TWR114-treated tomato plants. Moreover, the colonization capacity of both isolates in the same regions was also investigated.

In chapter two, we evaluated the biocontrol effect of the combined application of TWR114 and TCR112 against bacterial wilt. In the first pot experiment, the effect of several inoculum ratios (i.e., 1:1, 1:2, and 2:1) of the TWR114+TCR112 were tested for their biocontrol ability against bacterial wilt under glasshouse conditions. In the second pot experiment, the effect of inoculum concentrations (i.e., original concentration [ca. 9×10^8 CFU/ml], 2-fold [ca. 4.5×10^8 CFU/ml] and 10-fold [ca. 9×10^7 CFU/ml] dilutions of the original concentration) of the TWR114+TCR112 (at a ratio of 2:1) were tested for their biocontrol effect against the wilt disease. The

population dynamics of *R. pseudosolanacearum*, TWR114, and TCR112 in several regions of TWR114+TCR112-treated tomato plants and the individually-treated plants was monitored.

In chapter three, we investigated the different biocontrol mechanisms of TWR114 and TCR112. The production of siderophore, indole-3-acetic acid, protease, polygalacturonase, and hydrogen cyanide by these isolates was examined using *in vitro* assay. The effect of TWR114 and TCR112 individual treatments and their combination on the expression of six defense-related marker genes (i.e., *PR1-a*, *GluA*, *GluB*, *Osmotin*-like *Le4*, and *LoxD*) in the roots of tomato plants was examined. The expression levels of these genes were determined by using quantitative real-time PCR at 5 and 7 days after treatment (2 and 4 days post-challenge inoculation, respectively) in pathogen-uninoculated and -inoculated plants. To identify the genetic traits possibly involved in the biocontrol activity and to assess their taxonomical relationships, the genomes of TWR114 and TCR112 were sequenced and analyzed. Genome relatedness was computed using the average nucleotide identity and genome-to-genome distance (*in silico* DNA-DNA hybridization) analysis. Pan- and core-genomic analysis of *Mitsuaria* and non-pathogenic *Ralstonia* isolates was performed to identify shared and unique genetic components in TWR114 and TCR112 isolates. Additionally, a core-genome based phylogenetic analysis was constructed to obtain a higher resolution classification of our isolates.

Chapter 1

Screening of biocontrol bacteria for controlling tomato bacterial wilt

Screening of biocontrol bacteria for controlling tomato bacterial wilt

Abstract

In this chapter, we aimed to identify potential biocontrol agents capable of suppressing tomato bacterial wilt caused by *Ralstonia pseudosolanacearum*. In total, 442 bacteria were isolated from the rhizosphere soil of tomato, Chinese chive, and Welsh onion. Based on the results of the *in vitro* antibacterial activity assay, 276 isolates were selected and further evaluated using a tomato seedling bioassay. Nineteen isolates that belonged to that the genera *Ralstonia* and *Mitsuaria* exhibited a relatively higher disease suppression (>50% reduction in disease severity) than the other isolates. The isolate TCR112 of *Ralstonia* and 11 isolates of *Mitsuaria* were assessed for their biocontrol effect in a series of pot experiments. Among the isolates, TCR112 (identified as non-pathogenic *Ralstonia* sp.) and TWR114 (identified as *Mitsuaria* sp.), which showed a consistent disease suppression in pot experiments, were selected as final candidates for further evaluation under field conditions. The results showed that soil drenching at weekly intervals with isolates TCR112 and TWR114 reduced the wilt incidence in the first year by 57.2% and 85.8%, and in the second year by 57.2% and 35.3%, respectively, indicating that these isolates were promising biocontrol agents of tomato bacterial wilt. The isolates effectively reduced the pathogen population in the rhizosphere and crown of pot grown tomatoes. Monitoring the population dynamics of biocontrol isolates revealed that both isolates have stable rhizosphere and endophytic colonization capacities. This is the first study reporting the potential of *Mitsuaria* as a biocontrol agent against tomato bacterial wilt.

1. Introduction

Bacterial wilt is caused by *Ralstonia solanacearum* (228), *R. pseudosolanacearum*, and *R. syzygii* subsp. *indonesiensis* (formerly classified as *R. solanacearum*) (178), and is the second most destructive bacterial disease of plants worldwide (126). Bacterial wilt affects the yield of many solanaceous plants, such as tomato (*Solanum lycopersicum*), potato (*S. tuberosum*), tobacco (*Nicotiana tabacum*), and eggplant (*S. melongena*) (75). Five million hectares of tomatoes are estimated to be grown annually worldwide, producing >170 million tons (56). In Japan, tomatoes are grown on a total area of 12 thousand hectares, with an annual production of 740 thousand tons (56), and thus are listed as the second most important crop after rice.

The management of tomato bacterial wilt is difficult owing to the viability, adaptability, and genetic diversity of the responsible pathogen (53). In Japan, the current countermeasures used against bacterial wilt include chemical controls and cultural practices. However, chemical controls using soil fumigants such as chloropicrin are potentially harmful to the environment and have not been efficient in eradicating *R. solanacearum* (177). Cultural practices through commercially grafted seedlings (grafting resistant rootstock with susceptible scion) restrict pathogen multiplication and movement in the rootstock, thereby suppressing the infection and wilting in the scion, and through an anaerobic/reductive soil disinfestation (RSD) method reduces the pathogen population in the soil and is widely adopted in Japan (137). However, grafting is expensive, requires more labor, and result in the production of fruits of inferior quality (taste, color, and sugar contents) (110). Furthermore, new virulent races of the pathogen might overcome the resistance, resulting in colonization and migration of the pathogen into susceptible scions and

causing wilt symptoms (140). Moreover, for the RSD method, achieving sufficient disinfection in the deep soil layers where the pathogen might localize is difficult (137). Thus, other alternative or supplementary methods for controlling bacterial wilt are required. The biological control method of using beneficial microorganisms has been proposed as an effective, safe, and sustainable approach.

R. solanacearum is well adapted to grow and survive in the bulk soil for many years in the absence of susceptible host plants (165). When the pathogen encounters a susceptible host, it enters the root via wounded parts or natural openings such as lateral root emergence points and colonizes the root cortex (42). Therefore, antagonistic rhizobacteria were thought to be the best choice of biocontrol agents (BCAs) for controlling tomato bacterial wilt. Indeed, several studies in the past have successfully obtained rhizobacteria such as *Pseudomonas* spp. (112), *Bacillus* spp. (105), and *Flavobacterium johnsoniae* and *Chryseobacterium daecheongens* (80) that have strong biocontrol ability against bacterial wilt under laboratory and/or greenhouse conditions. In Japan, rhizospheric *Pseudomonas fluorescens* isolates were previously commercialized as a biocontrol product against bacterial wilt (Cell Nae Genki, Taki Chemical, Kakogawa, Japan); however this product was abolished and no longer exists, therefore it is necessary to develop new biopesticides against bacterial wilt.

Many researchers have screened rhizobacteria from host plants susceptible to pathogen infection to identify promising candidates as BCAs to control soil-borne diseases, as these bacteria have high affinity for the roots of host plant. We assumed that bacteria inhabiting the rhizosphere of non-host plants, particularly companion plants, are also a good source of BCAs. Intercropping has long been used for controlling soil-borne diseases. Companion plants used for intercropping

enhance antagonist populations in soil and reduce pathogen attack on host plants (77). Intercropping with *Allium* plants, such as Welsh onion, Chinese chive, and garlic, has been reported to suppress soil-borne diseases including bacterial wilt of tomato (106, 235). Nishioka et al. (146) have shown that antagonistic bacteria inhabiting the rhizosphere of *Allium* spp. play an important role in the suppression of cucumber Fusarium wilt. Although the mechanisms of bacterial wilt suppression due to *Allium* intercropping are unknown, this suppression can be attributed to the accumulation of antagonistic bacteria. Therefore, *Allium* spp. were thought to be a reservoir of potential BCAs.

In this study, we isolated antagonistic rhizobacteria from tomato and *Allium* plants, and then screened their biocontrol potential against tomato bacterial wilt to develop a new biocontrol product.

2. Materials and methods

2.1. Isolation of rhizobacteria

Bacteria were isolated from the rhizosphere soil of tomato (*S. lycopersicum* cv. Ohgata-Fukuju), Chinese chive (*Allium tuberosum* Rottler ex Spreng., cv. Super green belt), and Welsh onion (*Allium fistulosum* L., cv. Kujo-hoso), grown in fields at Gifu University (Yanagido, Gifu city, Gifu Prefecture, Japan). For isolating the bacteria from the rhizosphere, 3-month-old plants (tomato, Chinese chive, and Welsh onion) were uprooted, and loosely adhering soil was gently removed. Then, roots of each plant were suspended in sterile distilled water (SDW) and shaken on a rotary shaker at 150 rpm for 15 min. Serial dilutions of the soil suspension were

spread on the surface of tryptic soy agar medium and incubated at 30°C for 24 h. The purified colonies were suspended in 10% (w/v) skim milk (Difco, Sparks, MD, USA) supplemented with L-glutamic acid monosodium salt (16.5 g/l) and kept at −80°C until use.

2.2. Bacterial isolates and culture conditions

R. pseudosolanacearum isolate VT0801 (isolated from an infested tomato field in Tsu city, Mie prefecture, Japan) was used as the challenging pathogen. *R. pseudosolanacearum* and rhizobacterial isolates were cultured in casamino acid-peptone-glucose broth medium (76) and nutrient broth (NB) medium (Nissui Pharmaceutical Co., Tokyo, Japan), respectively, at 30°C for 24 h with shaking at 200 rpm.

2.3 In vitro antibacterial activity

The antibacterial activity was assessed using the agar well diffusion assay (166). A 70-µl aliquot of 24-h-old culture broth (approximately 10^7–10^8 cells/ml) of each rhizobacterial isolate was applied to 7-mm-diameter well on solidified King's B medium supplemented with washed cell suspension of isolate VT0801 and incubated at 30°C for 24 h. The inhibition of VT0801 growth was assessed based on the production of a clear halo zone surrounding the wells. Three replicates were used for each bacterial isolate.

2.4. Evaluation of disease suppression using tomato seedling bioassay

Rhizobacterial isolates that exhibited antibacterial activity in the agar well diffusion assay were further screened for their disease suppressive activity against bacterial wilt using tomato seedling bioassay as described previously (6), with some modifications. Seeds of susceptible tomato (cv. Ponderosa) were surface sterilized with 70% (v/v) ethanol for 1 min, followed by 2% sodium hypochlorite for 5 min, and then thoroughly rinsed with SDW. After germination, 10 seeds were sown into a flat-bottom glass tube (25 mm × 100 mm; AGC Techno Glass Co. Ltd., Shizuoka, Japan) that contained 3.4 g of sterile vermiculite (autoclaved twice at 24-h intervals). The cells of rhizobacterial isolates harvested from 24 h were washed twice with SDW. A 2-ml aliquot of cell suspension of each isolate, adjusted to OD_{600} = 0.1 (ca. 10^8 CFU/ml) was added the above seeded tubes, followed by inoculation with 2 ml of pathogen suspension (ca. 8 × 10^5 CFU/ml). The control treatment was prepared using 2 ml of SDW instead of the rhizobacterial cell suspension. All tubes were maintained in a controlled environmental chamber (Biotron, standard, Nippon Medical and Chemical Instruments Co., Ltd., Osaka, Japan) at 28°C under a 12-h light/12-h dark cycle for 7 days. In trial 1, three seedling tubes were used for each isolate. In trial 2, three tubes were used for each isolate, and the experiment was repeated thrice. The disease severity of the tomato seedlings was visually score on a scale of 0–2, where 0 represent no symptoms, 1 indicate small areas of the hypocotyl showing necrosis, 2 indicates wilted seedling or large areas of the seedling showing necrosis. The disease suppressive efficacy was calculated using the following formula: disease suppressive efficacy = [(mean disease scale of the control treatment) − (mean disease scale of bacterial treatment)/(mean disease scale of control treatment)] ×100%.

2.5. Evaluation of selected rhizobacterial isolates in pot experiments

2.5.1. Growth of plant and bacterial inoculation

The rhizobacterial isolates selected in the above seedling bioassay were evaluated for their biocontrol effect in a series of pot experiments (trial 1 to 3). As described later, we selected 1 isolate of *Ralstonia* and all of the *Mitsuaria* isolates, except for the TCR127 isolate, for pot experiments.

Tomato seeds (cv. Ponderosa) were surface sterilized and germinated as described above. The seeds were then sown in plastic trays (Bee pot Y-49; Canelon Kaka Co. Ltd., Japan) that contained a commercial potting soil mix “New star bed” (Zen-Noh, Tokyo, Japan) and grown in a glasshouse maintained at 30°C with a relative humidity of 70% until the seedlings reached fourth-leaf stage. Seedlings were transplanted into vinyl pots (9 cm in diameter) comprising three layers: top and bottom layers, each containing 150 g of commercial potting soil mix, and middle layer containing 20 g of river sand. Rhizobacterial cells were harvested from 24-h-old cultures, washed twice, and diluted with SDW to obtain a concentration of ca. 3×10^8 CFU/ml. In trials 1 and 2, tomato plants were treated by bottom watering with the cell suspension of each rhizobacterial isolate (100 ml per pot) to obtain a final concentration of ca. 1×10^8 CFU/g soil. Plants treated with an equal volume of SDW without the rhizobacteria were used as controls. One day after treatment, both control plants and those treated with rhizobacteria were challenged with 100 ml of VT0801 washed cell suspension (ca. 4×10^7 CFU/ml) to obtain a final concentration of ca. 1×10^7 CFU/g soil. The inoculated plants were maintained in the same glasshouse for 14 days. In trial 3, tomato plants were treated as above with the cell

suspension of rhizobacteria isolates (ca. 9 × 10^8 CFU/ml) to obtain a final concentration of ca. 3 × 10^8 CFU/g soil. Three days after the treatment, plants were challenged with 100 ml of VT0801 cell suspension (ca. 2 × 10^7 CFU/ml) and grown for 14 days under the same glasshouse conditions. Five plants were used for each treatment in trial 1. By contrast, each treatment consisted of three replicates of nine plants per replicate and five replicates of ten plants per replicate in trial 2 and trial 3, respectively.

2.5.2. Development of disease symptoms

The symptoms of tomato bacterial wilt were monitored daily on the basis of a disease scale that ranged from 0 to 4, as described by Kempe and Sequeira (96), where 0 = no wilt symptoms (healthy), 1 = up to 25% of the leaves wilted, 2 = 25%–50% of the leaves wilted, 3 = 50%–75% of the leaves wilted, and 4 = 75%–100% of the leaves wilted. The disease incidence, disease severity and the area under disease severity progress curve (AUDPC) were calculated using the following formulas:

Disease incidence = {[total number of diseased plants (scale 1–4) in the treatment/total number of plants investigated)]} × 100

Disease severity = [(the number of diseased plants in each scale × disease scale)/(total number of plants investigated × the highest disease scale)] × 100.

AUDPC was calculated on the basis of disease severity using the trapezoid integration of disease progress curve over time according to the following formula: AUDPC = $\sum [0.5 (x_i + x_{i-1})] (t_i - t_{i-1})$, where x_i and x_{i-1} are disease severity at time t_i and t_{i-1}, respectively, and t_i and t_{i-1} are consecutive evaluation dates, with t_i and t_{i-1} equal to 1.

2.6. Quantification of R. pseudosolanacearum

Tomato plants were treated with two final candidate isolates (TCR112 and TWR114) and challenged with *R. pseudosolanacearum* VT0801 as in trial 3 of pot experiments. The pathogen multiplication in the rhizosphere and crown (basal part of hypocotyl) of tomato plants was determined at 1, 3, 5, 7, and 14 days after challenge inoculation. Samples were obtained from a total of three plants at each time point. Rhizosphere soil samples were serially diluted with SDW. Crown samples of tomato plants (2 cm in length) were surface sterilized with 100% ethanol and flamed as described previously (Wei et al., 2013). The samples were then homogenized using mortar and pestle, and used to prepared serial dilutions in SDW. Dilutions of rhizosphere soil and crown homogenate were spread in triplicates onto the surface of modified semi-selective medium South Africa (M-SMSA) (61). Typical colonies of *R. pseudosolanacearum* that appeared elevated fluidal with a pink center were counted after incubation for 3 days at 30°C. The experiment was repeated thrice. The population was expressed as log colony-forming units per gram (wet weight) of soil (log CFU/g wet soil) or tissue (log CFU/g fresh tissue).

2.7. Colonization capacity of isolates TCR112 and TWR114

The populations of the isolates TCR112 and TWR114 in the rhizosphere and crown of tomato plants were simultaneously enumerated with the pathogen population. Dilutions of the rhizosphere soil and crown homogenate, used for pathogen enumeration, were spread onto the surface of isolation media that were

optimized for each isolate. A preliminary survey of the antibiotic resistance of the isolates revealed that TCR112 and TWR114 had resistance to six (kanamycin, ampicillin, hygromycin B, gentamicin, tobramycin and streptomycin) and three (kanamycin, ampicillin and hygromycin B) antibiotics, respectively. Accordingly, these antibiotics were added in respective combination to 1/10-strength TSA medium (5 mg/L each). Moreover, cycloheximide (50 mg/L) was also added to both media to prevent fungal contamination. These inoculated plates were incubated at 30°C for 48 h, and the number of representative colonies of each isolate (Fig. 1) was counted. The experiment was repeated thrice.

2.8. Field experiments

Field experiments were conducted in an experimental field at Gifu University, from August to October in 2016 and from September to October in 2017. Before transplanting, 30 tons/ha of organic fertilizer (60% of cow manure, 20% of pig manure, and 20% of horse manure) and 2.3 tons/ha of chemical fertilizer ($N:P_2O_5:K_2O$ in the ratio of 12:9:10 supplemented with Mg:B ratio of 2:0.1) (Nittofc Co., Ltd., Japan) was added to the soil. Moreover, limestone was added at a rate of 2.3 tons/ha (55.4% CaO, pH 9.5) (Shinko Kogyo Co., Ltd., Japan). Furthermore, the population of the pathogen in the field was quantified for both years. Soil samples were obtained from 12 different locations distributed across the field. Ten grams of bulk soil was used to prepared serial dilutions in SDW. Dilution of bulk soil was spread onto the surface of M-SMSA medium and incubated as described earlier. The population was expressed as log colony-forming units per gram (dry weight) of soil (log CFU/g dry soil).

The field (11.5 m × 6.6 m) comprised eight rows, and each row (10.8 m length, 0.8 m width) was divided into three plots (3.6 m length). There were three and four replicate plots per treatment arranged in a randomized complete block design in the first and second year, respectively. Six tomato plants were transplanted in each plot with distances of 0.55 m between the plants. Standard agronomic practices were performed to grow tomato plants.

Field experiments comprised three treatments: (1) control, (2) TCR112, and (3) TWR114. Fourth-leaf-stage tomato seedlings (cv. TY Misora 86) grafted with the rootstock (cv. Magnet, moderately resistant to *R. solanacearum* and highly resistant to *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *F. oxysporum* f. sp. *lycopersici*, *Verticillium dahlia*, and *Pyrenochaeta lycopersici*) and planted in vinyl pots containing commercial potting soil mix (300 g). These plants were then treated with TCR112 and TWR114 (final concentration ca. 3×10^8 CFU/g soil) as described earlier. Tomato plants treated with SDW were served as control. All the plants were grown in a greenhouse at 28–30°C. After reaching the eight–ninth-leaf stage, the tomato plants were transplanted into the field, and then, 300 ml of the cell suspension (ca. 3×10^8 CFU/ml) of each isolate or the same volume of distilled water was applied around the stem base of each plant. TCR112 and TWR114 were applied at weekly intervals until 42 and 28 days in the first and second year of the experiments, respectively. During the experiment, the number of wilted plants was recorded daily and disease incidence was calculated as described above. Moreover, at the end of the experiment in first year (8 days after the final application with the candidate isolates), three healthy plants from each treatment (one plant from each plot) were used to estimate the population of the TCR112 and TWR114 in the rhizosphere and crown tissues as described above.

2.9. Identification of selected rhizobacterial isolates

2.9.1 Partial and complete sequence of the 16S rRNA gene

Bacterial isolates showing *in vitro* antibacterial activity was tentatively identified based on the partial sequence of 16S rRNA gene, according to a protocol described previously (147). The isolates TCR112 and TWR114 were further identified by sequencing the full-length 16S rRNA gene. Primers 27f and 1492r (Lane, 1991), were used for sequencing the PCR products. PCR amplification and DNA sequencing were performed using the same conditions as described previously (147). The 16S rRNA gene sequences were compared with those of type strains in the EzBioCloud database (https://www.ezbiocloud.net/) (234). Additionally, the 16S rRNA gene sequences of the representative strains were downloaded from the GeneBank database and aligned with the sequences of TCR112 and TWR114 isolates. A phylogenetic tree was constructed with the neighbor-joining method using MEGA version 7.0.26 (201). The 16S rRNA gene sequences of the isolates were deposited in GeneBank database under the accession numbers MG877646–MG877664.

2.9.2 Multilocus sequence analysis

An additional phylogenetic analysis based on multiple protein coding genes was carried out to better characterize the final candidate isolate TCR112 at the species level.

Five protein-coding housekeeping genes *gdhA* (glutamate dehydrogenase, NADP-specific, oxidoreductase protein), *mutS* (methyl-directed DNA mismatch repair

protein), *leuS* (Leucyl-tRNA synthetase), *rplB* (50S ribosomal subunit protein L2), and *gyrB* (DNA gyrase, subunit B), were selected for the multilocus sequence analysis (MLSA) scheme of the isolate TCR112. Sets of primers used to amplify fragments of these five genes are listed in Table 1. PCR amplifications were carried out using One-Taq Hot Start 2X Master Mix with GC Buffer (New England BioLabs) to help the amplification of the GC-rich sequences. Primer concentrations (forward and reverse) were 0.2 µM and 10% One-Taq High GC enhancer was added to help the amplification of extremely difficult amplicons. The amplification conditions were at 94°C for 2 min (initial denaturation), followed by 30 cycles at 94°C for 1 min, the appropriate annealing temperature (Table 1), and an extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. The amplification products were purified using GenElute PCR Clean-Up Kit (Sigma, MO, USA), as described in the manufacturer's instructions. The cycle sequencing reaction was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and the same primers used for the amplification process except for the *gdhA* gene (Table 1). The cycle sequencing conditions were 96°C for 1 min (initial denaturation), followed by 25 cycles at 96°C for 1 min, 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Amplification and sequencing were performed in ABI PRISM 3100 Genetic Analyzers (Applied Biosystems, CA, USA). The raw sequences were assembled and then manually inspected and corrected using Chromas pro (version 2.1.6, Technelysium Pty. Ltd, Tewantin, Queensland, Australia). The partial sequences of the housekeeping genes of other representative strains were downloaded from the GeneBank database. The consensus sequence of each gene was aligned with the sequences of the other representative strains using CLUSTAL W within MEGA software (201). Gene alignments were concatenated with Geneious R11 software

platform (version 11.0.3; Biomatters Ltd., New Zealand). The phylogenetic tree was constructed using the maximum likelihood in PhyML (version 3.0, http://www.atgc-montpellier.fr/phyml/) (69). Bootstrap support with 1000 replicates was generated to estimate the reliability of the clusters. The protein-coding housekeeping genes sequences of TCR112 isolate were deposited in GeneBank database under the accession numbers MG878974–MG878978.

2.9.3 Physiological and biochemical characterization

The growth of TCR112 and TWR114 was tested by culturing the isolates on nutrient agar (NA) plates and incubating them under various temperature conditions (4°C, 15°C, 25°C, 30°C, 37°C, and 42°C). Tolerance to NaCl was tested on NA plates containing 0%, 1%, 3%, and 5% NaCl (w/v). Urease production was detected using urea agar as previously described by Christensen (35). Starch hydrolysis was examined using starch agar medium as previously described by Atlas et al., (15). Briefly, the bacterial isolates were grown on NA medium supplemented with 2% starch and incubated at 30°C for 4 days. After incubation, the zone of hydrolysis was detected by flooding the plates with iodine solution.

Additional biochemical tests were performed to assess the carbon source utilization pattern of TCR112 and TWR114 isolates. Briefly, overnight bacterial cultures were adjusted to an optical density of 0.1 (OD_{600}) and were grown in 96-well microtiter plates in 150-µl one-quarter strength of M63 minimal medium (VWR International, LLC, Solon, Ohio, USA) supplemented with each carbon source with a final concentration of 10mM. After 48-h growth at 30°C with agitation (200 rpm), the OD_{570} was measured using a Tecan Sunrise microplate reader (Tecan Austria

GmbH, Grödig, Austria). Wells with an OD_{570}>0.05 were scored as positive for growth on a given substrate.

2.10. Data analysis

The data of pot experiments were compared using Student's *t*-test (*P*<0.05). The data of bacterial counts were transformed into logarithm numbers and compared by Student's *t*-test (*P*<0.05). All analyses were performed using SigmaPlot 11.0 software (Systat Software Inc., USA).

3. Results

3.1. Isolation of antibacterial rhizobacteria

In total, 442 bacteria were successfully isolated from rhizosphere soil samples and used as a pool for antibacterial screening (Table 2). Of these isolates, 276 (62.4%) exhibited weak-to-very strong antibacterial activity against *R. pseudosolanacearum* in the agar well diffusion assay (Table 3 and Fig. 2A) and were selected for subsequent tomato seedling bioassay. By analyzing a partial sequence of 16S rRNA gene, these antibacterial isolates were assigned to 24 genera, including *Burkholderia*, *Pseudomonas*, *Mitsuaria, Acinetobacter, Arthrobacter*, *Achromobacter*, and *Ralstonia* (Table 3).

3.2. Tomato seedling bioassay

The suppressive effect of selected antibacterial isolates against tomato bacterial wilt was examined using the tomato seedling bioassay. In the first trial, 56 of 276 isolates reduced the disease severity in the treated plants compared with that in the untreated control (Table 3). In addition, rhizobacterial isolates from Chinese chive and Welsh onion plants exhibited stronger suppressive effect than those isolated from tomato plants (Fig. 3B). Among the 56 isolates, 19 showed 50–100% reduction in disease severity (Table 3). Therefore, the disease suppressive effect of these 19 isolates was further evaluated in the second trial of the seedling bioassay. All the tested isolates exhibited strong suppressive effects (ranging from 68.5% to 95.9% reduction in disease severity) (Table 3). These isolates comprised two genera, namely *Ralstonia* (isolates TCR111, TCR112, TCR113, TCR123, TCR124, TCR133, TCF143, and TCF148) and *Mitsuaria* (isolates TCR103, TCR127, TCR156, TCR158, TCR159, TCR167, TWR114, TWR120, TWR137, TWR165, and TWR167).

3.3. Evaluation of biocontrol efficacy of selected rhizobacteria in pot experiments

Because none of the isolates belonging to genus *Mitsuaria* has been reported as a biocontrol agent against *R. pseudosolanacearum*, we evaluated the biocontrol efficacy of all of our *Mitsuaria* isolates against tomato bacterial wilt in trials 1 and 2 of pot experiments. Furthermore, TCR112 was selected from eight *Ralstonia* isolates for trial 3, because this isolate showed the highest suppressive effect, both in the first and second trial of seedling bioassay (Table 3). In trial 1 performed in a glasshouse with 11 *Mitsuaria* isolates, ten isolates reduced disease severity, which was expressed as AUDPC (Table 4). In particular, four isolates, TCR103, TCR127,

TCR159, and TWR114, showed the lowest AUDPC. Therefore, the biocontrol effect of these four isolates was again evaluated in trial 2. In this trial, the highest reduction of AUDPC was achieved using the isolate TWR114 (45.7%)(Table 4); thus, this isolate was selected for the trial 3.

The evaluation of biocontrol efficacy of the final candidate isolates TCR112 and TWR114 in trial 3 proved that the isolates were highly effective in suppressing disease severity of tomato bacterial wilt, as shown by the significant reduction of AUDPC values of 66.4% and 55.3%, respectively (Table 5 and Fig. 4).

3.4. Quantification of R. pseudosolanacearum

The *R. pseudosolanacearum* population was effectively reduced following the treatment with TCR112 and TWR114 in the rhizosphere and crown of tomato plants (Fig. 5). In TCR112-treated plants, the pathogen was not detected in both the rhizosphere and crown at 1 and 3 days post-challenge inoculation (dpi), whereas in the untreated control plants, the pathogen was detected in the rhizosphere (4.4 and 5.8 log CFU/g wet soil) and crown (1.7 and 3.9 log CFU/g fresh tissue). Subsequently, the pathogen population reached a detectable level in TCR112-treated plants; however, the population densities were significantly lower in the rhizosphere (2.7 and 2.6 log CFU/g wet soil) and crown (2.7 and 2.6 log CFU/g fresh tissue) of TCR112-treated plants than in the rhizosphere (8.8 and 9.5 log CFU/g wet soil) and crown (8.6 and 9.4 log CFU/g fresh tissue) of untreated control plants at 5 and 7 dpi, respectively (Fig. 5A and 5C). The pathogen population was significantly reduced in the rhizosphere of TWR114-treated plants (4.0, 4.3, and 5.4 log CFU/g wet soil) compared with that of the untreated control plants (4.9, 7.0, and 9.1 log

CFU/g wet soil) at 1, 3, and 5 dpi, respectively (Fig. 5B). The pathogen was not detected or was significantly reduced in the crown of TWR114-treated plants (2.2 log CFU/g fresh tissue) compared with that of the untreated control plants (3.3 and 8.3 log CFU/g fresh tissue) at 1 and 5 dpi, respectively (Fig. 5D).

3.5. Colonization capacity of the isolates TCR112 and TWR114

The isolates TCR112 and TWR114 were successfully recovered from both the rhizosphere and crown of tomato plants during the growth period under the glasshouse conditions (Fig. 6). The colonization of the isolate TCR112 was relatively stable throughout the experiment, with a mean population of 6.5 log CFU/g wet soil and 3.5 log CFU/g fresh tissue in the rhizosphere and crown, respectively (Fig. 6A). In contrast, the isolate TWR114 showed an increased colonization throughout the experiment. At 1 dpi (4 days after bacterial treatment), the population densities of TWR114 steadily increased from 5.0 log CFU/g wet soil and 2.0 log CFU/g tissue to 7.3 log CFU/g wet soil and 4.3 log CFU/g tissue, respectively, in the rhizosphere and crown, respectively, at 14 dpi (Fig. 6B). TCR112- and TWR114-like colonies were not detected in both the rhizosphere and crown of untreated control tomato plants.

3.6. Evaluation of TCR112 and TWR114 in field experiments

In the field experiments, the mean initial pathogen population were 3.6 ± 0.2 (log CFU/g dry soil) and 3.0 ± 0.3 (log CFU/g dry soil) in 2016 and 2017, respectively (Fig. 7).

In two consecutive years, the wilt incidence was considerably suppressed in plots drenched treated with the biocontrol isolates at weekly intervals (Fig. 8). In the first year, the wilt incidence at the end of field experiment (50 days after transplanting) was reduced by 57.2% and 85.8% in plots drenched with TCR112 and TWR114, respectively (Fig. 8). Similarly, the wilt incidence at the end of field experiment in the second year (30 days after transplanting) was reduced by 57.2% and 35.3% in plot drenched with TCR112 and TWR114, respectively (Fig. 8).

At the end of the experiment in the first year, we enumerated the populations of both biocontrol isolates. Consequently, both isolates (TCR112 and TWR114) were successfully recovered from the rhizosphere (5.7 and 6.2 CFU/g wet soil, respectively) and crown (4.0 and 5.8 CFU/g fresh tissue, respectively) of the tomato plants (Fig. 9).

3.7. Characterization and identification of the biocontrol isolates TCR112 and TWR114 and the pathogen VT0801

The analysis of full-length 16S rRNA sequence of biocontrol isolates and the pathogen, indicated that TCR112 (approximately 1,230 bp) were 99.8% similar to those of *R. pickettii* (accession number: JOVL01000020) and TWR114 (approximately 1,200 bp) shared 99.3% similarity with *Mitsuaria chitosanitabida* (accession number: BCYP01000048), whereas VT0801 (approximately 880 bp) were shared 99.6% similarity with *R. pseudosolanacearum* (accession number: KC757037). To clarify the phylogenetic position of the biocontrol isolates and the pathogen, a phylogenetic tree was constructed on the basis of partial or complete 16S rRNA gene sequences (Figs. 10 and 11). As a result, the isolates TCR112,

TWR114, and VT0801 showed a clear distinction from the known type strains of *R. pickettii* ATCC 27511^T, *M. chitosanitabida* 3001^T, and *R. pseudosolanacearum* UQRS 461^T, respectively (Figs. 10 and 11).

In order to construct a higher resolution phylogenetic tree for the isolate TCR112, an MLSA scheme using five housekeeping genes (*gdhA*, *mutS*, *leuS, rplB*, and *gyrB*) was applied. The lengths of the five protein-coding genes were: *gdhA* = 503 bp, *mutS* = 640 bp, *leuS* = 700 bp, *rplB* = 639 bp, and *gyrB* = 345 bp. The MLSA phylogenetic tree revealed that the isolate TCR112 have a clear distinction from its closest relative type strains of *R. pickettii* (Fig. 12).

The growth of TWR114 occurred at 15–37°C and 0–1.0% NaCl (w/v), being optimal at 25–30°C and 0.5% NaCl (w/v). The growth of TCR112 occurred at 15–42°C and 0–1.0% NaCl (w/v), being optimal at 25–30°C and 0.5% NaCl (w/v). The TWR114 showed positive starch hydrolysis, whereas, it had negative urease and β-glucosidase activity. The TCR112 showed negative starch hydrolysis, while it showed positive urease and β-glucosidase activity. Both isolates did utilize sucrose, D-fructose, D-mannose, D-xylose, D-galactose, N-Acetyl-D-glucosamine, and L-Arabinose, but did not utilize capric acid (Table 7).

Based on the above characteristics, TWR114 was identified as a *Mitsuaria* sp. and TCR112 was identified as a *Ralstonia* sp., and VT0801 was identified as *R. pseudosolanacearum*.

Table 1. Primers used for the amplification and sequencing of protein-coding genes in the non-pathogenic *Ralstonia* sp. TCR112.

Gene	Primer Name	Sequence (5'-3')	Annealing T°	Reference	Template size (bp)
gdhA	GdhAF	GATGGATGACGGCCGCATCG	61	30	1056
	GdhAR	TGAACGCCGCCGTCCGCAG			
	gdhA486-F†	GCCGGACGTGAACACCAAC	50	220	639
	gdhA1124-R†	GAGAAATCCTGCACCCACTCGAAA			
mutS	mutS-RsF.1570	ACAGCGCCTTGAGCCGGTACA	62	220	758
	mutSRsR1926	GCTGATCACCGGCCCGAACAT			
leuS	leuS27-F	GGTCGAACAGCAGGCGCAGCAA	65	220	793
	leuS819-R	GGCGCAGAAGGTCACGCCCA			
rplB	rplB39-F	CCGCTCGATGGTGAAGGTCGT	60	220	766
	rplB804-R	CATGCTGGTCGTGCGCTTGTTG			
gyrB	gyrB1F	GACAACGGCCGCGGSATTCC	60	206	432
	gyrB2R	CACGCCGTTGTTCAGGAASG			

† Sequencing primers

Table 2. Total number of bacteria successfully isolated from the rhizosphere soil of Alliums and tomato plants.

Host plant	Chinese chive	Welsh onion	Tomato
Total number of bacteria	154	141	147

Table 3. Selected bacterial isolates used for the seedling bioassay and their disease suppressive effect against bacterial wilt in tomato seedlings.

Isolate	Antibacterial activity[†]	Source[‡]	Reduction in disease severity (%)[§]		Closest hit (accession number)	Identity (%)
			Trial 1[#]	Trial 2[†]		
TTR103	+	T	0.00	NT	*Paracoccus huijuniae* (EU725799)	99.9
TTR108	+	T	0.00	NT	*Pseudomonas* sp. TCU-HL1 (CP015992)	99.1
TTR111	+	T	47.9	NT	*Pseudomonas* sp. TCU-HL1 (CP015992)	99.1
TTR115	+	T	43.8	NT	*Pseudomonas brassicacearum* subsp. *neoaurantiaca* (EU391388)	99.1
TTR116	+	T	0.00	NT	*Bacillus pseudomycoides* (NVOR01000041)	99.5
TTR118	+	T	0.00	NT	*P. huijuniae* (EU725799)	99.8
TTR132	++	T	0.00	NT	*Flavobacterium hibisci* (KX263317)	99.2
TTR135	+	T	0.00	NT	*Pseudomonas simiae* (AJ936933)	92.1
TTR136	+	T	0.00	NT	*Staphylococcus caprae* (AB009935)	99.4
TTR139	+	T	0.00	NT	*Cupriavidus oxalaticus* (AF155567)	91.0
TTR144	++	T	0.00	NT	*Paenibacillus cucumis* (KU201962)	99.3
TTR163	+	T	0.00	NT	*P. cucumis* (KU201962)	99.0
TTR168	+	T	0.00	NT	*Arthrobacter oryzae* (AB279889)	99.4
TTR171	+	T	0.00	NT	*C. oxalaticus* (AF155567)	91.0
TTR172	+	T	0.00	NT	*C. oxalaticus* (AF155567)	91.0
TTR174	+	T	20.8	NT	*Pseudomonas umsongensis* (NIWU01000003)	99.8
TTR178	+	T	0.00	NT	*C. oxalaticus* (AF155567)	91.0
TTR179	+	T	10.4	NT	*P. umsongensis* (NIWU01000003)	99.4
TTR188	+	T	0.00	NT	*Streptomyces tanashiensis* (AJ781362)	100
TTR191	+	T	0.00	NT	*P. huijuniae* (EU725799)	100
TTR192	+	T	0.00	NT	*Streptomyces tuirus* (AB184690)	97.8
TTR197	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TTF101	++	T	0.00	NT	*Burkholderia cenocepacia* (JJOA01000042)	99.6
TTF102	++	T	0.00	NT	*Burkholderia contaminans* (LASD01000006)	99.7
TTF103	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.2
TTF104	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.5
TTF105	++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TTF106	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.2
TTF107	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.2
TTF108	++	T	0.00	NT	*Burkholderia puraquae* (NBYX01000050)	100
TTF110	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TTF111	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.8
TTF112	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.8
TTF113	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.8
TTF114	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TTF115	+++	T	0.00	NT	*B. puraquae* (NBYX01000050)	100
TTF116	++	T	0.00	NT	*B. cenocepacia* (JTDP01000003)	100

TTF118	++	T	0.00	NT	*Burkholderia dolosa* (JX986970)	81.9
TTF119	+++	T	0.00	NT	*B. puraquae* (NBYX01000050)	100
TTF120	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.6
TTF121	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TTF122	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.8
TTF124	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.8
TTF125	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TTF126	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.7
TTF127	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.6
TTF128	++	T	5.00	NT	*Burkholderia ambifaria* (CP000442)	98.3
TTF129	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TTF130	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TTF131	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.3
TTF132	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.7
TTF133	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.3
TTF134	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.2
TTF135	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.9
TTF136	++++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.8
TTF137	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.9
TTF138	++	T	0.00	NT	*B. contaminans* (LASD01000006)	98.7
TTF139	+	T	0.00	NT	*B. contaminans* (LASD01000006)	99.8
TTF140	++	T	0.00	NT	*B. contaminans* (LASD01000006)	99.8
TTF141	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.1
TTF142	++	T	0.00	NT	*Burkholderia ubonensis* (EU024179)	95.6
TTF143	++	T	0.00	NT	*B. ubonensis* (EU024179)	95.8
TTF144	++	T	0.00	NT	*B. ubonensis* (EU024179)	96.0
TTF145	++	T	0.00	NT	*B. ubonensis* (EU024179)	96.1
TTF146	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.1
TTF147	+++	T	0.00	NT	*B. ambifaria* (CP000442)	99.8
TTF148	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.1
TTF149	+++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TTF150	++	T	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TCR101	++	C	8.3	NT	*B. puraquae* (NBYX01000050))	97.9
TCR102	+	C	0.00	NT	*Sphingobium* sp. YL23 (ASTG01000050)	96.6
TCR103	+	C	76.7	94.8 ± 7.6‡‡	*Mitsuaria chitosanitabida* (BCYP01000048)	99.1
TCR104	++	C	0.00	NT	*Streptomyces roseofulvus* (AB184327)	98.4
TCR106	+++	C	3.3	NT	*B. cenocepacia* (JJOA01000042)	91.9
TCR107	++	C	0.00	NT	*Novosphingobium aromaticivorans* (AKFJ01000034)	97.6
TCR108	+	C	0.00	NT	*Sphingobium* sp. YL23 (ASTG01000050)	97.1
TCR109	+++	C	6.67	NT	*B. cenocepacia* (JJOA01000042)	98.5
TCR110	+	C	0.00	NT	*Pantoea septica* (MLJJ01000077)	96.4
TCR111	+	C	63.3	73.3 ± 6.9#	*Ralstonia pickettii* (JOVL01000020)	100

TCR112	+	C	100.0	90.0 ± 5.8	*R. pickettii* (JOVL01000020)	99.8
TCR113	+	C	90.0	85.6 ± 3.0	*R. pickettii* (JOVL01000020)	99.8
TCR114	+++	C	0.00	NT	*B. ambifaria* (CP000442)	92.7
TCR116	++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	98.2
TCR117	++	C	0.00	NT	*Burkholderia stabilis* (CP016444)	98.3
TCR118	+	C	0.00	NT	*A. oryzae* (AB279889)	98.0
TCR119	+++	C	16.7	NT	*B. ambifaria* (CP000442)	100
TCR120	+	C	16.7	NT	*Pseudomonas nitroreducens* (AM088474)	98.1
TCR121	++	C	0.00	NT	*Bacillus pseudomycoides* (NUQE01000007)	100
TCR122	+	C	21.7	NT	*Pseudomonas asplenii* (LBME01000002)	98.8
TCR123	+	C	86.7	83.9 ± 4.6	*R. pickettii* (JOVL01000020)	100
TCR124	++	C	83.3	82.7 ± 2.5	*R. pickettii* (JOVL01000020)	100
TCR125	+	C	0.00	NT	*Arthrobacter nicotinovorans* (X80743)	100
TCR126	+	C	20.8	NT	*P. nitroreducens* (AM088474)	98.1
TCR127	+	C	86.7	91.4 ± 2.4	*M. chitosanitabida* (BCYP01000048)	99.3
TCR130	++	C	0.00	NT	*P. nitroreducens* (AM088474)	99.2
TCR131	+	C	0.00	NT	*B. puraquae* (NBYX01000050)	100
TCR132	++	C	0.00	NT	*Burkholderia vietnamiensis* (CP009631)	90.7
TCR133	+	C	60.0	72.2 ± 6.2	*R. pickettii* (JOVL01000020)	99.4
TCR135	+	C	0.00	NT	*R. pusense* (jgi.1102370)	100
TCR136	++	C	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TCR137	++	C	0.00	NT	*B. stabilis* (CP016444)	99.6
TCR138	+	C	0.00	NT	*Bacillus megaterium* (JJMH01000057)	99.7
TCR140	+	C	0.00	NT	*Pseudoxanthomonas mexicana* (AF273082)	100
TCR141	+	C	0.00	NT	*B. puraquae* (NBYX01000050)	100
TCR142	++	C	0.00	NT	*B. puraquae* (NBYX01000050)	100
TCR143	++	C	0.00	NT	*P. huijuniae* (EU725799)	100
TCR144	+	C	0.00	NT	*Arthrobacter oxydans* (X83408)	100
TCR145	+	C	0.00	NT	*Sphingobium quisquiliarum* (ATHO01000107)	99.2
TCR146	+	C	0.00	NT	*B. puraquae* (NBYX01000050)	100
TCR147	+	C	0.00	NT	*Burkholderia ubonensis* (EU024179)	92.1
TCR148	+	C	23.3	NT	*P. mexicana* (AF273082)	100
TCR149	++	C	0.00	NT	*Delftia lacustris* (jgi.1102360)	100
TCR151	+++	C	10.4	NT	*Pseudomonas donghuensis* (AJJP01000212)	100
TCR152	++	C	20.8	NT	*P. donghuensis* (AJJP01000212)	100
TCR153	+	C	0.00	NT	*A. nicotinovorans* (X80743)	82.3
TCR154	+	C	13.3	NT	*P. asplenii* (LBME01000002)	98.8
TCR155	+++	C	15.0	NT	*B. puraquae* (NBYX01000050)	99.8
TCR156	+	C	96.7	94.8 ± 1.0	*M. chitosanitabida* (BCYP01000048)	99.2
TCR157	+	C	0.00	NT	*P. huijuniae* (EU725799)	99.8
TCR158	+	C	76.7	95.9 ± 0.8	*M. chitosanitabida* (BCYP01000048)	99.3
TCR159	+	C	83.3	95.9 ± 6.6	*M. chitosanitabida* (BCYP01000048)	99.3

TCR161	+	C	16.7	NT	*Delftia lacustris* (jgi.1102360)	82.3
TCR162	+	C	0.00	NT	*Pseudomonas entomophila* (AE015451)	97.2
TCR163	++	C	0.00	NT	*D. lacustris* (jgi.1102360)	100
TCR164	+	C	0.00	NT	*P. entomophila* (AE015451)	97.8
TCR165	++	C	0.00	NT	*D. lacustris* (jgi.1102360)	100
TCR166	++	C	8.3	NT	*B. puraquae* (NBYX01000050)	98.7
TCR167	+	C	93.3	87.8 ± 4.0	*M. chitosanitabida* (BCYP01000048)	99.3
TCR168	+	C	0.00	NT	*Pseudomonas umsongensis* (NIWU01000003)	99.4
TCR169	+	C	0.00	NT	*Pseudomonas graminis* (MDEN01000035)	100
TCR170	+	C	0.00	NT	*P. graminis* (MDEN01000035)	100
TCR171	+	C	3.3	NT	*B. puraquae* (JJOA01000042)	100
TCR172	+	C	0.00	NT	*P. umsongensis* (NIWU01000003)	99.8
TCR173	+	C	0.00	NT	*R. pusense* (jgi.1102370)	100
TCR174	+	C	0.00	NT	*Microbacterium hydrothermale* (HM222660)	81.9
TCR175	++	C	14.6	NT	*P. nitroreducens* (AM088474)	99.0
TCR176	++	C	5.00	NT	*B. puraquae* (NBYX01000050)	99.8
TCR178	++	C	0.00	NT	*Staphylococcus epidermidis* (L37605)	99.6
TCR179	+	C	0.00	NT	*P. umsongensis* (NIWU01000003)	99.6
TCR180	+	C	28.3	NT	*Acidovorax soli* (jgi.1085893)	99.0
TCR181	+	C	1.7	NT	*Rhizobium radiobacter* (AJ389904)	100
TCR182	+	C	0.00	NT	*P. huijuniae* (EU725799)	99.8
TCR184	+	C	0.00	NT	*P. huijuniae* (EU725799)	100
TCR188	+	C	0.00	NT	*Arthrobacter enclensis* (JF421614)	75.4
TCR189	++	C	1.67	NT	*B. puraquae* (NBYX01000050)	99.7
TCR190	++	C	0.00	NT	*B. pseudomycoides* (NUQE01000007)	100
TCR193	+	C	0.00	NT	*P. huijuniae* (EU725799)	100
TCR194	+	C	0.00	NT	*Mycobacterium agri* (PDCP01000163)	95.5
TCR196	+	C	0.00	NT	*P. nitroreducens* (AM088474)	98.8
TCR197	+	C	0.00	NT	*A. nicotinovorans* (X80743)	98.7
TCR198	++	C	0.00	NT	*D. lacustris* (jgi.1102360)	100
TCR199	+	C	0.00	NT	*B. pseudomycoides* (ACMX01000133)	100
TCR200	+	C	0.00	NT	*Pseudomonas entomophila* (AE015451)	100
TCF101	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.0
TCF102	+	C	0.00	NT	*Achromobacter xylosoxidans* (CP006958)	99.0
TCF103	+	C	0.00	NT	*Achromobacter pulmonis* (CP006958)	99.4
TCF104	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.2
TCF105	+++	C	0.00	NT	*B. puraquae* (NBYX01000050)	98.8
TCF106	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.6
TCF107	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.9
TCF109	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TCF110	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.9
TCF111	+++	C	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TCF112	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	100

TCF113	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	98.8
TCF114	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TCF115	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.3
TCF116	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	96.9
TCF117	+	C	0.00	NT	*Cupriavidus respiraculi* (AF500583)	94.9
TCF118	+	C	0.00	NT	*A. xylosoxidans* (CP006958)	99.8
TCF119	+	C	0.00	NT	*A. xylosoxidans* (CP006958)	99.6
TCF121	+	C	0.00	NT	*A. pulmonis* (CP006958)	99.4
TCF122	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TCF123	+++	C	0.00	NT	*B. puraquae* (NBYX01000050)	98.3
TCF124	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TCF125	+++	C	0.00	NT	*B. puraquae* (NBYX01000050)	100
TCF126	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.6
TCF127	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	98.5
TCF128	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.6
TCF129	+++	C	0.00	NT	*B. puraquae* (NBYX01000050)	99.4
TCF130	+	C	0.00	NT	*A. xylosoxidans* (CP006958)	99.6
TCF131	++	C	0.00	NT	*B. puraquae* (NBYX01000050)	98.7
TCF133	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TCF134	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TCF135	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.9
TCF136	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.9
TCF137	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.9
TCF138	+++	C	0.00	NT	*B. puraquae* (NBYX01000050)	98.5
TCF139	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TCF140	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TCF141	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TCF142	+++	C	0.00	NT	*B. puraquae* (NBYX01000050)	98.5
TCF143	++	C	96.7	79.0 ± 6.2	*Ralstonia mannitolilytica* (AJ270258)	99.5
TCF144	++	C	0.00	NT	*A. xylosoxidans* (CP006958)	99.8
TCF145	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TCF146	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.9
TCF147	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.2
TCF148	++	C	76.7	79.0 ± 3.3	*R. mannitolilytica* (AJ270258)	99.5
TCF149	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TCF150	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TCF151	+++	C	0.00	NT	*B. puraquae* (NBYX01000050)	100
TCF152	++	C	0.00	NT	*A. xylosoxidans* (CP006958)	99.8
TCF153	+++	C	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TWR102	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR103	+	W	0.00	NT	*Ochrobactrum lupini* (NNRN01000040)	100
TWR108	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR109	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	100

TWR110	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.7
TWR112	+	W	0.00	NT	*R. pusense* (jgi.1102370)	100
TWR114	+	W	50.0	68.5± 11.6	*M. chitosanitabida* (BCYP01000048)	99.3
TWR115	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR117	+++	W	5.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR118	+	W	0.00	NT	*R. pusense* (jgi.1102370)	100
TWR120	++	W	66.7	81.7 ± 7.5	*M. chitosanitabida* (BCYP01000048)	99.3
TWR123	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR124	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.6
TWR125	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR127	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR130	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR136	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR137	+	W	90.0	86.2 ± 3.1	*M. chitosanitabida* (BCYP01000048)	99.3
TWR138	+++	W	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TWR141	+++	W	31.7	NT	*B. puraquae* (NBYX01000050)	99.8
TWR143	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR144	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR146	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR148	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.7
TWR150	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR151	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR155	+	W	5.00	NT	*R. pusense* (jgi.1102370)	99.7
TWR156	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR158	+	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR159	++	W	0.00	NT	*O. lupini* (NNRN01000040)	100
TWR163	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR164	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR165	+	W	80.0	86.2 ± 3.1	*M. chitosanitabida* (BCYP01000048)	99.3
TWR166	+	W	0.00	NT	*A. nicotinovorans* (X80743)	99.4
TWR167	+	W	70.0	79.0 ± 5.9	*M. chitosanitabida* (BCYP01000048)	99.3
TWR172	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR173	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR174	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR176	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR177	+	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR180	+++	W	0.00	NT	*O. lupini* (NNRN01000040)	100
TWR183	+	W	0.00	NT	*B. cenocepacia* (JJOA01000042)	97.7
TWR184	+++	W	0.00	NT	*Streptomyces phaeoluteigriseus* (MPOH01000466)	100
TWR189	++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWR190	+	W	0.00	NT	*A. nicotinovorans* (X80743)	99.6
TWR192	+++	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8

TWR193	+++	W	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TWR194	+	W	0.00	NT	*B. puraquae* (NBYX01000050)	99.8
TWF101	++	W	15.0	NT	*Acinetobacter radioresistens* (BAGY01000082)	95.8
TWF102	++	W	36.7	NT	*A. radioresistens* (BAGY01000082)	99.8
TWF105	++	W	0.00	NT	*B. contaminans* (LASD01000006)	99.8
TWF107	++	W	0.00	NT	*B. contaminans* (LASD01000006)	99.3
TWF108	+++	W	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.3
TWF109	+	W	0.00	NT	*A. nicotinovorans* (X80743)	99.5
TWF110	++	W	11.7	NT	*A. radioresistens* (BAGY01000082)	99.8
TWF115	++	W	0.00	NT	*B. contaminans* (LASD01000006)	98.8
TWF116	+++	W	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TWF118	++	W	6.7	NT	*A. radioresistens* (BAGY01000082)	99.7
TWF119	++	W	10.0	NT	*A. radioresistens* (BAGY01000082)	99.0
TWF120	++	W	6.7	NT	*A. radioresistens* (BAGY01000082)	99.6
TWF123	++	W	0.00	NT	*A. radioresistens* (BAGY01000082)	99.8
TWF125	++	W	5.0	NT	*A. radioresistens* (BAGY01000082)	99.2
TWF126	+++	W	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.9
TWF127	++	W	21.7	NT	*P. nitroreducens* (AM088474)	98.8
TWF128	+	W	11.7	NT	*A. radioresistens* (BAGY01000082)	99.6
TWF129	+++	W	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.9
TWF130	++	W	0.00	NT	*B. contaminans* (LASD01000006)	99.1
TWF132	+++	W	0.00	NT	*B. contaminans* (LASD01000006)	99.3
TWF133	++	W	0.00	NT	*A. radioresistens* (BAGY01000082)	99.8
TWF134	++	W	0.00	NT	*A. radioresistens* (BAGY01000082)	99.8
TWF137	+++	W	0.00	NT	*B. cenocepacia* (JJOA01000042)	100
TWF138	++	W	0.00	NT	*B. contaminans* (LASD01000006)	99.8
TWF139	+++	W	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.9
TWF140	+++	W	0.00	NT	*B. cenocepacia* (JJOA01000042)	99.8
TWF141	+++	W	0.00	NT	*B. cenocepacia* (JJOA01000042)	98.9

[†]Antibacterial activity: + weak (<5 mm), ++ moderately (6-10 mm), +++ strong (11-15 mm), ++++ very strong (>15 mm)

[‡]Source of isolation: T = tomato, C = Chinese chive, W = Welsh onion

[§]Reduction in disease severity was calculated using the following formula: reduction of disease severity (%) = [(mean disease severity of control treatment) − (mean disease severity of bacterial treatment)/(mean disease severity of control treatment)] ×100%.

[#]Trial 1 included three tubes for each treatment, and the experiment was conducted once.

[††]Trial 2 included three tubes for each treatment and the experiment was conducted thrice.

[‡‡]Each value represents a mean ± standard error.

NT: Not tested

Table 4. Biocontrol efficacy of *Mitsuaria* isolates on tomato bacterial wilt in trials 1 and 2 of pot experiments

Isolate	Reduction of AUDPC (%)[†]	
	Trial 1[‡]	Trial 2[§]
TCR103	81.6	−7.2
TCR156	6.9	NT[#]
TCR158	26.4	NT
TCR159	80.5	9.4
TCR167	−29.9	NT
TWR114	77.0	45.7
TWR120	56.3	NT
TWR137	49.4	NT
TWR165	12.6	NT
TWR167	57.5	NT

[†]AUDPC was calculated using the trapezoid integration of disease severity progress curve over time according to the following formula: AUDPC = $\sum [0.5 (x_i + x_{i-1})] (t_i - t_{i-1})$, where x_i and x_{i-1} are disease severity at time t_i and t_{i-1}, respectively, and t_i and t_{i-1} are consecutive evaluation dates, with t_i and t_{i-1} equal to 1. Reduction of AUDPC was calculated using the following formula: reduction of AUDPC (%) = [(mean AUDPC of the control treatment − mean AUDPC of the bacterial treatment)/mean AUDPC of the control] × 100

[‡]Trial 1 included five tomato plants in each treatment.

[§]Trial 2 had three replicates, and each replicate included nine tomato plants.

[#]NT: not tested.

Table 5. Biocontrol effect of non-pathogenic *Ralstonia* sp. TCR112 and *Mitsuaria* sp. TWR114 against bacterial wilt in tomato plants grown under glasshouse conditions

Treatment[†]	Disease severity[‡]	AUDPC (Reduction of AUDPC)[§]
Experiment 1		
Control	81.0 ± 9.8a[#]	580.5 ± 104.2a (66.4%)
TCR112	36.5 ± 10.1b	195.3 ± 73.8b
Experiment 2		
Control	92.5 ± 4.7a	603.0 ± 82.5a (55.3%)
TWR114	52.5 ± 5.6b	269.8 ± 58.2b

[†]Plants were inoculated with 100 ml of the cell suspension of TCR112 (experiment 1) or TWR114 (experiment 2) to obtain a final concentration of ca. 3×10^8 CFU/g soil. While in the control, plants were treated with the same volume of sterile distilled water. Each treatment had 10 plants, and the experiment was repeated five times.

[‡]Disease severity = [(the number of diseased plants in each scale × disease scale)/(total number of plants investigated × the highest disease scale)] × 100.

[§]Reduction of AUDPC (%) = [(mean AUDPC of the control treatment − mean AUDPC of bacterial treatment)/mean AUDPC of the control] × 100

[#]Each value represents a mean ± standard error. Values with the same lower case letters in a row within the column are not significantly different at $P<0.05$ (Student's *t*-test).

Table 6. Comparison of physiological properties of TWR114 and the type strains *Mitsuaria chitosanitabida* 3001^T and IAM14711^T.

Characteristic	TWR114	3001 ^{T†}	IAM 14711 ^{T‡}
Maximum temperature for growth (°C)	37	34	37
β-Glucosidase	−	NA	+
Utilization of:			
Sucrose	+	−	NA
D-Fructose	+	−	NA
D-Mannose	+	−	−
D-Xylose	+	−	NA
D-Galactose	+	−	NA
N-Acetyl-D-glucosamine	+	−	−
L-Arabinose	+	−	−
Capric acid	−	NA	+

† Data from Amakata et al. (2005)

‡ Data from Gomila et al. (2007)

+, Positive; −, negative; NA, not available

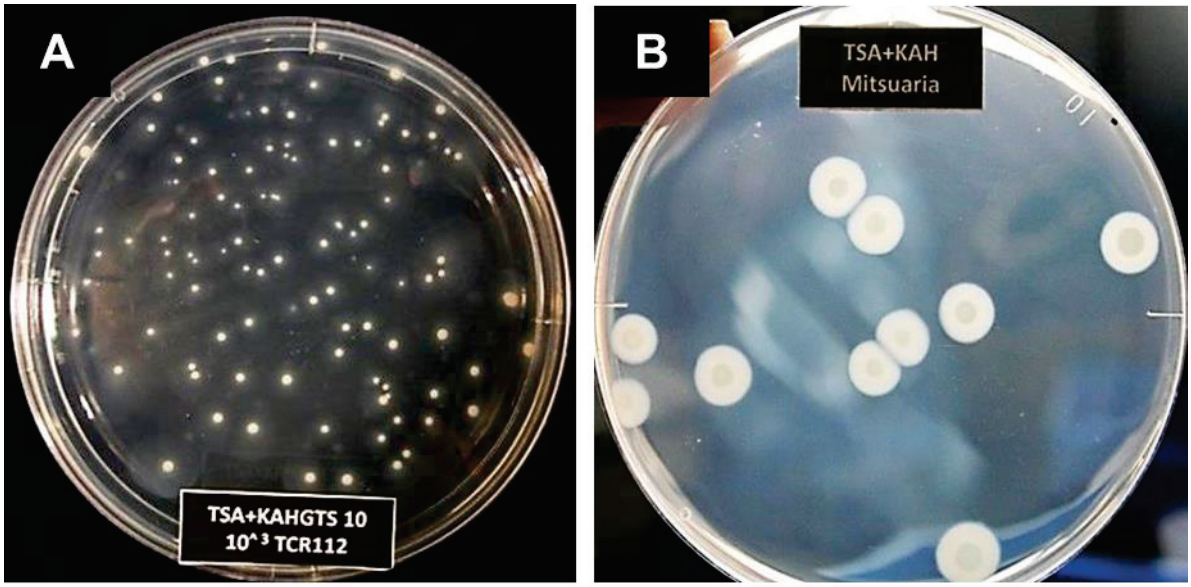


Figure 1. Typical colonies of non-pathogenic *Ralstonia* sp. TCR112 (A) and *Mitsuaria* sp. TWR114 (B) after incubating for 48 h on 1/10 TSA supplemented with the selected antibiotics that are optimized for its enumeration.

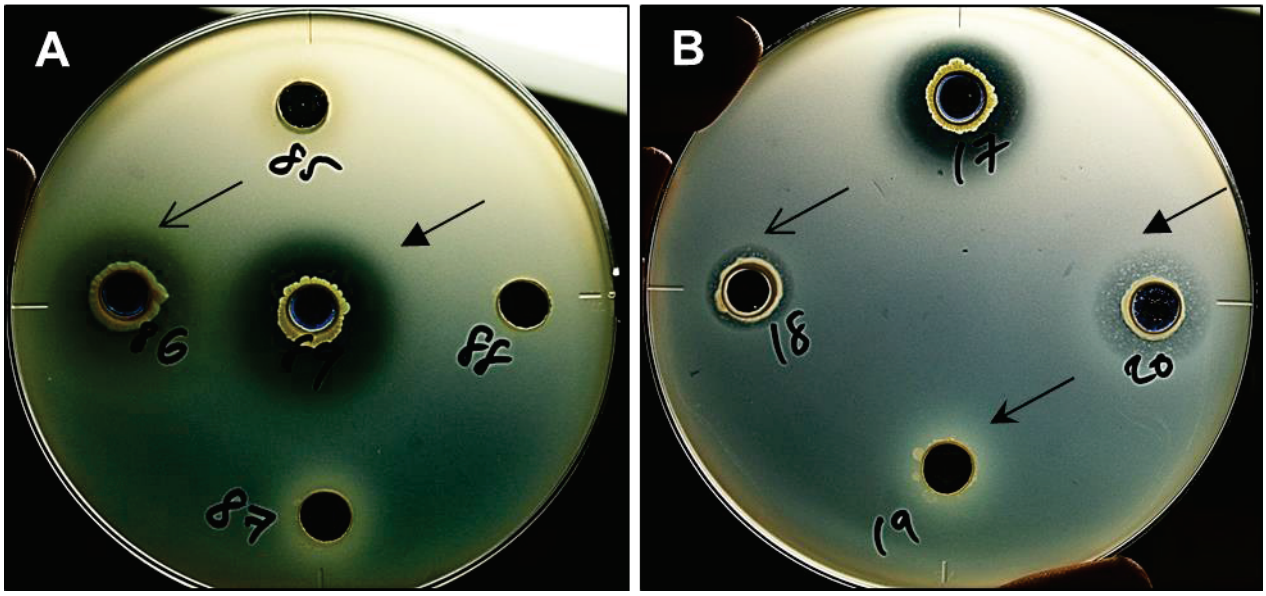
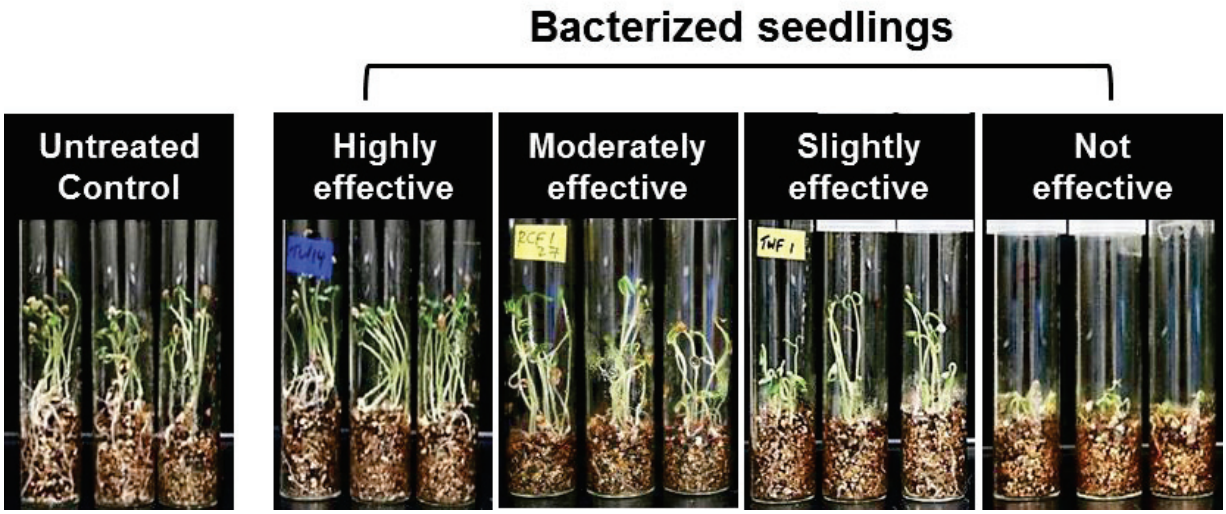


Figure 2. The antibacterial activity of rhizobacterial isolates against *Ralstonia pseudosolanacearum* using the agar well diffusion assay. Photos were taken after incubating the agar plates at 30°C for 48 h. (A) Very strong activity (close arrowhead) and strong activity (open arrowhead). (B) Intermediate activity (close arrowhead), weak activity (open arrowhead), and no activity (stealth arrowhead).

A


Bacterized seedlings
Untreated Control
Highly effective
Moderately effective
Slightly effective
Not effective

B

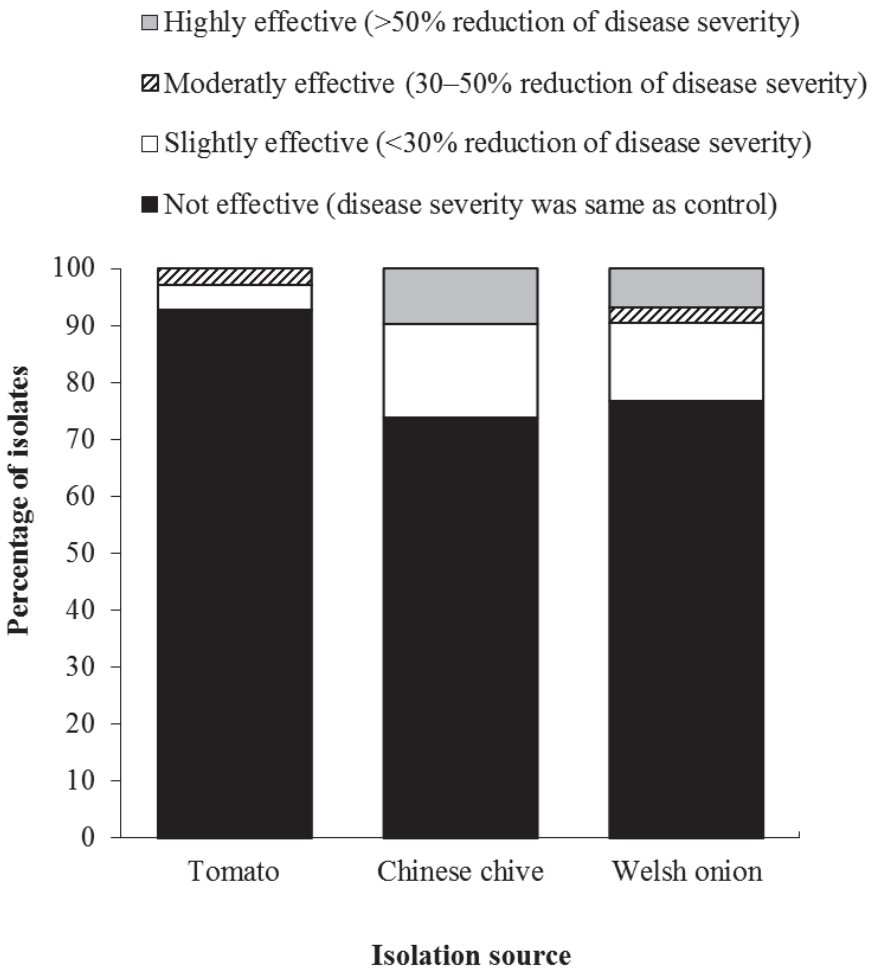


Figure 3. (A) Disease suppressive effect of rhizobacterial isolates against bacterial wilt in tomato seedling bioassay. (B) Differential suppressive effect of rhizobacterial isolates from tomato, Chinese chive, and Welsh onion plants against tomato bacterial wilt using seedling bioassay.

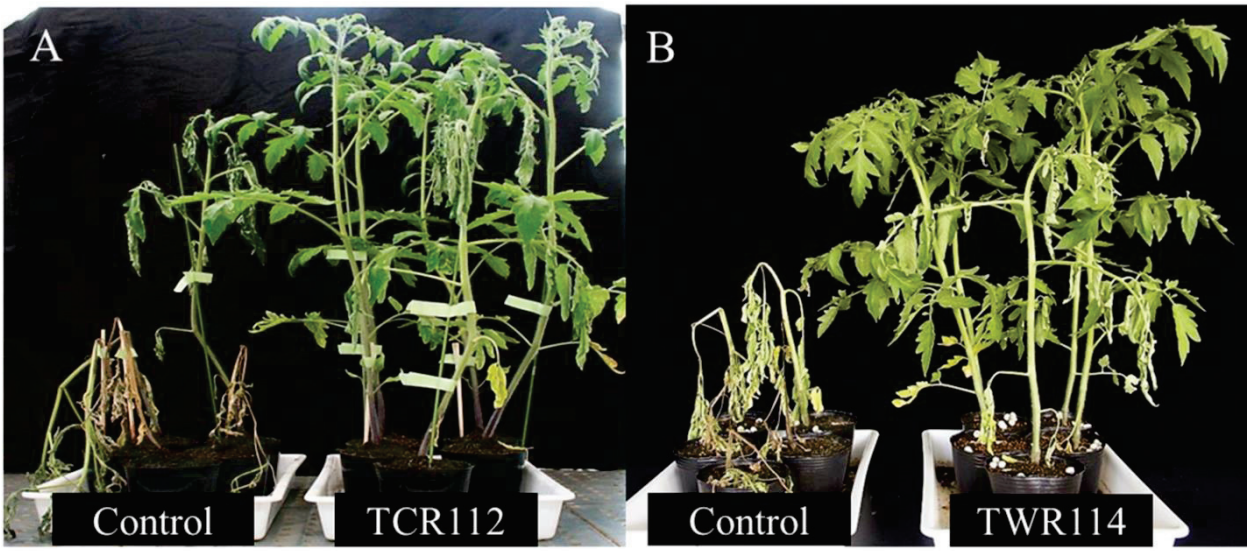


Figure 4. Suppression of bacterial wilt in tomato plants by treatment with the non-pathogenic *Ralstonia* sp. TCR112 (A) and *Mitsuaria* sp. TWR114 (B). The pot on the left side in each photo was untreated, and the pot on the right side was treated with each biocontrol isolate. Photos were taken at 14 days after inoculation with *R. pseudosolanacearum*.

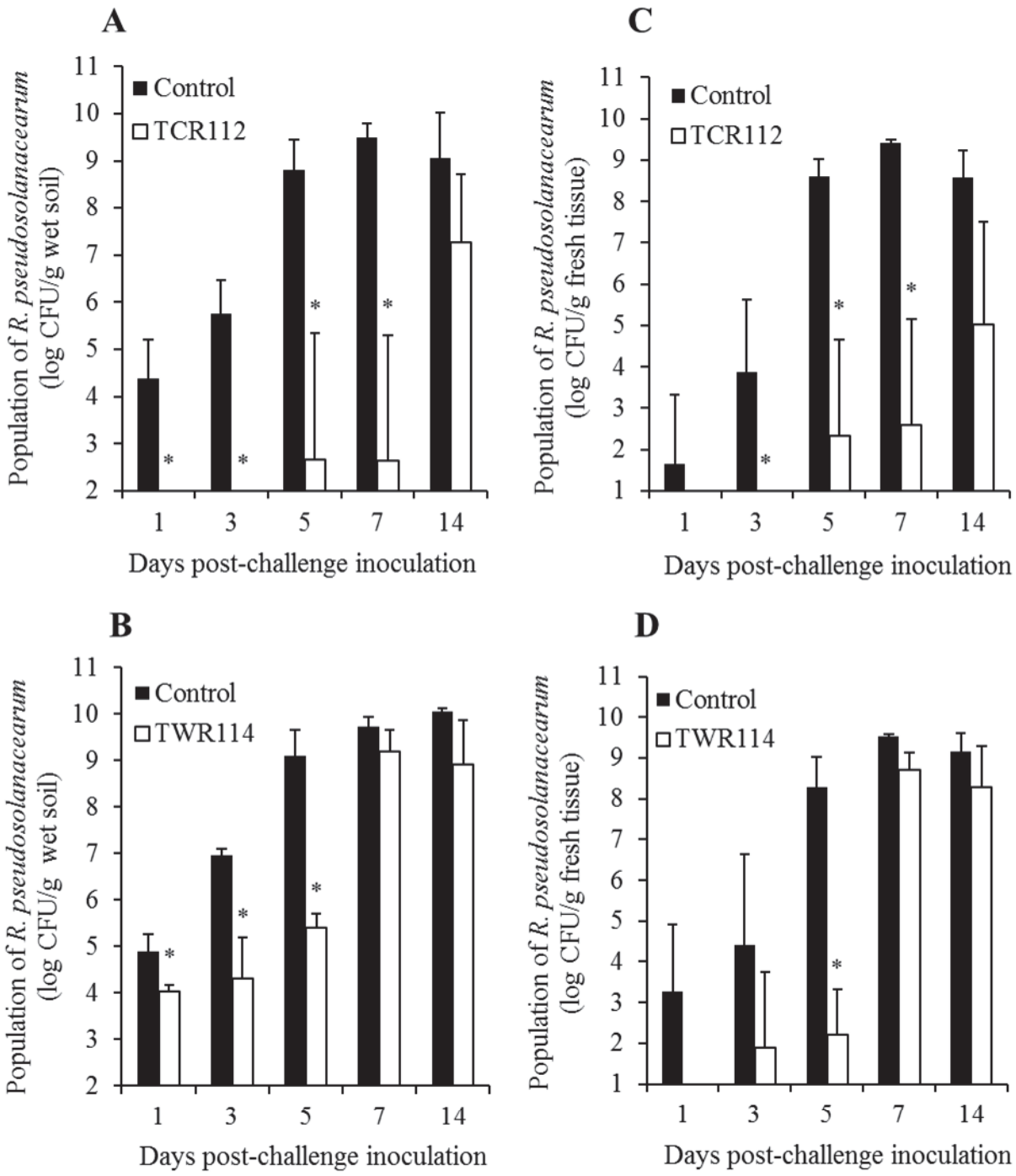


Figure 5. Population dynamics of *Ralstonia pseudosolanacearum* in the rhizosphere soil (A and B) and crown (C and D) of tomato plants treated with the biocontrol isolates non-pathogenic *Ralstonia* sp. TCR112 (upper graphs) and *Mitsuaria* sp. TWR114 (lower graphs). The initial density of the pathogen in the soil was

approximately ca. 1 × 10^7 CFU/g wet soil. An asterisk indicates a statistically significant difference between the control and biocontrol bacterial treatment at $P<0.05$ (Student's *t*-test).

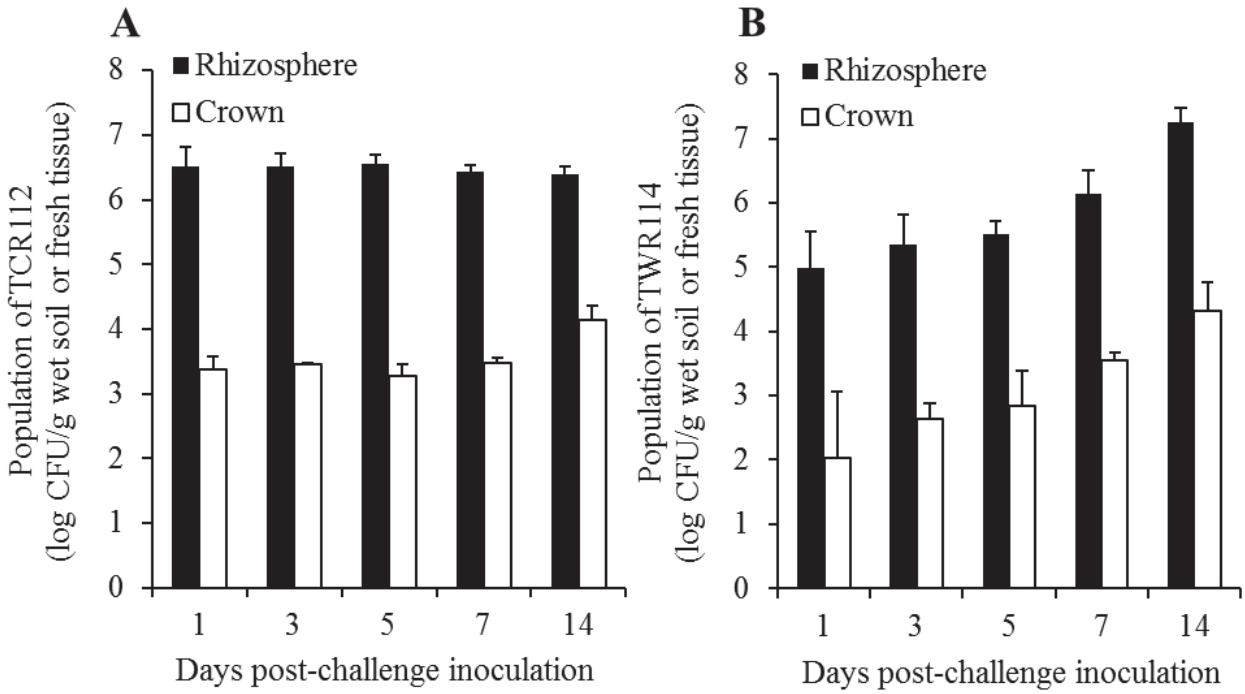
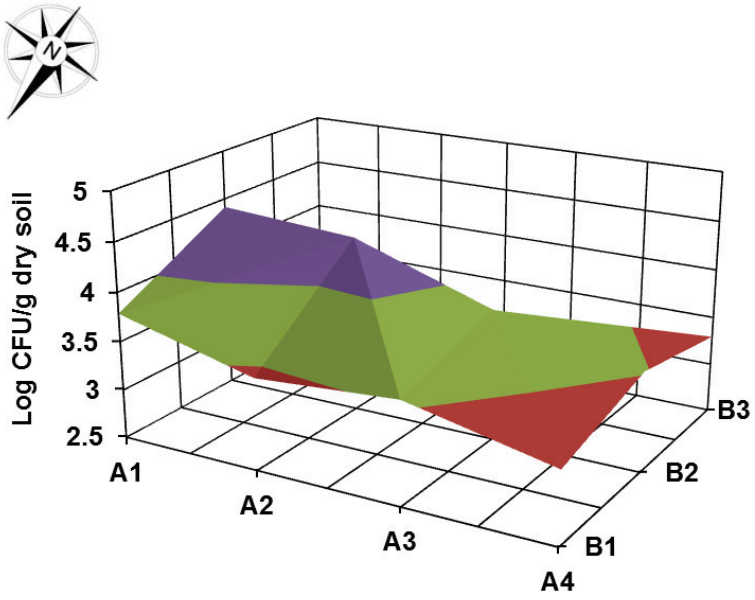


Figure 6. Population dynamics of non-pathogenic *Ralstonia* sp. TCR112 (A) and *Mitsuaria* sp. TWR114 (B) in the rhizosphere soil and crown of tomato plants grown under glasshouse conditions. The initial density of the biocontrol isolates was approximately ca. 3×10^8 CFU/g soil.

2016



2017

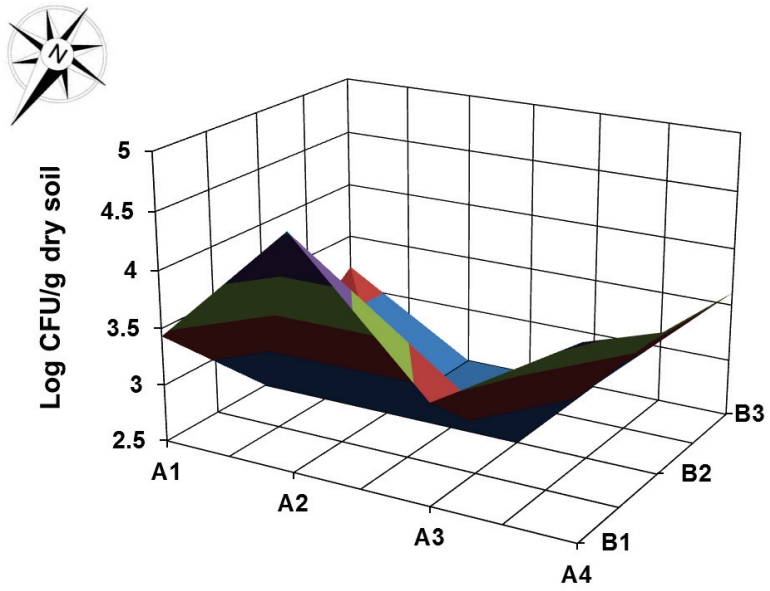


Figure 7. Population of *Ralstonia pseudosolanacearum* in two consecutive years (2016 and 2017) of the field experiments, determined at the time of transplanting of tomato plants.

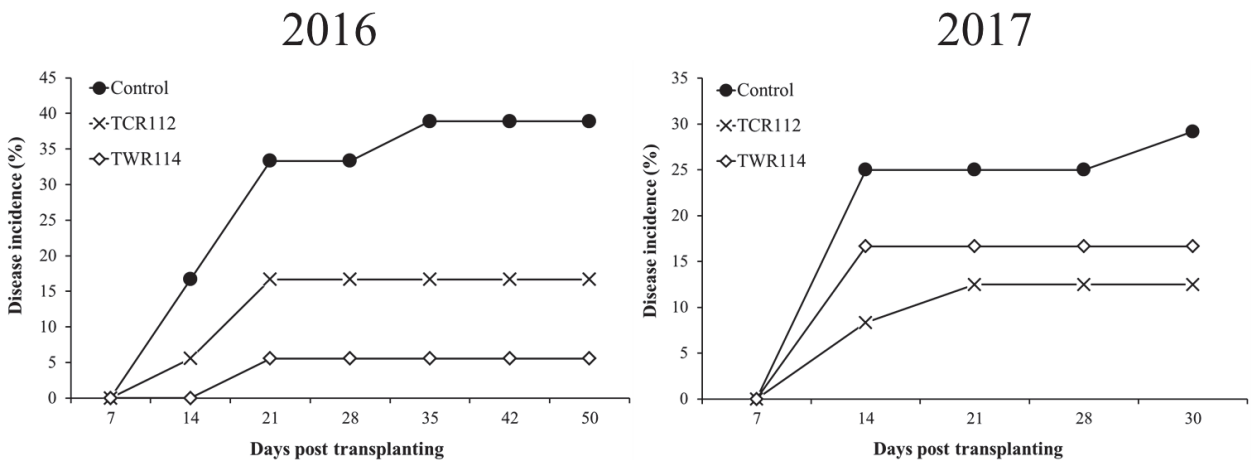


Figure 8. Effect of weekly drenching of non-pathogenic *Ralstonia* sp. TCR112 and *Mitsuaria* sp. TWR114 on the incidence of tomato bacterial wilt in field experiments performed in two consecutive years from August to October in 2016 and from September to October in 2017. Tomato plants were inoculated with 300 ml of the cell suspension (ca. 3×10^8 CFU/ml) of TCR112 and TWR114 at weekly intervals. In the control, plants were treated with the same volume of DW. First year (2016) and second year (2017) consisted of three and four replicate plots per treatment, respectively, and each replicate included 6 tomato plants. Disease incidence was calculated as follows; disease incidence = {[total number of diseased plants (scale 1–4) in the treatment/total number of plants investigated)]} × 100.

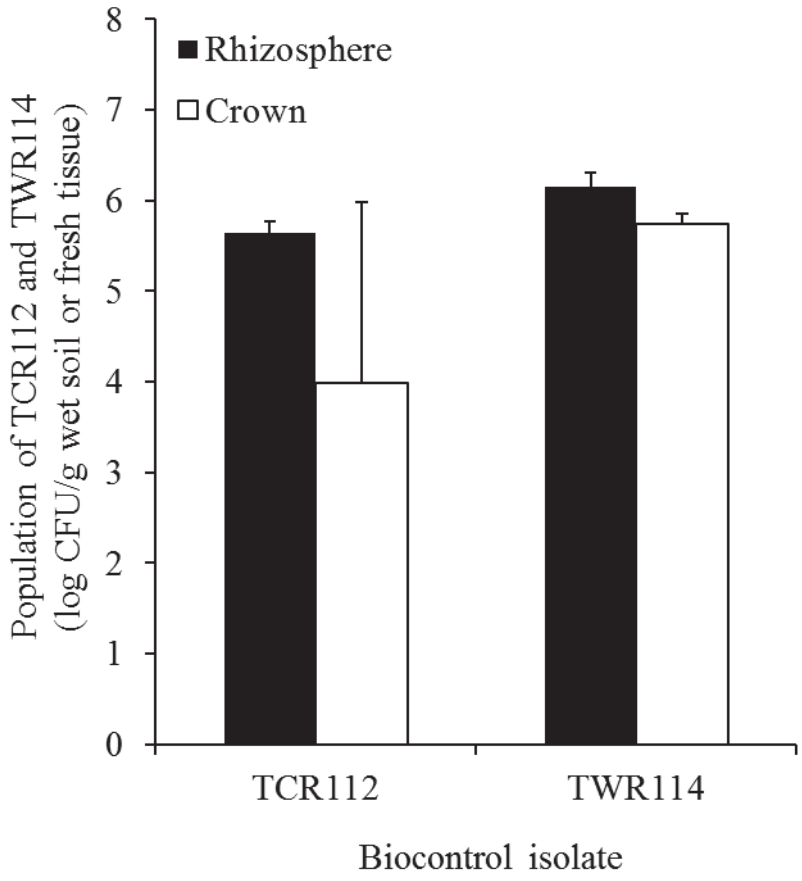


Figure 9. Populations of non-pathogenic *Ralstonia* sp. TCR112 and *Mitsuaria* sp. TWR114 in the rhizosphere and crown of tomato plants grown for 50 days (8 days after the final application of candidate isolates) in the first year of field experiment.

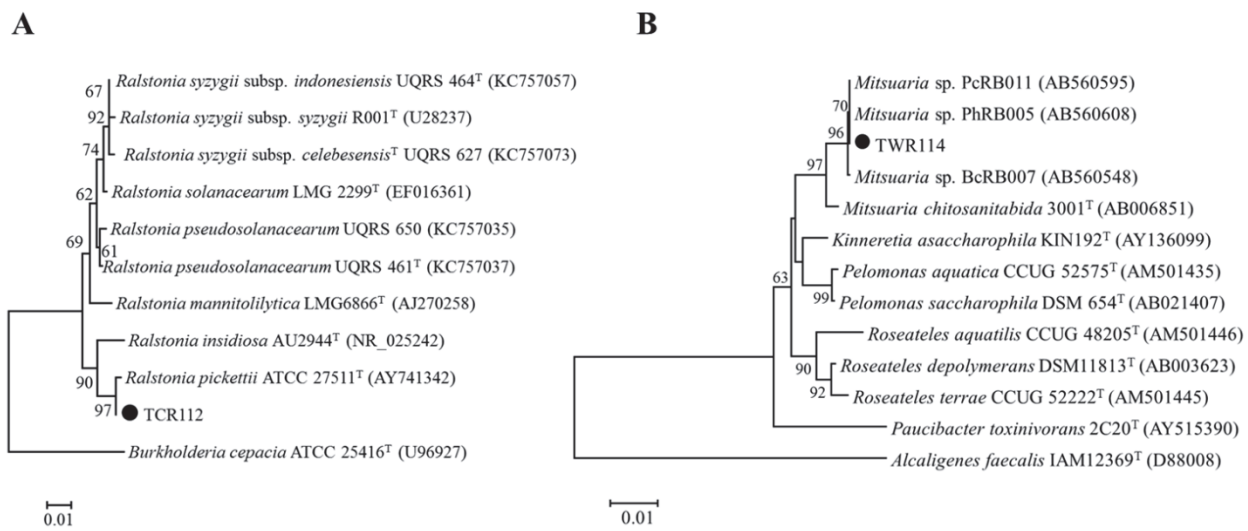


Figure 10. Phylogenetic position of final candidate isolates TCR112 (A) and TWR114 (B) based on complete 16S rRNA gene sequence analysis. The sequences of representative strains of other species of the order *Burkholderiales* are included in the dendrogram. Bootstrap values of ≥60% (1000 replicates) are shown next to the branches. Accession numbers for each sequence are shown in parentheses. Scale bar shows the number of base substitutions per site.

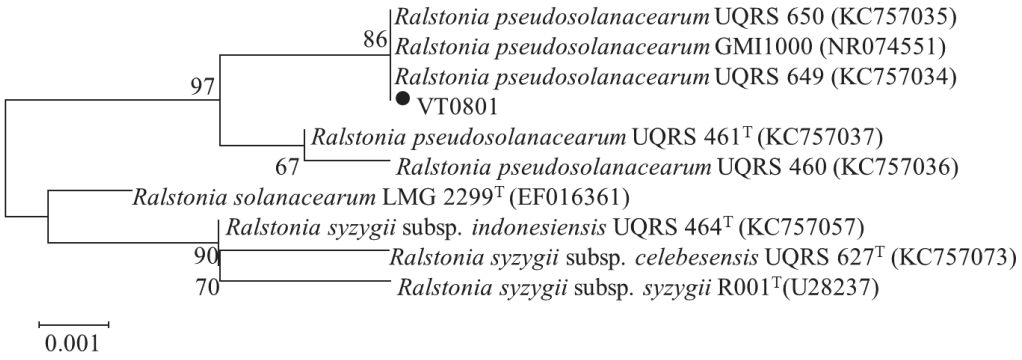


Figure 11. Phylogenetic position of the pathogen *Ralstonia pseudosolanacearum* based on partial 16S rRNA gene sequence analysis. The sequences of representative strains of the pathogenic *Ralstonia* species are included in the dendrogram. Bootstrap values of ≥60% (1000 replicates) are shown next to the branches. Accession numbers for each sequence are shown in parentheses. Scale bar shows the number of base substitutions per site.

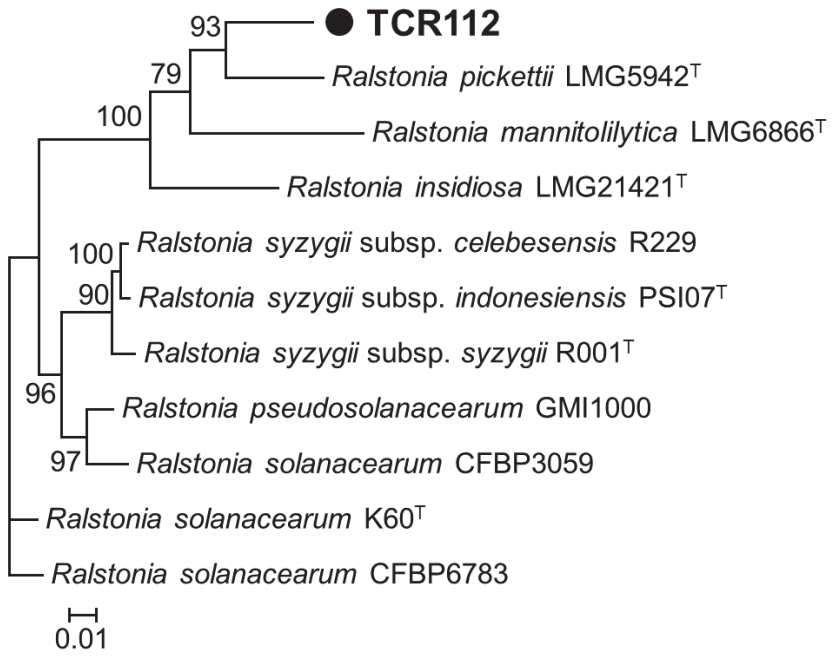


Figure 12. Maximum likelihood tree based on the concatenated partial *gdhA*, *mutS*, *leuS, rplB*, and *gyrB* sequences of the final candidate isolate TCR112 and other members of *Ralstonia* species. Bootstrap values after 1000 replicates are expressed as percentages. The tree was constructed with PhyML (version 3.0). Bootstrap values of ≥60% (1000 replicates) are shown next to the branches. Scale bar shows the number of base substitutions per site.

4. Discussion

As we expected, we could obtain many bacterial isolates which have high suppressive effect against tomato bacterial wilt from the rhizospheres of Chinese chive and Welsh onion (Fig. 3B), which have been used as companion plants to suppress bacterial wilt (152, 235). The isolates that gave over 50% reduction of disease severity in the tomato seedling bioassay were discovered from these two plants, but not from tomato. This suggests that alliums are a good source for isolating rhizobacteria to suppress tomato bacterial wilt.

Interestingly, the 19 isolates selected based on the disease suppressive effect in the seedling bioassays were classified as either non-pathogenic *Ralstonia* or *Mitsuaria* on the basis of 16S rRNA gene sequence analysis (Table 3). To our knowledge, only few studies have described the biocontrol effect of non-pathogenic *Ralstonia* spp. against soil-borne diseases including tomato bacterial wilt (32, 47, 217). *Mitsuaria* isolates have been recently reported to have biocontrol effect against *Rhizoctonia solani* and *Pythium aphanidermatum* in tomato and soybean (23), but there was no report on the suppressive effect of *Mitsuaria* species against bacterial diseases. Therefore, this is the first study to describe the biocontrol capacity of the genus *Mitsuaria* against bacterial wilt. In this study, two isolates TCR112 and TWR114 were selected as potential biocontrol agent. According to the phylogenetic analysis based on the full-length 16S rRNA sequence, the isolate TCR112 was found to be a species closely-related to *R. pickettii* (Fig. 10A). However, it was reported that the application of 16S rRNA gene as a phylogenetic marker is insufficient resolution at the genus level of *Ralstonia* species (65). Therefore, additional phylogenetic analysis based on multiple protein coding genes was carried

out to better characterize the isolate TCR112 at species level. Results revealed that the isolate TCR112 was clearly separated from the type strain of *R. pickettii* LMG5942^T (Fig. 12), thus might be a representative of a new species. Likewise, the *Mitsuaria* isolate TWR114 was clearly separated from the known-type strains of *M. chitosanitabida* (Fig. 10B). Moreover, TWR114 had the ability to produce β-glucosidase and use some sugars (Table 6), which could not be produced or used by the known-type strains of *M. chitosanitabida* 3001^T (12) and *M. chitosanitabida* IAM 14711^T (67). To date, the genus *Mitsuaria* comprises only one species of *M. chitosanitabida*; therefore, our isolate might represent a new species of this genus. This is in agreement with that reported by Someya et al. (190) who found a clear distinction between several isolates of *Mitsuaria* and the known-type strain of *M. chitosanitabida* 3001^T and thus suggested that they might represent a new species of this genus. We found that the isolate TWR114 was grouped in the same clade with some of their isolates (PcRB011 and BCR007) (Fig. 10B).

The non-pathogenic *Ralstonia* sp. TCR112 and *Mitsuaria* sp. TWR114 showed a remarkable biocontrol effect in pot experiments. Our results clearly demonstrated that treatment with these isolates effectively suppressed bacterial wilt up to 2 weeks in tomato plants in the glasshouse under high pathogen pressure (approximately 10^7 CFU/g soil) (Table 5). Moreover, the field experiments in two consecutive years proved that weekly drenching with these two isolates provided considerable protection to tomato plants against bacterial wilt even in a naturally infested field (Fig. 8). When comparing between 2016 and 2017, the protection level in the second year was relatively lower than that in the first year. This might be due to the effect of torrential rainfall. Unfortunately, we had torrential rainfall twice, one of which was caused by typhoon, in the first 3 weeks of September of 2017, and our field was

heavily flooded. This unsolicited flooding might reduce the population of our isolates in the rhizosphere, thereby resulted in the decrease in the biocontrol effect. In Japan, tomato growers often suffer from bacterial wilt and other soil-borne diseases simultaneously. Therefore, the growers prefer to use grafted seedlings having multiple resistances against several diseases. Tomato seedlings used in the field experiments were grafted on the rootstock which is highly resistant against *F. oxysporum*, *V. dahliae*, and *P. lycopersici*, while its resistance level against bacterial wilt is unstable and not adequate. Actually, the incidence of bacterial wilt on the untreated seedlings reached more than 25% within 3 weeks after transplanting in our field experiments. The fact that both the isolates considerably reduced the wilt incidence on this grafted seedlings suggesting that our isolates can be used as BCAs to compensate for a shortage of bacterial wilt resistance of the rootstocks which have a high level of resistance against other soil-borne diseases. However, in order to commercialize our isolates as practical biocontrol products, it would be necessary to develop more sophisticated and cost effective application methods. As Yuliar et al. (238) pointed out, the poor performance due to inconsistent colonization or requirement of uneconomically high rates of inoculums are an important disadvantages of BCAs. Wei et al. (217) stated that the population ratio of biocontrol *R. pickettii* QL-A6 to *R. solanacearum* in the rhizosphere soil affected the biocontrol efficacy. Therefore, attempts such as provisioning of sugar sources (145) may increase our biocontrol isolates to pathogen ratio and enhance the biocontrol efficacy.

The population of *R. pseudosolanacearum* in tomato rhizosphere was considerably decreased by the treatment with the isolates TCR112 and TWR114 (Fig. 5), indicating that both isolates have an ability to suppress the multiplication of the pathogen in rhizosphere soil. Our results demonstrated that both the isolates

have rhizosphere colonization capacity (Figs. 6 and 9). We suggest that the colonization ability of these isolates might contribute to suppress the multiplication of *R. pseudosolanacearum* in the rhizosphere. It is generally assumed that rhizosphere and rhizoplane colonization are key factors for BCAs to suppress soil-borne diseases (164), including bacterial wilt (80). Additionally, maintaining the BCA population in the rhizosphere at $>10^6$ CFU/g dry soil is critical for controlling bacterial wilt (236). In this study, *in vitro* antibacterial activity of the rhizobacteria isolates, against pathogen was tested as a primary criterion for screening candidate biocontrol isolates. Although all of the selected isolates have antibacterial activity, they showed varying level of antagonism against *R. pseudosolanacearum* (Table 3). With regard to this, the antibacterial activity of the isolates TCR112 and TWR114 was noticeably weak (narrow and fuzzy inhibition zone), suggesting that antibiotic productivity of these isolates or inhibitory activity of their antibiotics are low. Therefore, although the antibiosis may partially be involved, other mechanisms such as competition for the nutrients and antimicrobial enzyme mediated antagonism may play an important role in the suppression of pathogen in tomato rhizosphere.

The virulence factors of *R. solanacearum* have been suggested to be regulated by quorum sensing which are expressed during exponential growth only when cell densities exceed 10^7 cells/ml (38). Moreover, previous study indicated that a specific threshold of the pathogen population must be surpassed to induce wilt symptoms, where it must exceed 8 log CFU/g tissue in the above-ground regions of tomato plants (83). In this study, it was found that the pathogen multiplication in crown tissues was maintained below this threshold level for at least up to 5 dpi and 14 dpi by the treatment with TWR114 and TCR112, respectively (Fig. 6B and 6D). Our results demonstrated that both isolates can colonize the inside crown tissues (Figs. 7

and 9). We speculated that these endophytically colonizing isolates prevented the pathogen multiplication in the stem xylem vessels by the induction of disease resistance. Bacterial endophytes have often reported to systemically enhance resistance against pathogens by the activation of the defense system or prime the inducible defense responses of host plants through various eliciting factors (100).

In conclusion, the findings from this study clearly demonstrate that non-pathogenic *Ralstonia* sp. TCR112 and *Mitsuaria* sp. TWR114 are potential BCAs capable of suppressing tomato bacterial wilt. Further research will be conducted to better understand the detailed mechanism of disease suppression of our isolates.

Chapter 2

Establishment of an effective application method of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112

Establishment of an effective application method of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112

Abstract

In chapter 1, we identified *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 as potential biocontrol agents capable of suppressing tomato bacterial wilt. However, to commercialize our isolates as practical biocontrol products, it is necessary to develop a cost-effective application method for maximizing their biocontrol performance. Therefore, we investigated whether the combined application of TWR114 and TCR112 would enhance the biocontrol effect against bacterial wilt. In a pot experiment, all the tested inoculum ratios (i.e., 1:1, 1:2 and 2:1) of the TWR114+TCR112 treatment significantly suppressed the incidence of bacterial wilt, even at 28 days post-challenge inoculation (dpi) (13–47% wilt incidence), while the incidence of bacterial wilt of plants treated with the individual isolates reached more than 60% within 10–12 dpi. The population of *Ralstonia pseudosolanacearum* in the rhizosphere and above-ground regions was considerably decreased by the TWR114+TCR112 treatment compared with that in the individual treatments. Moreover, the pathogen population in the above-ground regions of TWR114+TCR112-treated plants decreased to an undetectable level at 28 dpi. The combination of TWR114 and TCR112 exhibited a synergistic suppressive effect, resulting in enhanced biocontrol efficacy against tomato bacterial wilt. The combination of both isolates may represent a very promising approach for controlling tomato bacterial wilt in the future.

1. Introduction

Bacterial wilt caused by the soil-borne pathogens *Ralstonia solanacearum*, (228) *R. pseudosolanacearum* and *R. syzygii* subsp. *Indonesiensis* (178) is ranked as the second most destructive bacterial disease of plants worldwide (126). Together, these pathogens infect more than 200 plant species belonging to more than 50 different plant families, including some important solanaceous crops such as tomato (*Solanum lycopersicum* L.), potato (*S. tuberosum* L.), tobacco (*Nicotiana tabacum* L.), eggplant (*S. melongena* L.), and *Capsicum* spp. Direct crop losses can reach up to 90% in tomato and potato, and 70% in tobacco (53). Pathogenic *Ralstonia* penetrate roots via natural openings and wounding, thereafter moving into the xylem vessels where they blocks the translocation of water resulting in wilting and subsequently death of the plants (10).

The biological control of bacterial wilt using antagonistic bacteria has been accomplished (26, 33, 225, 229). Most of these studies reported the biocontrol efficacy of single biocontrol agents (BCAs) against bacterial wilt in pot and/or field experiments. Yuliar et al. (238) pointed out that the biocontrol effect exhibited by single BCAs can sometimes be low and/or last for only a short period of time, thus requiring uneconomically high rates of inoculums or repeated applications in the field. They considered that these points are the most important disadvantages of BCAs in controlling bacterial wilt. Therefore, sophisticated methods for improving the biocontrol of wilt disease are required. The approach of using a combination of several BCAs to control plant diseases was proposed as an effective way to overcome some of these drawbacks (191). Actually, there has been increasing

interest among researchers in using the combination of BCAs to exploit potential synergistic effects on plant health (181, 224).

Many previous studies reported that the combined application of multiple microbes may enhance the biocontrol efficacy and reliability against bacterial wilt on tomato (88, 90), tobacco (117, 237), bell pepper (*Capsicum annuum* L.) (116), and *Coleus* (*Coleus forskohlii* Briq.) (186) plants. Moreover, the combination may also lead to broad-spectrum protection against multiple pathogens (46, 89, 170) and may improve the growth, yield and quality of different crops (122, 179, 192).

Recently, we identified *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112, originally isolated from Welsh onion (*Allium fistulosum* L.) and Chinese chive (*A. tuberosum Rottler ex Spreng*), respectively, as potential BCAs capable of suppressing tomato bacterial wilt (Chapter 1). In that chapter, we reported that single application of these individual isolates effectively suppressed bacterial wilt for up to 2 weeks in tomato plants under glasshouse conditions. Moreover, weekly drenching with each of these two isolates provided considerable protection to field-grown tomatoes against bacterial wilt. However, to commercialize our isolates as practical biocontrol products, it is necessary to develop a cost-effective application method for maximizing their biocontrol performance. Therefore, in the present chapter, we investigated whether the combined application of TWR114 and TCR112 would enhance the biocontrol effect against tomato bacterial wilt.

2. Materials and methods

2.1 Bacterial isolates, culture conditions, and inoculum preparation

The biocontrol bacteria TWR114 and TCR112, and the pathogen *R. pseudosolanacearum* isolate VT0801 were used throughout this study. TWR114 and TCR112 isolates were cultured in nutrient broth (Nissui Pharmaceutical Co., Tokyo, Japan). VT0801 isolate was cultured in casamino acid-peptone-glucose broth medium (76). Both media were incubated at 30°C for 24 h with shaking at 200 rpm. The cells of TWR114 and TCR112 were harvested by centrifugation at 10,000 rpm for 10 min, washed twice and resuspended in sterile distilled water (SDW) to a final concentration of ca. 9×10^8 CFU/ml. The cells of VT0801 was harvested as mentioned earlier, washed twice and resuspended in 10mM $MgCl_2$ to a final concentration of ca. 2×10^7 CFU/ml.

2.3 Growth conditions of tomato plants

Seeds of tomato (cv. Ponderosa, susceptible to bacterial wilt) were surface-sterilized with 70% (v/v) ethanol for 1 min, followed by 1% (v/v) sodium hypochlorite for 5 min and then thoroughly rinsed with SDW. After germination on a moist filter paper, the seeds were sown in plastic trays (Bee pot Y-49; Canelon Kaka Co. Ltd., Japan) containing a commercial potting soil mix “Saika Ichiban” (Ibigawa Kogyo Co. Ltd., Japan) and grown in a glasshouse (maintained at 30°C, relative humidity of 70%) until the seedlings reached the four-leaf stage. Tomato seedlings were then transplanted into vinyl pots (9 cm in diameter) comprising three layers: top and bottom layers, each containing 150 g of commercial potting soil mix; and a middle layer, containing 20 g of river sand, and grown in the same glasshouse.

2.4 Evaluation of biocontrol effect of combined application of TWR114 and TCR112 in pot experiments

2.4.1 Effect of inoculum ratios

For the TWR114+TCR112 treatment, cell suspensions of TWR114 and TCR112 (ca. 9 × 10^8 CFU/ml) were mixed thoroughly at ratios of 1:1, 1:2, and 2:1 (v/v), before the treatment of tomato plants by bottom watering (100 ml/pot). For individual treatments, a cell suspension of each isolate was applied (100 ml/pot) to obtain a final concentration of 3 × 10^8 CFU/g wet soil. The plants treated with an equal volume of SDW without the bacteria were used as controls. Three days after treatment, both control plants and those treated with the isolates were challenged with 100 ml of a VT0801 washed cell suspension to obtain a final concentration of 7 × 10^6 CFU/g wet soil. The inoculated plants were maintained in the same glasshouse as mentioned above for 28 days. Each treatment had five plants and the experiment was repeated three times.

2.4.2 Effect of inoculum concentration

Plants were treated with the combination of TWR114 and TCR12 (100 ml/pot) at a ratio of 2:1 (v/v, selected from the above pot experiment) using the original inoculum concentration (ca. 9 × 10^8 CFU/ml) or using 2-fold (ca. 4.5 × 10^8 CFU/ml) and 10-fold (ca. 9 × 10^7 CFU/ml) dilutions of the original concentration. The plants treated with an equal volume of SDW were used as controls. At 3 days after treatment, both control plants and those treated with the isolates were challenged with the pathogen as mentioned above. The inoculated plants were maintained in the same glasshouse

for 28 days. Each treatment included five plants and the experiments were repeated three times.

2.4.3 Disease assessment

The number of wilted plants was recorded daily and disease incidence and the area under the disease progress curve (AUDPC) were calculated using the following formulas:

Disease incidence = (total number of diseased plants in the treatment/total number of plants investigated) × 100.

AUDPC = $\sum$ [0.5 ($x_{i+1} + x_i$)] ($t_{i+1} - t_i$), where x_{i+1} and x_i are disease incidences at times t_{i+1} and t_i, respectively, and t_{i+1} and t_i are consecutive evaluation dates, with t_{i+1} and t_i equal to 1.

2.5 Quantification of R. pseudosolanacearum

Tomato plants were treated with the combination of TWR114 and TCR112 at a ratio of 2:1 using the original inoculum concentration (ca. 9×10^8 CFU/ml), or with the individual isolates and then challenged with *R. pseudosolanacearum* VT0801 as described in the above pot experiments. The pathogen multiplication in the rhizosphere soil, crown (basal part of hypocotyl), mid-stem (immediately above the cotyledon), and upper stem (approximately 1 cm above the first true leaves) regions of symptomless plants (i.e., did not show any signs of wilt disease) was determined at 1, 3, 5, 7, and 28 days post-challenge inoculation (dpi). In addition, the pathogen multiplication in the bulk soil was simultaneously enumerated using separate pots without any transplanted tomatoes. Samples were obtained from three pots or plants

that were treated with the combination of TWR114 and TCR112 and the individual isolates and an untreated control at each time point. The rhizosphere soils tightly attached to the roots were harvested and serially diluted with SDW. Bulk soil samples were serially diluted with SDW. The above-ground samples (crowns, mid-stems, and upper stems; each 2 cm in length) of tomato plants were surface sterilized with 100% ethanol and flamed for 5 s as described previously (Chapter 1). The samples were then homogenized using a sterile mortar and pestle, and used to prepare serial dilutions in SDW. Dilutions of rhizosphere soil and tissue homogenates were spread in triplicate onto the surface of the modified semi-selective medium South Africa (61). Typical colonies of *R. pseudosolanacearum* that appeared elevated and fluidal with a pink center were counted after incubation for 3 days at 30°C. The experiment was repeated three times. The size of the bacterial populations was expressed as log colony-forming units per gram (wet weight) of soil (log CFU/g wet soil) or tissue (log CFU/g fresh tissue).

2.6 Quantification of TWR114 and TCR112 isolates

The population dynamics of TWR114 and TCR112 in the bulk soil and rhizosphere and above-ground (crown, mid-stem, and upper stem) regions of tomato plants were simultaneously enumerated with the pathogen enumeration. Dilutions of the bulk and rhizosphere soils and tissue homogenates, used for the pathogen enumeration, were spread in triplicate onto the surface of the isolation media that were optimized for each isolate, as described previously (Chapter 1). These inoculated plates were incubated at 30°C for 48 h and the number of representative

colonies of each isolate was counted (Chapter 1). The experiment was repeated three times.

2.7 Field experiment

Field experiment was conducted in an experimental field at Gifu University, from June to July in 2018. Before transplanting, 30 tons/ha of organic fertilizer (60% of cow manure, 20% of pig manure, and 20% of horse manure) and 2.3 tons/ha of chemical fertilizer ($N:P_2O_5:K_2O$ in the ratio of 12:9:10 supplemented with Mg:B ratio of 2:0.1) (Nittofc Co., Ltd., Japan) was added to the soil. Moreover, limestone was added at a rate of 2.3 tons/ha (55.4% CaO, pH 9.5) (Shinko Kogyo Co., Ltd., Japan).

Prior transplanting, the population of the pathogen in the field was quantified. Soil samples were obtained from 12 different locations distributed across the field. Ten grams of bulk soil was used to prepared serial dilutions in SDW. Dilution of bulk soil was spread onto the surface of M-SMSA medium and incubated as described earlier. The population was expressed as log colony-forming units per gram (dry weight) of soil (log CFU/g dry soil).

The field (11.5 m × 6.6 m) comprised seven rows, and each row (10.8 m length, 0.8 m width) was divided into three plots (3.6 m length). There were three and four replicate plots arranged in a randomized complete block design for the TWR114+TCR112-treatments and untreated control, respectively. Six tomato plants were transplanted in each plot with distances of 0.55 m between the plants. Standard agronomic practices were performed to grow tomato plants.

Field experiments comprised four treatments: (1) control, (2) TWR114+TCR112 [2-weeks interval], (3) TWR114+TCR112 [3-weeks interval], and (4)

TWR114+TCR112 [4-weeks interval]. Fourth-leaf-stage tomato seedlings (cv. Momotaro-8, highly resistant to *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici*, *Verticillium dahlia* and moderately resistant to *R. solanacearum*), were planted in vinyl pots containing commercial potting soil mix (300 g). These plants were then treated with TWR114+TCR112 as described earlier. Tomato plants treated with SDW were served as control. All the plants were grown in a greenhouse at 28–30°C. After reaching the eight–ninth-leaf stage, the tomato plants were transplanted into the field, and then, 300 ml of the cell suspension (ca. 3 × 10^8 CFU/ml) of TWR114+TCR112 or the same volume of distilled water was applied around the stem base of each plant. The TWR114+TCR112 treatment was performed at 2-, 3-, and 4-weeks intervals until 30 days of the experiments. During the experiment, the number of wilted plants was recorded daily and disease incidence was calculated as described above.

2.8 In vitro compatibility test between TWR114 and TCR112 isolates

Both biocontrol isolates were tested for their compatibility with each other using the agar well diffusion assay. Three milliliters of the TWR114 or TCR112 washed cell suspension was added to 100 ml of molten King's B agar medium before its solidification and poured into square Petri dishes (100 × 100 mm). After agar solidification, 7 mm diameter wells were cut out using a sterile cork borer. A 70-µL aliquot of culture broth of TWR114 or TCR112 isolate was added to the wells. The inhibition of TWR114 and TCR112 growth was assessed based on the production of a clear halo zone surrounding the wells. Three replicates were used for each bacterial isolate.

2.9 Statistical analysis

Differences among treatments in the biocontrol studies were analyzed using Tukey's multiple-comparison test ($P<0.05$). The data of the populations of the pathogen and biocontrol bacteria were transformed into logarithmic values before analysis using Tukey's multiple-comparison test ($P<0.05$) and Student *t*-test ($P<0.05$), respectively. Throughout the bacterial population studies, the minimum detection limit was 2.5 log CFU/g wet soil and 1.5 log CFU/g fresh tissue in the rhizosphere and above-ground regions, respectively. All analyses were performed using BellCurve for Excel (version 2.13; Social Survey Research Information Co. Ltd., Tokyo, Japan).

3. Results

3.1 Biocontrol effect of combined application of TWR114 and TCR112 in pot experiments

The effects of the TWR114+TCR112 treatment at different inoculum ratios on the control of tomato bacterial wilt were examined under glasshouse conditions. The results showed that all of the tested ratios (i.e., 1:1, 1:2, and 2:1) of the TWR114+TCR112 treatment significantly suppressed the incidence of bacterial wilt even at 28 dpi (13–47% wilt incidence), whereas the incidence of bacterial wilt of plants treated with the individual isolates reached more than 60% within 10–12 dpi (Figs. 1 and 2A). Among the three ratios, that of 2:1 was associated with the greatest

reduction of wilt incidence, which was expressed as AUDPC (93% reduction) (Fig. 2B). Therefore, this ratio was further evaluated in another pot experiment. In this experiment, all tested concentrations (i.e., original concentration, 2-fold diluted, and 10-fold diluted) of the TWR114+TCR112 treatment at a ratio of 2:1 significantly reduced the disease incidence, with the original concentration (ca. 9×10^8 CFU/ml) being proven to be the most effective, as demonstrated by it achieving the highest reduction of AUDPC (100%) (Fig. 3). Accordingly, the TWR114+TCR112 treatment at a ratio of 2:1 using the original concentration was used throughout the following experiments.

3.2 Quantification of R. pseudosolanacearum

We monitored the pathogen population in the bulk soil (i.e., with no plants) and symptomless plants treated with biocontrol bacteria and untreated control at 1, 3, 5, 7, and 28 dpi. At 28 dpi, all of the plants treated with TWR114 alone, and TCR112 alone and untreated control were completely wilted, and thus pathogen populations in these plants were only investigated in the bulk soil. In the bulk soil, the pathogen population was not affected by any type of treatments compared with that in the untreated control (Fig. 4A). In the rhizosphere of tomato plants, the pathogen population was considerably reduced by the TWR114+TCR112 treatment and the individual treatments compared with that in the untreated control at 3 and 5 dpi (Fig. 4B). Subsequently, at 7 dpi, the pathogen population reached densities similar to those in the untreated control (around 7 log CFU/g wet soil). However, the pathogen population drastically decreased to less than 4 log CFU/g wet soil in the TWR114+TCR112 treatment at 28 dpi. The population densities in the above-ground

regions of plants treated with TWR114+TCR112 and individual isolates were significantly lower than that of untreated plants at 5 dpi (Fig. 4C, 4D, and 4E). At 7 dpi, the populations in the individual treatments reached densities similar to that in the untreated control, whereas in the TWR114+TCR112 treatment, the population density was kept considerably lower than untreated control. In the TWR114+TCR112 treatment, the pathogen population in the above-ground regions was reduced to an undetectable level (<1.5 log CFU/g fresh tissue) at 28 dpi.

3.3 Quantification of the TWR114 and TCR112 biocontrol isolates

Both isolates were successfully recovered from all regions (bulk soil, rhizosphere, crown, mid-stem, and upper stem) of tomato plants in the TWR114+TCR112 treatment and individual treatments during the growth period under glasshouse conditions, except at 28 dpi (31 dat), where TCR112 was not detected in the upper stem region of TWR114+TCR112-treated plants (Figs. 5 and 6). The colonization of both isolates in the bulk soil showed decreased colonization throughtout the experiment, where at 1 dpi (3 dat) their population densities steadliy decreased from about 6 log CFU/g wet soil to about 5 log CFU/g wet soil (Figs. 5A and 6A). In contrast, the colonization of TWR114 and TCR112 in all treatments was relatively stable in the rhizosphere (ranged from 6.5 to 7.5 log CFU/g wet soil and 5.6 to 7.2 log CFU/g wet soil, respectively) and crown (ranged from 3.0 to 4.7 log CFU/g fresh tissue and 1.9 to 4.0 log CFU/g fresh tissue, respectively) (Figs. 5B, 5C, 6B, and 6C). In the mid-stem, TWR114 and TCR112 persisted at about 3 log CFU/g tissue until 7 dpi (10 dat) (Figs. 5D and 6D). However, at 28 dpi (31 dat), population densities of TWR114 and TCR112 were decreased to about 2 and 1 log CFU/g fresh tissue,

respectively. Similarly, both isolates established populations of about 1 to 2 log CFU/g fresh tissue in the upper stem and persisted until 7 dpi (10 dat), and considerably decreased at 28 dpi (31 dat) (Figs. 5E and 6E). At most time points of sampling in all regions, there were no significant differences between the population of both isolates in the TWR114+TCR112 treatment and the individual treatments (Figs. 5 and 6).

3.4 Evaluation of TWR114+TCR112 in field experiment

In the field experiment, the mean initial pathogen population was 2.9 ± 0.3 (log CFU/g dry soil) (Fig. 7).

The wilt incidence was suppressed in plots drenched treated with TWR114+TCR112 at 2-weeks, 3-weeks, and 4-weeks intervals (Fig. 8). The wilt incidence at the end of the field experiment (35 days after transplanting) was reduced by 21.2–33.3% in plots drenched with TWR114+TCR112 compared with the untreated plots.

3.5 In vitro compatibility between TWR114 and TCR112

TWR114 isolate exhibited antibacterial activity against the TCR112 isolate, as evidenced by the presence of an inhibition zone around the well in the agar well diffusion assay, whereas TCR112 isolate did not show any activity towards TWR114, as indicated by the absence of any such inhibition zone (Fig. 9).

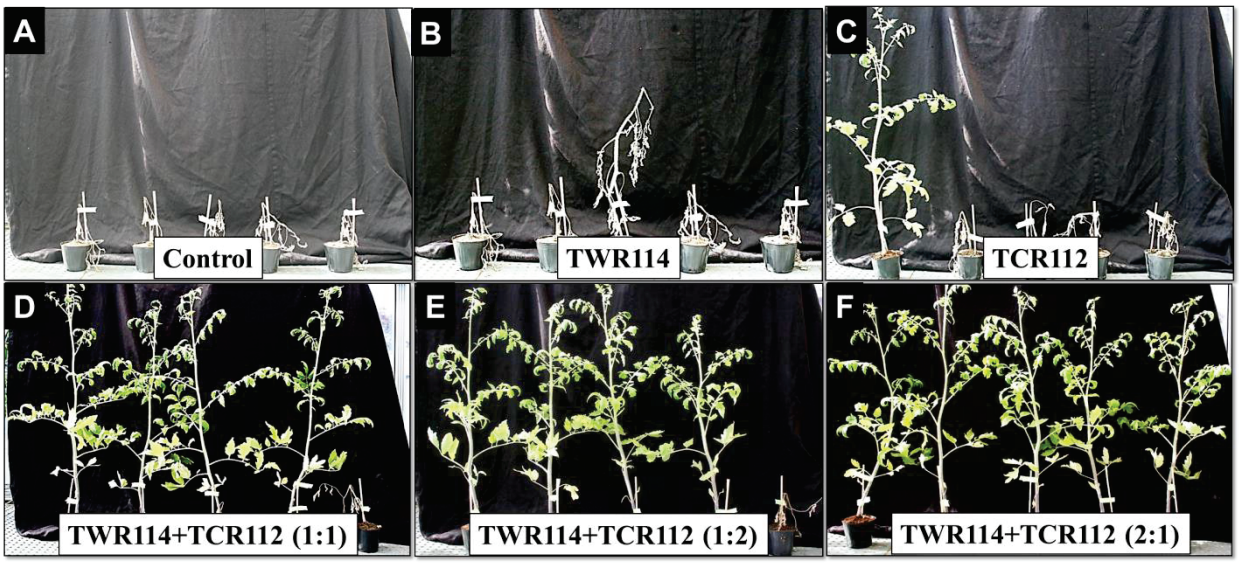


Figure 1. Suppression of tomato bacterial wilt by the combined application of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 at different inoculum ratios. (A) Untreated control. (B) TWR114. (C) TCR112. (D) TWR114+TCR112 (ratio 1:1). (E) TWR114+TCR112 (ratio 1:2). (F) TWR114+TCR112 (ratio 2:1). Photos were taken at 28 days post inoculation with *Ralstonia pseudosolanacearum*.

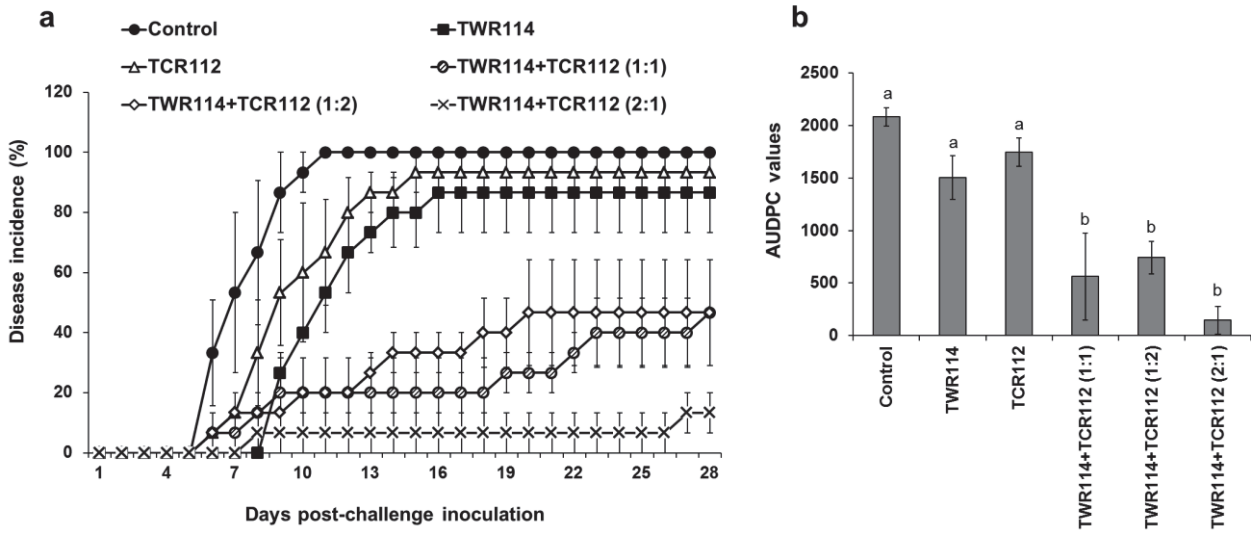


Figure 2. Effect of inoculum ratios of the combined application of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 on the biocontrol of bacterial wilt in tomato plants grown under glasshouse conditions. (A) Disease incidence of tomato bacterial wilt over time in different treatments post inoculation with *Ralstonia pseudosolanacearum*. (B) Wilt incidence expressed as area under the disease progress curve (AUDPC). Bars represent mean ± standard error of three independent experiments. Different letters indicate significant differences among treatments according to Tukey's test at $P<0.05$.

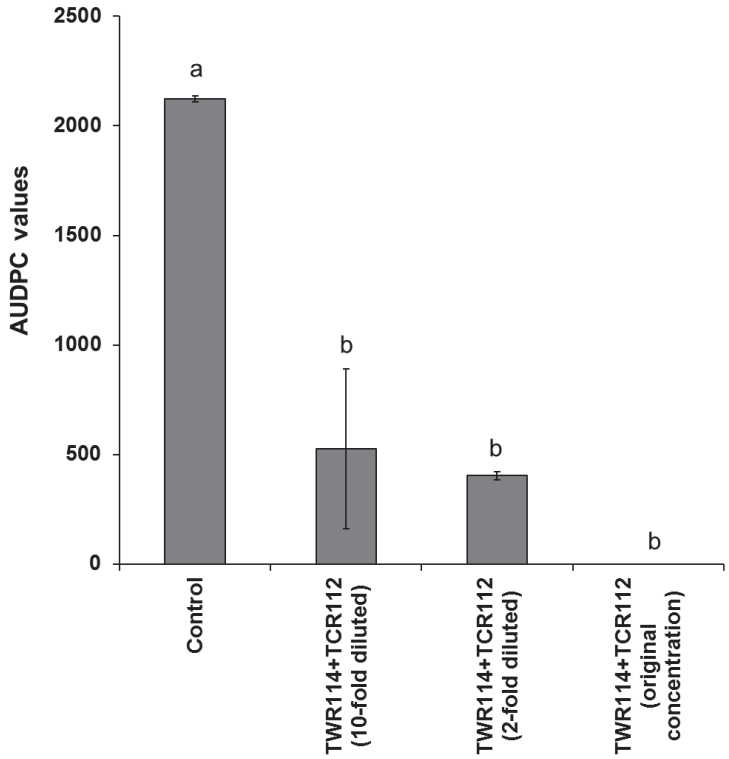


Figure 3. Effect of inoculum concentration in the combined application at a ratio of 2:1 of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 on the incidence of tomato bacterial wilt expressed as area under the disease progress curve (AUDPC). Bars represent mean ± standard error of three independent experiments. Different letters indicate significant differences among treatments according to Tukey's test at $P<0.05$.

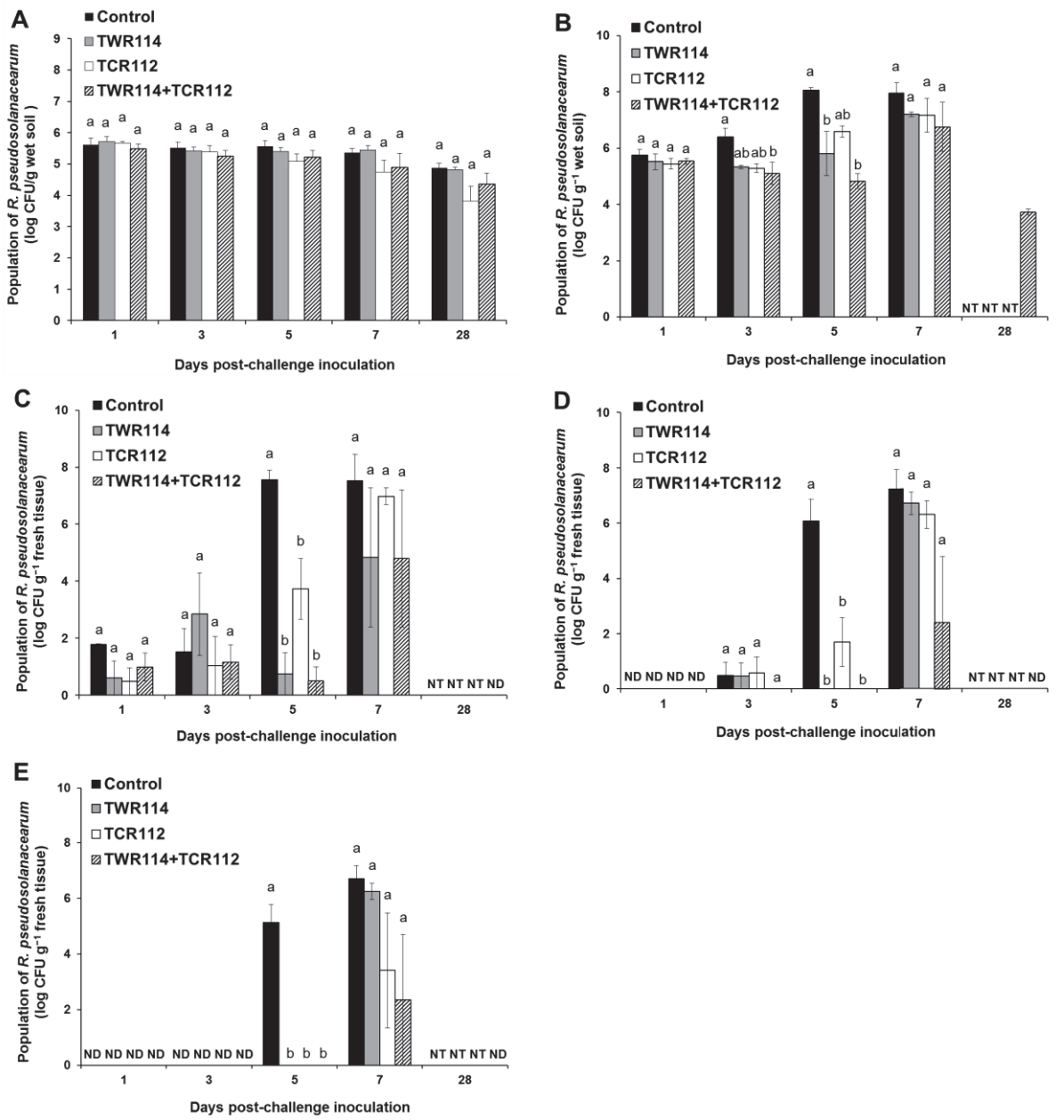


Figure 4. Population dynamics of *Ralstonia pseudosolanacearum* in soil or tomato plants treated with the combination of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 and the individual isolates. (A) Bulk soil. (B) Rhizosphere soil. (C) Crown. (D) Mid-stem. (E) Upper Stem. Bars represent the mean ± standard error of three independent experiments. Different letters represent significant differences among treatments according to Tukey's test at $P<0.05$. NT = not tested. ND = not detected.

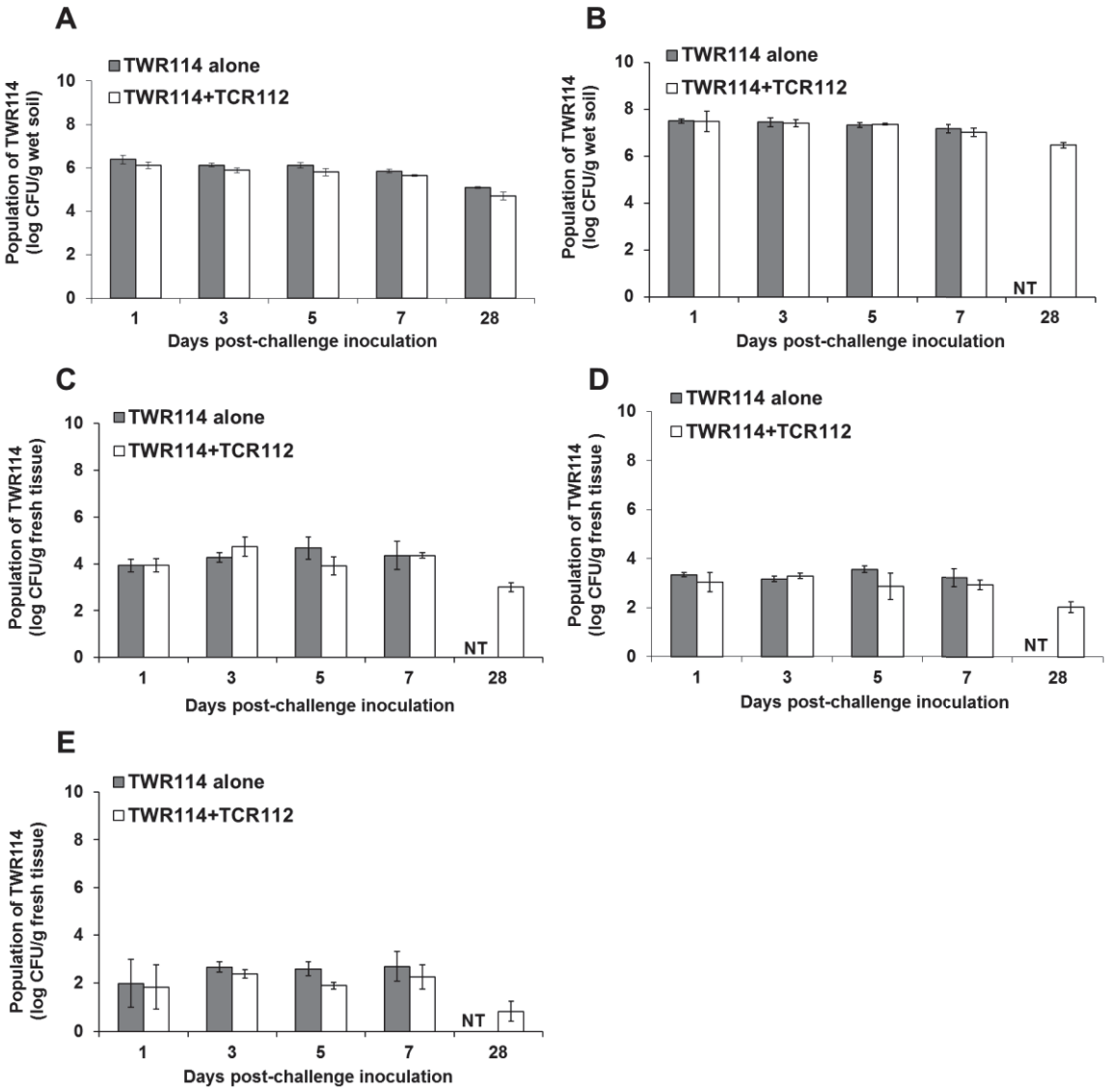


Figure 5. Population dynamics of *Mitsuaria* sp. TWR114 in the soil or tomato plants treated with the combination of TWR114 and non-pathogenic *Ralstonia* sp. TCR112 and the individual isolate. (A) Bulk soil. (B) Rhizosphere soil. (C) Crown. (D) Mid-stem. (E) Upper Stem. Bars represent mean ± standard error of three independent experiments. An asterisk indicates significant difference between the biocontrol bacterial treatments according to Student's *t*-test at $P<0.05$. NT = not tested. ND = not detected.

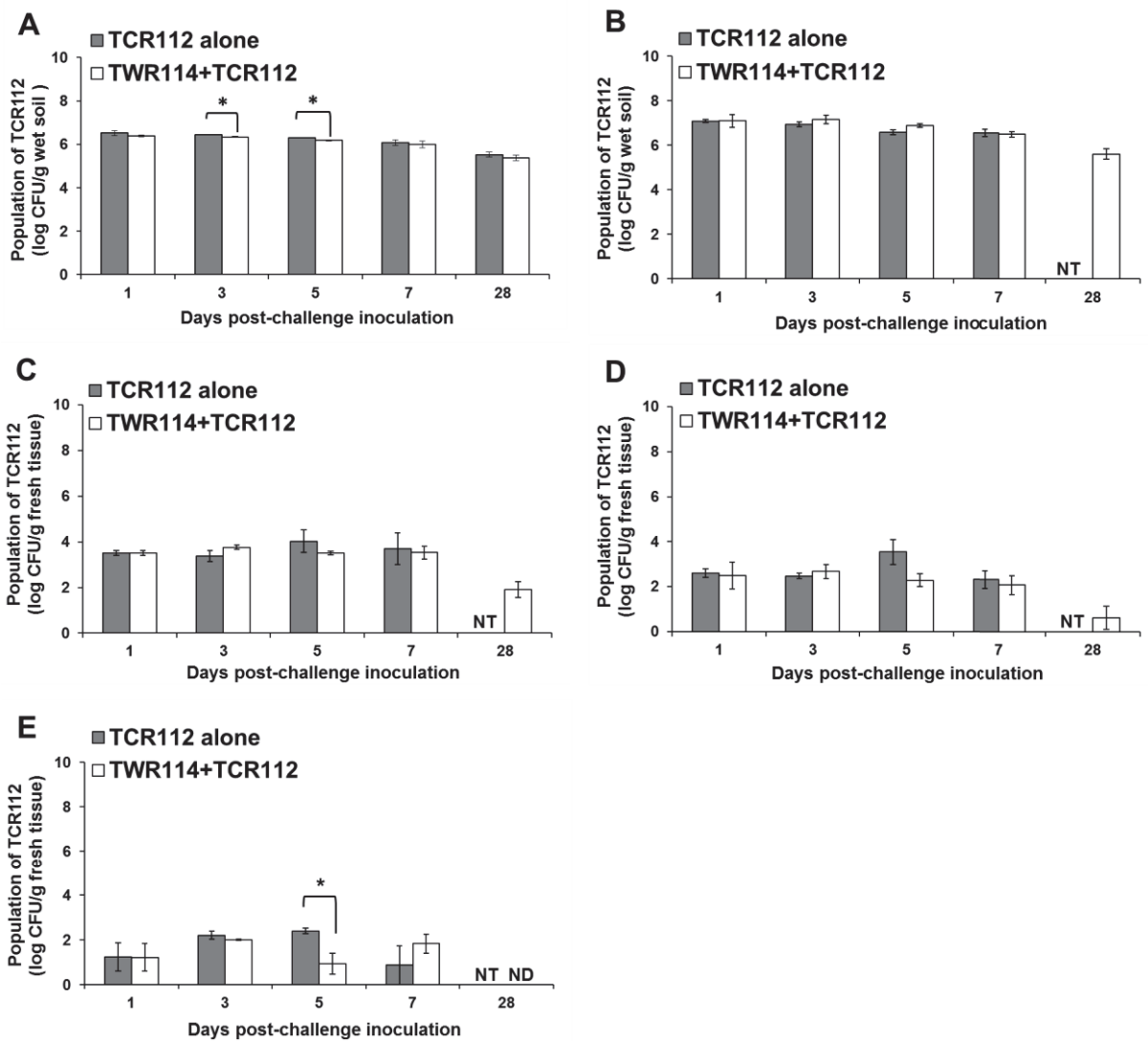


Figure 6. Population dynamics of non-pathogenic *Ralstonia* sp. TCR112 in the soil or tomato plants treated with the combination of *Mitsuaria* sp. TWR114 and TCR112 and the individual isolate. (A) Bulk soil. (B) Rhizosphere soil. (C) Crown. (D) Mid-stem. (E) Upper Stem. Bars represent the mean ± standard error of three independent experiments. An asterisk indicates significant difference between the biocontrol bacterial treatments according to Student's *t*-test at $P<0.05$. NT = not tested. ND = not detected.

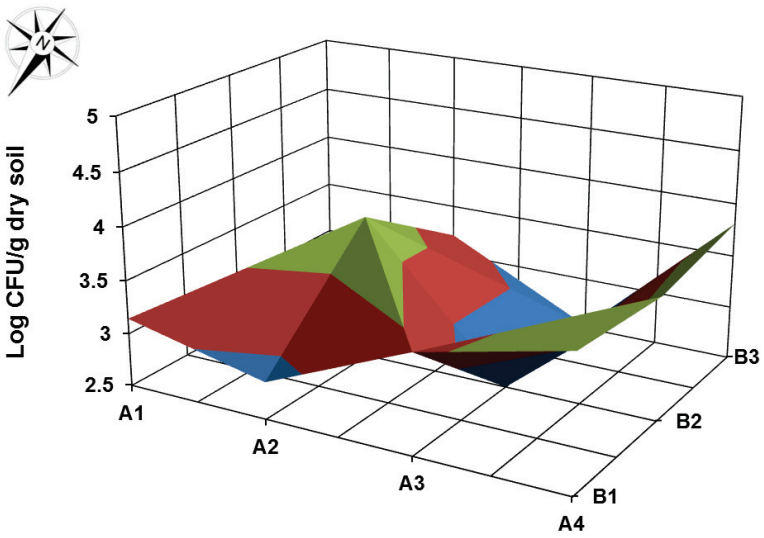


Figure 7. Population of *Ralstonia pseudosolanacearum* in the field experiment 2018 determined prior transplanting of tomato plants.

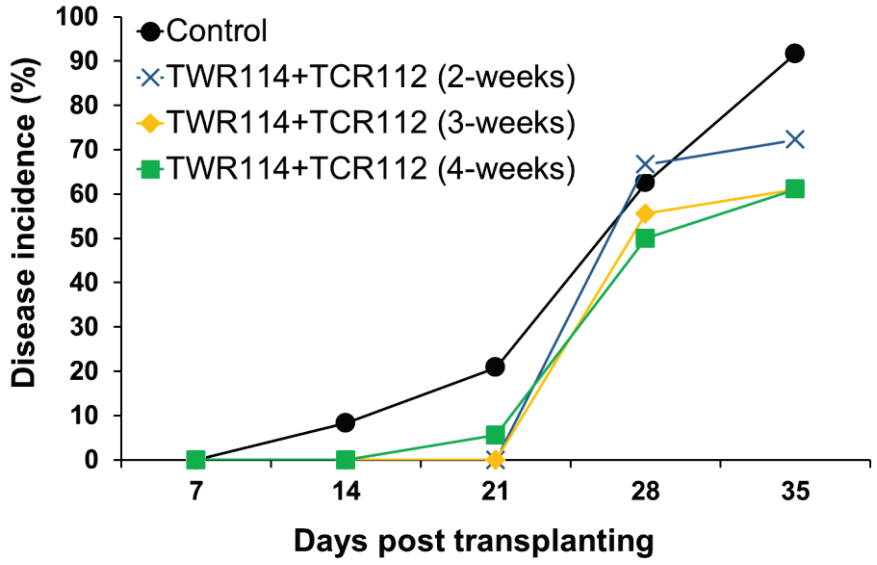


Figure 8. Effect of drenching of combination of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 on the incidence of tomato bacterial wilt in a field experiments conducted from May to July in 2018. Tomato plants were inoculated with 300 ml of the cell suspension (ca. 3×10^8 CFU/ml) of TWR114+TCR112 (at 2:1 ratio) at 2-weeks, 3-weeks, and 4-weeks intervals. In the control, plants were treated with the same volume of DW. The experiment consisted of three and four replicate plots for bacterial treatments and untreated control, respectively, and each replicate included 6 tomato plants. Disease incidence was calculated as follows; disease incidence = {[total number of diseased plants (scale 1–4) in the treatment/total number of plants investigated)]} × 100.

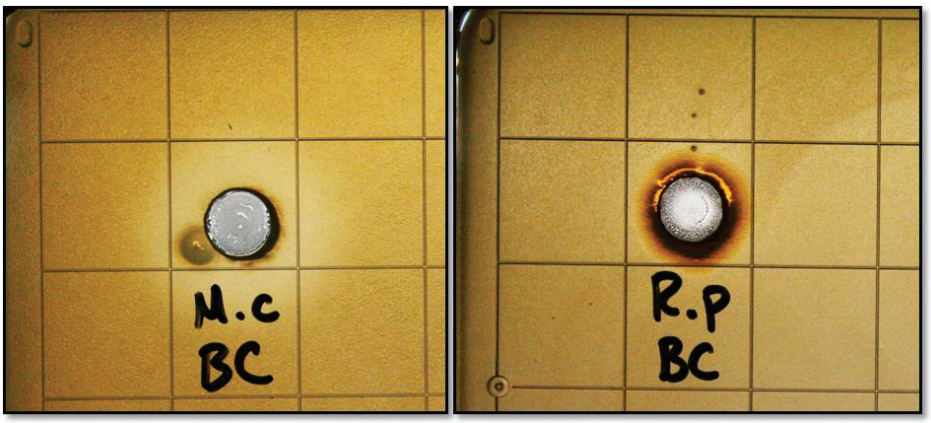


Figure 9. *In vitro* compatibility test between the biocontrol isolates *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 using the agar well diffusion assay. The left image represents the antibacterial activity of TWR114 against TCR112, while the right image represents the activity of TCR112 against TWR114. Photos were taken after incubating the agar plates at 30°C for 48 h.

4. Discussion

In this chapter, we aimed to establish a cost-effective method for applying the isolates *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 in order to maximize their biocontrol effect against tomato bacterial wilt. For this purpose, we investigated whether the combined application of TWR114 and TCR112 can enhance the biocontrol effect. Earlier studies showed that a combination of BCAs resulted in improved biocontrol performance against several soil-borne diseases including bacterial wilt compared with the application of one agent alone (88, 116, 174). However, in some cases the combination failed to show any enhanced performance (71, 139). Our results clearly demonstrated that TWR114+TCR112 treatment could exert a synergistic suppressive effect on the biocontrol of wilt disease. A single application of TWR114+TCR112 achieved a more intense and prolonged biocontrol effect, lasting for at least 28 dpi, compared with it lasting less than 14 dpi upon the application of TWR114 or TCR112 alone (Figs. 1 and 2). Based on this result, the use of TWR114 and TCR112 in combination will be able to reduce drenching frequency and total dose of the isolates to at least one-fourth of individual application. Moreover, TWR114+TCR112 treatment did not show any adverse effect on the growth of pathogen-uninoculated tomato plants even after 28 dat (data not shown). Therefore, combined application of these two isolates is thought to be a cost-effective and practical biocontrol method.

Generally, biocontrol studies involving the application of multiple bacteria in a mixture have used a 1:1 ratio (20, 88, 196). In this study, although all of the TWR114+TCR112 treatments at different ratios exhibited an improved biocontrol effect compared to the individual treatments, the efficacy of TWR114+TCR112

treatment at a 1:1 ratio was not the highest among the treatments. Actually, the TWR114+TCR112 treatment at a 2:1 ratio showed a superior biocontrol effect compared with the other two ratios of 1:1 and 1:2 (TWR114 and TCR112, respectively) (Figs. 1 and 2). These results suggest that the TWR114 isolate should be dominant in this bacterial consortium to maximize the effect of controlling tomato bacterial wilt. However, the reason why this specific ratio conferred the best biocontrol performance is still unclear. Similarly, Singh et al. (184) showed that the combined application of *Paenibacillus* sp. Pb300 and *Streptomyces* sp. 385 was more effective against Fusarium wilt of cucumber than their individual application. They also found that the use of antagonist ratios of 4:1 and 3:2 provided better disease suppression than the use of 1:4 and 2:3 (Pb300 and 385, respectively).

We tested several inoculum concentrations of TWR114+TCR112 treatment at a 2:1 ratio for their biocontrol ability under glasshouse conditions. Although the original concentration (ca. 9×10^8 CFU/ml) exhibited the best biocontrol effect, the other two lower concentrations (i.e. 2- and 10-fold diluted concentrations) also achieved significant reductions of the wilt incidence in a dose-dependent manner (Fig. 3). Therefore, we will evaluate the effectiveness of these different treatments under natural field conditions in the future.

Roberts et al. (174) defined compatible microbes as microbes that, when combined, do not have diminished disease suppression or reduced persistence *in planta* relative to the same isolates applied individually. Moreover, it was assumed that the compatibility among BCAs *in vitro* is an important criterion for obtaining a positive and improved biocontrol effect (59, 157, 174). We found that TWR114 has *in vitro* antibacterial activity towards TCR112, indicating that TWR114 was incompatible with TCR112 under *in vitro* conditions (Fig. 9). However, both isolates in the

TWR114+TCR112 treatment established population densities at levels similar to those with the individual treatments (Figs. 5 and 6). These data suggest that, although the combination of TWR114 and TCR112 was incompatible *in vitro*, it was not *in planta*. We previously found that TWR114 has antibacterial activity against the pathogenic *Ralstonia* (Chapter 1), and thus assumed that TWR114 produces some antibacterial compounds that suppress *Ralstonia* species. However, *in planta*, TWR114 suppressed the population density of the pathogen only, while it did not affect that of TCR112 (Figs. 4 and 6), indicating that the TWR114 isolate suppressed the pathogen multiplication in tomato rhizosphere perhaps not via antibiosis-mediated antagonism but via other mechanisms such as competition for nutrients. Recently, Wu et al. (221) showed that the competitive ability of BCAs to use certain components of tomato root exudates directly affected not only the population density of *R. solanacearum* but also its pathogenicity, thus efficiently suppressing the incidence of bacterial wilt.

The population of *R. pseudosolanacearum* in the rhizosphere and above-ground regions of tomato plant, particularly in the mid-stem and upper stem, was considerably decreased by the combined treatment of TWR114 and TCR112 compared with the levels upon their individual treatments (Fig. 4). Interestingly, although the pathogen population in the above-ground regions of TWR114+TCR112-treated plants increased to 2.4–4.8 log CFU/g fresh tissue at 7 dpi, its population decreased to an undetectable level (<1.5 log CFU/g fresh tissue) at 28 dpi (Fig. 4C, 4D, and 4E). This may have been due to the enhanced defense responses upon the TWR114+TCR112 treatment. It was previously suggested that the priming of defense responses by treatment with the rhizobacterium *Pseudomonas putida* could reduce the population of *R. solanacearum* in root tissues of tomato plants (4).

In conclusion, the findings from the present study clearly demonstrate that the combination of the biocontrol isolates *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 exerts a synergistic suppressive effect, resulting in enhanced biocontrol efficacy against tomato bacterial wilt. We succeeded in establishing a cost-effective method for applying our isolates, which may support their future development and commercialization as new biocontrol products for controlling tomato bacterial wilt. More studies are still necessary to evaluate the effectiveness of the TWR114+TCR112 treatment under natural field conditions.

Chapter 3

Biocontrol mechanisms of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112

Biocontrol mechanisms of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112

Abstract

In this chapter, we aimed to identify the mechanisms of disease suppression by *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 against tomato bacterial wilt. The *in vitro* assay for siderophore, indole-3-acetic acid, protease, and polygalacturonase production revealed that TCR112 produces the former three substances and TWR114 produces the latter three substances. Whole genome sequencing of TWR114 and TCR112 using MiSeq revealed that both isolates harbor several biosynthetic gene clusters encoding siderophore, protease, polygalacturonase, and antimicrobial compounds (e.g. bacteriocin and phenazine). Comparative genomic analyses (i.e., average nucleotide identity and *in silico* DNA-DNA hybridization) and core-genome based phylogenetic analysis showed that both isolates have a clear distinction for their closest relative type strains. The expression pattern of several tomato-defense genes were determined by qRT-PCR in plants treated with TWR114+TCR112 and the individual isolates. Upon pathogen inoculation, the expression of salicylic acid-, ethylene-, and abscisic acid-responsive genes were more strongly induced in the TWR114+TCR112-treated plants than in those treated with the individual isolates. Altogether, the results suggest that both isolates suppress tomato bacterial wilt by the combination of multiple biocontrol mechanisms such as antibiosis, production of siderophore and enzymes, competition for nutrients, and induced resistance. In addition, the isolates TWR114 and TCR112 might represent a novel species of the genus *Mitsuaria* and *Ralstonia*, respectively.

1. Introduction

The mechanisms employed by certain bacteria in the biocontrol of bacterial wilt are generally classified as; competition for nutrients and niches (92, 221), production of lytic enzymes (52, 183), siderophore-mediated competition for iron (168), antibiosis (81), and induce systemic resistance (ISR) (4, 94). Recently, Shen et al. (182) found that the biocontrol mechanism of *Bacillus pumilus* WP8 was not due to direct antagonism but instead via attenuation of the virulence of the pathogenic *Ralstonia solanacearum*. Tahir et al. (199) showed that in addition to ISR, volatile compounds from some *Bacillus* species can adversely affect the motility, chemotaxis, virulence, physiology and ultra-structure of *R. solanacearum*.

Many studies have been conducted to clarify the genetic contents of biocontrol agents (BCAs) by genome sequencing. Since it can provide advance knowledge that are particularly relevant to the mechanisms used by BCAs to suppress phytopathogens and to survive in the rhizosphere soil and tissues of plants (48, 160, 189, 199). The genome of BCAs such as *Bacillus* (49, 111), *Pseudomonas* (120), and *Serratia* (62, 144) has been successfully sequenced over the past decade. The use of whole genome sequencing has also been regarded as a promising avenue for the future of bacterial taxonomic and phylogenetic studies (63, 210). Comparative genomic analyses such as the average nucleotide identity (ANI) which is a similarity index between a given pair of genomes, and the genome to genome distance calculator, referred to as *in silico* DNA-DNA hybridization (*is*-DDH) have been proposed as a new standard for defining microbial species, and it is gaining wide acceptance (16, 101, 102, 133). In addition, comparative analyses of genome sequences are fundamental for defining the entire core- and pan-genomes of

different isolates from the same species. The core-genome is defined as the entire repertoire of translated genes conserved among all isolates. In turn, the pan-genome is the sum of the core genes and those within the accessory genome (207, 208). Recently, several pan-genomic studies have been carried out aiming to gain insight into the genomic and metabolic features as well as to study the taxonomic relationship of a bacterial species (25, 36, 98),

We previously identify *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 as effective BCAs against tomato bacterial wilt (Chapter 1). Moreover, we found that their combined application achieved a more intense and prolonged biocontrol effect, lasting for at least 28 dpi, compared with it lasting less than 14 dpi upon the application of TWR114 or TCR112 alone (Chapter 2). However, the exact mechanisms by which these isolates suppress the disease and why their combined application enhanced the biocontrol effect are still not well understood. Therefore, we investigated the biocontrol mechanisms of TWR114 and TCR112 isolates using *in vitro* tests and genome sequencing. In addition, the genome of both isolates was used to better assess their taxonomical relationship.

2. Materials and methods

2.1 Production of siderophore, indole-3-acetic acid, hydrogen cyanide, protease, and polygalacturonase by TWR114 and TCR112

The biocontrol bacteria TWR114 and TCR112 were used throughout this study (Chapter 1).

Siderophore production was detected using the overlaid chrome azurole S agar (O-CAS) method, as described by Pérez-Miranda et al. (155). In brief, 1 L of the overlay medium contained 60.5 mg chrome azurol S, 72.9 mg hexadecyl trimethyl ammonium bromide, 30.2 g piperazine-1,4-bis (2-ethane sulfonic acid), and 1 mM $FeCl_3$.6H2O in 10 ml of 10 mM HCl. Agarose (0.9%, w/v) was used as a gelling agent. Nutrient agar (NA) medium (NB solidified with 1.5% agar) was used to cultivate the isolates TWR114 and TCR112. Siderophore was detected by applying 10 ml of the overlay medium over NA plates that contained bacterial isolates that were previously cultivated for 3 days. After incubating the agar plates for 24 h, siderophore production was assessed by a change in color of the overlay medium from blue to purple or orange.

The indole-3-acetic acid (IAA) concentration was determined according to the method of Kurabachew and Hydra (105). In brief, 100 µL of the cell suspension of the isolates TCR112 and TWR114 (OD_{600} = 0.5), was inoculated in 10 ml tryptic soy broth medium (Difco, Sparks, MD, USA) that contained 0.5 mg L-tryptophan. The medium was incubated at 28°C for 48 h with continuous shaking at 200 rpm. After incubation, 2 ml cell-free culture supernatants of bacterial isolates were mixed with 2 ml Salkowski reagent (1 ml of 0.5 M $FeCl_3.6H_2O$ in 50 ml of 35% perchloric acid). After incubating in the dark for 30 min, IAA production was assessed by a change in color from yellow to pink. The optical density was determined by measuring the absorbance at 535 nm, and IAA concentration was estimated using a standard curve prepared from pure IAA.

Hydrogen cyanide (HCN) production was detected as described by Kurabachew and Hydra (105). The isolates TWR114 and TCR112 were cultured on NA medium that contained 4.4 g/L glycine. A 90-mm filter paper (Advantec Toyo Kaisha., Ltd.,

Tokyo, Japan) soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. The plates were then sealed with parafilm to avoid gas leakage. After incubation at 28°C for 4 days, HCN production was detected based on a change in color of the filter paper from yellow to orange.

Protease production was determined using skim milk agar medium Smibert and Krieg (187) that was prepared by mixing 28 g of skim milk in 500 ml DW. The mixture was thoroughly stirred and autoclaved at 115°C for 15 min. Likewise, 500 ml of the solution that contained 5 g casein enzymatic hydrolysate, 2.5 g yeast extract, 1 g glucose, and 1.5% agar was sterilized. For plating, both the solutions were mixed at 55°C and quickly poured into plates. The isolates TWR114 and TCR112 were spotted onto agar plates, and protease activity was accessed on the basis of the formation of a clear zone around the colony after incubation for 3 days at 30°C.

Polygalacturonase activity was determined on PYA medium (197), that contained 7 g polygalacturonic acid, 10 g yeast extract, and 1.5% agar, per liter, which was adjusted to pH 6.8 with 0.1 N NaOH. Polygalacturonase was detected after applying 10 ml of 5 N H_2SO_4 over the agar plates that contained the isolates TWR114 and TCR112, which were previously cultivated at 30°C for 3 days. The enzyme activity was identified based on the development of a clear zone around the bacterial colony after incubation for 10 min.

2.2 Genome sequencing of TWR114 and TCR112

2.2.1 DNA extraction

The genomic DNA of TWR114 and TCR112 were extracted using QuickGene Mini80 system and QuickGene DNA tissue kit S (Fujifilm Co., Tokyo, Japan) as

described by the manufacturer, and the DNA quality was assessed by agarose gel electrophoresis and measuring the A_{260}/A_{280} ratio by using a NanoVue plus Spectrophotometer (GE Healthcare Life Sciences, UK).

2.2.2 Genome sequencing and assembly

Genome sequences were determined by whole genome shotgun (WGS) sequencing using Illumina MiSeq platform with ~300-bp paired-end libraries (Illumina, CA, USA). The pair-end reads were trimmed and assembled *de novo* using Velvet (version 1.2.10) (240).

2.2.3 Genome annotation and components

The whole genome sequences of each isolate were submitted to the Microbial Genome Annotation Pipeline version 2.23 (http://www.migap.org/) for automatic annotation (193). In the pipeline, open reading frames (ORFs) were identified by MetaGene Annotator (version 1.0) (41, 149), and then predicted ORFs were used to search reference databases, including RefSeq, TrEMBL, and the COGs (clusters of orthologous groups of proteins) data set. Genes for tRNAs and rRNAs were identified by tRNAscan-SE (version 1.23) and rRNAmmer (version 1.2), respectively (193).

Gene clusters which encode biosynthetic pathways for secondary metabolites were predicted using the webserver antiSMASH 4.0.2 (215). Genomic Islands (GIs) were predicted by using the GI prediction method IslandPath-DIOMB (43). Clustered regularly interspaced short palindromic repeat sequences (CRISPRs) were found using CRISPRFinder (68). COG analysis was performed to generate functional annotations for coding sequences (205). Functional annotation was based on

RPSBLAST program searches against the COG database (222). The presence of antimicrobial resistance genes was inferred based on ResFinder 3.0 (239) and manual BLAST searches.

2.3 Comparative genomic analyses

Genome-based ANI and *is*-DDH analyses were used to evaluate the relatedness between the isolates TWR114 and TCR112 and other isolates belonging to same species. The whole genome sequences of these isolates were downloaded from the EzBioCloud web-server (https://www.ezbiocloud.net/), and their details are listed in Table 1. The pair-wise ANI values based on BLAST+ (27) among the genomes were calculated using the web-server JSpeciesWS (http://jspecies.ribohost.com/jspeciesws/) (173*)*. The pair-wise *in silico* DDH values among the genomes were computed using the server-based genome-to-genome distance calculator (version 2.1, http://ggdc.dsmz.de/distcalc2.php) (133), with BLAST+ for genome alignments (27).

2.4 Pan- and core- genome analyses and a core-genome-based phylogenetic analysis

Pan- and core-genome analyses were performed using a bacterial pan-genome analysis pipeline (BPGA, version 1.3) (31). The core-genome was extracted from the whole genomes of the isolates TWR114 and TCR112 and all representative strains using the USEARCH program (version 10.0) (51), with a 50% sequence identity cut-off, available in BPGA. The concatenated amino acid sequences of the core-genome

were aligned using the MUSCLE program (version 3.8.31). A core-genome-based phylogenetic tree with bootstrap values (1000 replicates) was constructed using the maximum likelihood method with MEGA software (version 7.0.26) (104).

2.5 COG analysis of Mitsuaria and *Ralstonia* pan-genomes

Clustering of functional genes derived from the whole genomes of TWR114 and TCR112 isolates and their closely related taxa was performed using the USEARCH program against the Clusters of Orthologous Group of Proteins database (COG) within the BPGA, with a default parameter setting. For the functional characterization of the genomes, functional genes derived from the genome sets for TWR114 or TCR112 were COG-categorized using the USEARCH program and the portions of genes assigned to each COG category were expressed as relative percentages.

2.6 Bacterial culture conditions and inoculum preparation

TWR114 and TCR112 isolates were cultured in nutrient broth (Nissui Pharmaceutical Co., Tokyo, Japan). VT0801 isolate was cultured in casamino acid-peptone-glucose broth medium (76). Both media were incubated at 30°C for 24 h with shaking at 200 rpm. The cells of TWR114 and TCR112 were harvested by centrifugation at 10,000 rpm for 10 min, washed twice and resuspended in sterile distilled water (SDW) to a final concentration of ca. 9×10^8 CFU/ml. The cells of VT0801 was harvested as mentioned earlier, washed twice and resuspended in 10mM $MgCl_2$ to a final concentration of ca. 2×10^7 CFU/ml.

2.7 Growth of plants and bacterial inoculation

Seeds of tomato (cv. Ponderosa, susceptible to bacterial wilt) were surface-sterilized with 70% (v/v) ethanol for 1 min, followed by 1% (v/v) sodium hypochlorite for 5 min and then thoroughly rinsed with SDW. After germination on a moist filter paper, the seeds were sown in plastic trays (Bee pot Y-49; Canelon Kaka Co. Ltd., Japan) containing a commercial potting soil mix “Saika Ichiban” (Ibigawa Kogyo Co. Ltd., Japan) and grown in a glasshouse (maintained at 30°C, relative humidity of 70%) until the seedlings reached the four-leaf stage. Tomato seedlings were then transplanted into vinyl pots (9 cm in diameter) comprising three layers: top and bottom layers, each containing 150 g of commercial potting soil mix; and a middle layer, containing 20 g of river sand. Tomato plants were grown in a chamber with a controlled environment (Biotron, standard model LH-241SP; Nippon Medical and Chemical Instruments Co. Ltd., Osaka, Japan) at 28°C under a 12-h light/12-h dark cycle.

For the TWR114+TCR112 treatment, cell suspensions of TWR114 and TCR112 (ca. 9×10^8 CFU/ml) were mixed thoroughly at a ratio of 2:1 (v/v), before the treatment of tomato plants by bottom watering (100 ml/pot). For individual treatments, a cell suspension of each isolate was applied (100 ml/pot) to obtain a final concentration of 3×10^8 CFU/g wet soil. The plants treated with an equal volume of SDW without the bacteria were used as controls. Three days after treatment (dat), both control plants and those treated with the isolates were challenged with 100 ml of a VT0801 washed cell suspension to obtain a final concentration of 7×10^6 CFU/g wet soil. The inoculated plants were maintained in the same glasshouse as mentioned above.

2.8 Analysis of tomato defense-related gene expression using quantitative real-time PCR

Tomato plants were treated with the combination of TWR114 and TCR112 at a 2:1 ratio, TWR114 alone, and TCR112 alone, and then challenged with *R. pseudosolanacearum* VT0801. The main root (100 mg) was sampled from plants inoculated without or with the pathogen at 5 dat (2 dpi) and 7 dat (4 dpi).

RNA extraction was performed as described previously by Suzuki et al. (198) with slight modifications. Samples were powdered in liquid nitrogen and the total RNA was extracted with the following extraction buffer [2% (w/v) of cetyltrimethylammonium bromide, 100 mM of Tris-HCl (pH 6.8), 25 mM of EDTA (pH 8.0), 1.4 M of NaCl, and 5% (v/v) of 2-mercaptoethanol added just before use and heated at 65°C for 10 min]. The resulting upper aqueous phase from centrifugation was re-extracted with a chloroform-isoamyl alcohol mixture (24:1, v/v). The collected supernatant was extracted with water-saturated phenol, guanidium thiocyanate, sodium acetate (pH 4.0) and chloroform. The upper phase was precipitated with isopropanol. The precipitated RNA was collected, washed twice with 75% ethanol, air dried briefly and dissolved in RNase-free water. RNA concentrations were measured with a NanoVue Plus Spectrophotometer (GE Healthcare Life Sciences, UK).

Five hundred nanograms of total RNA were used to synthesize the first-strand cDNA by ReverTra Ace qPCR RT Master Mix with a gDNA Remover (Toyobo, Co. Ltd., Osaka, Japan), following the manufacturer's protocol. The reverse transcription products (10 µl) were diluted with an equal volume of RNase-free water (Water

deionized & sterilized; Nacalai Tesque Inc., Kyoto, Japan) and used as templates for quantitative real-time PCR (qRT-PCR), performed using SYBR *Premix Ex TaqTM* II (Tli RNaseH Plus; Takara Bio Inc., Otsu, Japan). The qRT-PCR reaction mixtures were prepared in a total volume of 10 µl containing 3 µl of RNase-free water, 5 µl of 2× SYBR Premix, 1 µl of the cDNA template, and 0.5 µl of 10 µM of each forward and reverse gene specific primers (0.5 µM final concentration). The gene-specific primers used in this experiment are shown in Table 2. The reactions were performed with a LightCycler Nano Instrument (Roche Diagnostics, Mannheim, Germany) under the following conditions: an initial denaturation step of 95°C for 30 s, followed by 45 cycles of a three-step amplification profile of denaturation at 95°C for 10 s, primer annealing at 60°C for 10 s, and extension at 72°C for 60 s. The specific amplification was verified by melting curve analysis at the end of each qRT-PCR run over the temperature range of 60°C to 97°C. The housekeeping gene *β-tubulin* was used for normalization. The expression level of the target genes in different samples was calculated using the following formula $2^{-\Delta\Delta Cq}$ (118), and the expression level of each gene was given as a values relative to the untreated control plants (not inoculated with the pathogen). The qRT-PCR experiment was conducted once with three biological replicates for each treatment and three technical repetitions for each replicate.

2.9 Statistical analysis

Differences among treatments in the defense genes expression studies were analyzed using Tukey's multiple-comparison test ($P<0.05$). All analyses were

performed using BellCurve for Excel (version 2.13; Social Survey Research Information Co. Ltd., Tokyo, Japan).

3. Results

3.1 In vitro production of siderophore, IAA, protease, polygalacturonase, and HCN

In the O-CAS assay, the color change from blue to orange indicated that the isolate TCR112 produces siderophore, whereas the color remained unchanged in those plates cultivated with isolate TWR114, indicating that it was unable to produce siderophore (Fig. 1A). Both isolates showed positive reaction for protease activity (Fig. 1B). Polygalacturonase was produced by TWR114 only (Fig. 1C), whereas both the isolates did not produce HCN (data not shown). Both isolates could synthesize IAA at different concentrations, with a relatively higher IAA concentration produced by TCR112 (2.3 µg/ml) than by TWR114 (0.6 µg/ml) (Fig. 1D).

3.2 Genome features of TWR114 and TCR112

The draft genome sequence of TWR114 and TCR112 comprises 5,679,444 bp and 5,237,856 bp, respectively. The average GC content of the TWR114 and TCR112 genome were 69.8% and 64.3%, respectively. We predicted 6,464 and 5,638 protein coding sequences (CDSs), 46 and 50 tRNA sequences, in TWR114 and TCR112, respectively. In both isolates, we found three copies each of 16S, 23S, and 5S rRNA genes.

Six and nine genomic islands were predicted in the genome of TWR114 and TCR112, respectively. Four questionable CRISPRs were predicted in the genome of TWR114, whereas none were found in TCR112 (Table 3). The CDSs of TWR114 and TCR112 could be assigned to 21 and 23 COG families (Fig. 2). Except for the genes predicted to have general (0.10–0.11 abundance) or unknown functions (0.4–0.5 abundance), the largest group of genes were involved in amino acid transport and metabolism (0.16–0.17 abundance). No antimicrobial resistance genes were found in the genome of TWR114. In contrast, two resistance genes were found within the genome of TCR112 (Table 4); the sequences showed significant identity with the beta-lactamase genes bla_{OXA-22} (93.1%) and bla_{OXA-60} (96.32%)

3.3 Genome properties

3.3.1 Lytic enzymes

Among the CDSs of TWR114 isolate, we found two genes encoding homogalacturonan degrading genes polygalacturonase (*pglA*) and pectate lyase (*pel*). In addition, several genes encoding hydrolytic enzymes such as chitinase (*chi05*), chitosanase, cellulases (*Cel5F*), protease, and glucanase were also present. While, genes encoding polygalacturonase (*PehC*) and protease was found in TCR112.

3.3.2 Secondary metabolites

Sixteen biosynthetic gene clusters (BGCs) coding for putative secondary metabolites were predicted in the genome of TWR114: seven nonribosomal peptide synthetase, one of which might be a Delftibactin (28% gene similarity), three

arylpolyene, two N-acyl amino acid, two bacteriocin, one terpene, and one unclassified cluster. Whereas, only six BGCs were predicted in the genome of TCR112; two siderophore, one of which might be a desferrioxamine B siderophore (33% gene similarity), one bacteriocin, one arylpolyene, one terpene, and one phenazine cluster.

3.3.3 Secretion systems

We found that TWR114 genome contain the T3SS gene cluster and three effector genes, *ripA*, RSc1475 and putative gene. In addition, the TWR114 also contain T6SS gene cluster and two hcp and four vgrG translocator genes.

3.4 Comparative genomic analyses

The analysis of genome relatedness of ANI and *is*-DDH showed that closest isolate to TWR114 was *Mitsuaria* sp. PDC51. The isolate PDC51 had relatively higher values of ANI and DDH (98.2% and 91.1%, respectively) compared with that for the type strain *M. chitosanitabida* NBRC 102408^T (85.2% and 40.5%, respectively) (Table. 5). Likewise, the analysis revealed that the closest isolate to TCR112 was *Ralstonia* sp. UNC404CL21Col. The isolate UNC404CL21Col had relatively higher values of ANI and *is*-DDH (93.9% and 80.0%, respectively) than the type strain *R. pickettii* ATCC 27511^T (91.3% and 66.4%, respectively) (Table 6).

3.5 Pan- and core- genome analysis and a core-genome-based phylogenetic analysis

The pan-genome of TWR114 contained a total of 5,894 genes consisting of 1683 genes in the core-genome, 2825 genes in the dispensable genome, and 1334 genes in the unique genome (Table 7). The pan-genome of TCR112 contained a total of 5,083 genes consisting of 2112 genes in the core-genome, 2,296 genes in the dispensable genome, and 644 genes in the unique genome (Table 8).

To infer the phylogenetic relationships between the isolates TWR114 and TCR112 and their closely related species, a phylogenetic tree was constructed using the concatenated amino acid sequences of 1683 and 2112 genes in the core-genome, respectively. The core-genome-based phylogenetic tree revealed that both TWR114 and TCR112 have a clear distinction from their closest relative type strains of *M. chitosanitabida* and *R. pickettii*, respectively (Fig. 3).

3.6 COG analysis of Mitsuaria and *Ralstonia* pan-genomes

Core and accessory genes were searched to compare the distribution of their functional categories by using COGs database (Tatusov et al., 2003). The most abundant functions in the core genes of *Mitsuaria* and *Ralstonia* species were associated with the metabolism (40% and 42%, respectively) (Figs. 4 and 5). More specifically, energy production and conversion (C) and amino acid transport and metabolism (E) and coenzyme transport and metabolism (H) (Figs. 6 and 7). The functional category of information storage and processing was almost the same in both genes (core and accessory) of *Mitsuaria* species (Fig. 4). However, it was slightly higher in the accessory genes (27%) compared with that in the core genes (24%) of *Ralstonia* species (Fig. 4). Moreover, this functional category showed highly different proportions in the sub-categories. In the *Mitsuaria* and *Ralstonia* species,

the functions of translation, ribosomal structure, and biogenesis (J) and replication and recombination and repair (L) were more noticeable in the core genes compared with that in the accessory genes, whereas the function of transcription (K) was more abundant in the unique genes (Figs. 6 and 7). The functional category of the cellular processing and signaling was almost the same in the core and accessory genes of the *Mitsuaria* and *Ralstonia* species (Figs. 4 and 5).

3.7 Induction of tomato defense-related genes by TWR114 and TCR112 treatment

We investigated the effect of combined TWR114 and TCR112 treatment and the treatments with each of these individually on the expression of six defense-related genes in the roots of tomato plants. These genes were *PR-1a* and *GluA*, *GluB* and *Osmotin*-like, *Le4*, and *LoxD*, which are related to the salicylic acid (SA), ethylene (ET), abscisic acid (ABA), and jasmonic acid (JA) signaling pathways, respectively. The expression levels of these genes were determined by qRT-PCR at 5 and 7 dat (2 and 4 dpi, respectively) in pathogen-uninoculated and -inoculated plants. The results showed that, in the absence of pathogen inoculation, no or slight induction of the expression of ABA- or ET-responsive marker genes was observed in bacterized plants regardless of the type of treatment, whereas the expression of SA- and JA-responsive genes was strongly induced by the individual treatments compared with that with the TWR114+TCR112 treatment (Fig. 8). In pathogen-inoculated plants, the expression of SA-responsive marker genes *PR-1a* and *GluA* was significantly increased in the TWR114+TCR112 treatment compared with those in the individual treatments and pathogen-inoculated control at 2 dpi (Fig. 8a and 8b). The expression

of ET-responsive genes *GluB* and *Osmotin*-like was considerably increased by the TWR114+TCR112 treatment compared with that in the TWR114 and TCR112 individual treatments and pathogen-inoculated control at 4 dpi (Fig. 8c and 8d). The expression of the ABA-responsive gene *Le4* was strongly induced by the TWR114+TCR112 treatment and the TWR114 treatment compared with those by the TCR112 treatment and pathogen-inoculated control at 2 dpi (Fig. 8e). The JA-responsive gene *LoxD* was not induced by any of the treatments (Fig. 8f).

Table 1. General features of the genomes of *Mitsuaria* and non-pathogenic *Ralstonia* isolates used in this study.

Bacterial isolate	Total size (Mb)	Assembly level	GC (%)	No. of CDSs	Accession number
Mitsuaria sp. PDC51	5.85	Contig	70.00	4,933	GCA_900113225.1
Mitsuaria sp. HWN-4	5.74	Contig	69.50	4,917	GCA_002761755.1
Mitsuaria sp. H24L5A	6.66	Scaffold	67.80	7,267	GCA_000285635.1
Mitsuaria sp. 7	6.09	Complete	68.28	5,298	GCA_001653795.1
Mitsuaria chitosanitabida NBRC 102408^{T}	5.82	Contig	69.90	5,047	GCA_001598255.1
Ralstonia sp. UNC404CL21Col	5.09	Scaffold	64.60	4,655	GCA_000620465.1
Ralstonia sp. NFACC01	5.37	Scaffold	63.80	4,935	GCA_900115545.1
Ralstonia pickettii ATCC 27511^{T}	4.77	Scaffold	63.90	4,389	GCA_000743455.1
Ralstonia pickettii NBRC 102503^{T}	4.73	Contig	64.00	4,348	GCF_001544155.1
Ralstonia pickettii H2Cu2	5.20	Contig	64.00	4,796	GCA_001699795.1
Ralstonia pickettii H2Cu5	5.24	Contig	63.70	4,895	GCA_001699815.1
Ralstonia pickettii 5_2_56FAA	5.25	Scaffold	63.60	4,869	GCA_000227255.2
Ralstonia pickettii 5_7_47FAA	5.25	Scaffold	63.60	4,917	GCA_000165085.1
Ralstonia pickettii 12D	5.69	Complete	63.34	5,417	GCA_000023425.1
Ralstonia pickettii 12J	5.33	Complete	63.62	4,993	GCA_000020205.1
Ralstonia pickettii FDAARGOS_410	4.82	Complete	65.91	4,408	GCA_002393485.1

Table 2. The primers used in the quantitative real-time PCR analysis of tomato defense-related gene expression.

Target gene	Pathway[†]	Primer sequence (5′–3′)[‡]	Reference
PR-1a (pathogenesis-related protein-1a)	SA	F- TCTTGTGAGGCCCAAAATTC R- ATAGTCTGGCCTCTCGGACA	5
GluA (acidic extracellular β-1,3-glucanase)	SA	F- GGTCTCAACCGCGACATATT R- CACAAGGGCATCGAAAAGAT	5
GluB (basic intracellular β-1,3-glucanase)	ET	F- TCTTGCCCCATTTCAAGTTC R- TGCACGTGTATCCCTCAAAA	5
Osmotin-like	ET	F- TGTACCACGTTTGGAGGACA R- ACCAGGGCAAGTAAATGTGC	136
Le4 (desiccation protective protein)	ABA	F- ACTCAAGGCATGGGTACTGG R- CCTTCTTTCTCCTCCCACCT	129
LoxD (lipoxygenase D)	JA	F- CCTGAAATCTATGGCCCTCA R- ATGGGCTTAAGTGTGCCAAC	5
β-tubulin	HK	F- AACCTCCATTCAGGAGATGTTT R- TCTGCTGTAGCATCCTGGTATT	5

[†] The genes monitored are used as markers for the following signaling pathways: salicylic acid (SA), ethylene (ET), abscisic acid (ABA), and jasmonic acid (JA). *β-tubulin* was used as a housekeeping gene (HK) for normalization.

[‡] F = forward primer. R = reverse primer.

Table 3. Information about CRISPRs in the genome of *Mitsuaria* sp. TWR114.

CRISPR location		CRISPR length	DR consensus sequence	Spacer sequence
Start-End	26-116	90	GCCGGCCTTCTTCAGCGCCTCGG	TGGCCTCCCTCTTTCCCACTCTTTTGCCGGCAGGT
	772-857	85	GAGGGGGCCGGACCGTCCGCGTTTCGCT	CGGTGACGAAGAACTCGTCCCAGGTGGTGGGCAGCTTGGC
	3444-3562	118	CCCCCTCGAGGGGCAGCGAACGAAGTGAGCGTGGGGGC	GCATCGTCCGACGCCCGCCCGGCCGCCCGAAGGGCGTTGAGCA
	5049-5105	80	AGTAGCGGGAGCGAGTTGGTCTCCCATGGCT	AGTAGCGGGAGCGAGTTGGTCTCCCATGGCT

Table 4. Information about the antimicrobial resistance genes in the genome of non-pathogenic *Ralstonia* TCR112.

Resistance gene	Identity	Query/HSP	Contig	Position in contig	Phenotype	Accession no.
blaOXA-60	96.32	816/816	NODE_409_length_11041_cov_23.076895	1189..2004	Beta-lactam resistance	AY664505
blaOXA-22	93.09	825/825	NODE_689_length_24584_cov_26.483404	14214..15038	Beta-lactam resistance	AF064820

Table 5. ANI and *is*-DDH between the *Mitsuaria* sp. TWR114 and other isolates of the genus *Mitsuaria.*

Bacterial isolate	**ANI (%)**	***is*-DDH (%)**
Mitsuaria sp. PDC51	98.2	91.1
Mitsuaria sp. HWN-4	92.3	75.7
Mitsuaria sp. H24L5A	86.9	39.8
Mitsuaria sp. 7	86.1	37.0
Mitsuaria chitosanitabida NBRC 102408^{T}	85.2	40.5

Cut-off values: ANIb = <95%, *is*-DDH = <70%

Table 6. ANI and *is*-DDH between the non-pathogenic *Ralstonia* sp. TCR112 and other isolates of the genus *Ralstonia*.

Bacterial isolate	ANI (%)	*is*-DDH (%)
Ralstonia sp. UNC404CL21Col	93.9	80.0
Ralstonia sp. NFACC01	91.5	79.0
Ralstonia pickettii ATCC 27511 ^{T}	91.3	66.4
Ralstonia pickettii NBRC 102503 ^{T}	91.6	66.8
Ralstonia pickettii H2Cu2	91.1	64.9
Ralstonia pickettii H2Cu5	91.5	61.3
Ralstonia pickettii 5_2_56FAA	91.5	60.9
Ralstonia pickettii 5_7_47FAA	91.4	60.6
Ralstonia pickettii 12D	90.7	52.2
Ralstonia pickettii 12J	90.9	56.2
Ralstonia pickettii FDAARGOS_410	86.9	61.9

Cut-off values: ANIb = <95%, *is*-DDH = <70%

Table 7. Number of core, accessory, unique, and exclusively absent genes in six isolates belonging to the genus *Mitsuaria*.

Bacterial isolate	No. of core genes	No. of accessory genes	No. of unique genes	No. of exclusively absent genes
TWR114	2744	1766	1306	118
Mitsuaria sp. HWN-4	2744	1743	308	23
Mitsuaria sp. PDC51	2744	1908	182	1
Mitsuaria sp. H24L5A	2744	1847	2309	178
Mitsuaria sp. 7	2744	1424	967	410
Mitsuaria chitosanitabida NBRC 102408^{T}	2744	1403	790	209

Table 8. Number of core, accessory, unique, and exclusively absent genes in twelve isolates belonging to the genus *Ralstonia*.

Bacterial isolate	No. of core genes	No. of accessory genes	No. of unique genes	No. of exclusively absent genes
TCR112	3120	1247	672	60
Ralstonia sp. UNC404CL21Col	3120	1217	147	6
Ralstonia sp. NFACC01	3120	1308	311	13
Ralstonia pickettii ATCC 27511 ^{T}	3120	1080	4	0
Ralstonia pickettii NBRC 102503 ^{T}	3120	1073	4	2
Ralstonia pickettii H2Cu2	3120	1260	183	48
Ralstonia pickettii H2Cu5	3120	1432	99	12
Ralstonia pickettii 5_2_56FAA	3120	1480	4	1
Ralstonia pickettii 5_7_47FAA	3120	1481	10	1
Ralstonia pickettii 12D	3120	1387	587	3
Ralstonia pickettii 12J	3120	1371	153	0
Ralstonia pickettii FDAARGOS_410	3120	823	312	151

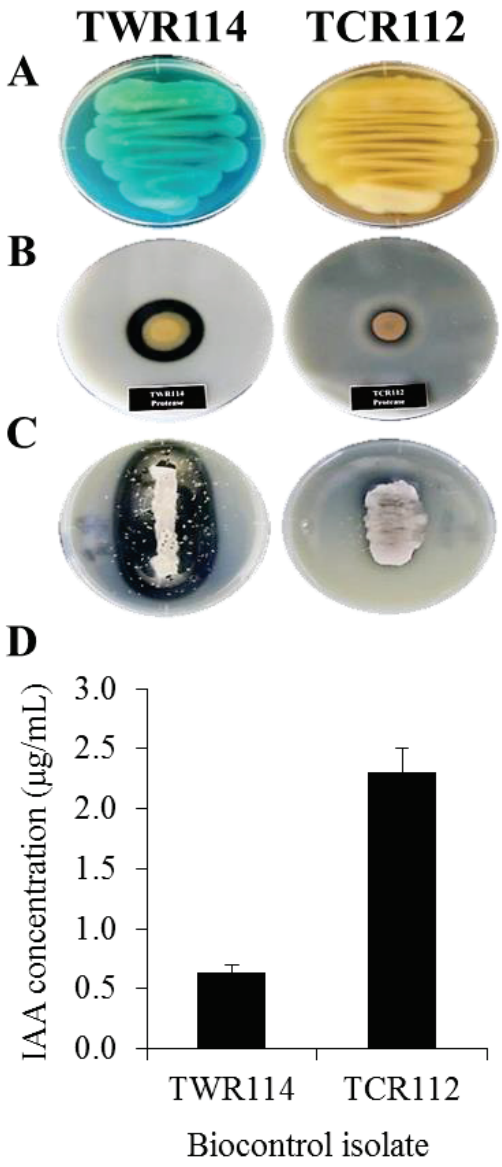


Figure 1. Biocontrol traits of *Mitsuaria* sp. TWR114 (left side photos) and non-pathogenic *Ralstonia* sp. TCR112 (right side photos). (A) Siderophore production detected by the overlaid chrome azurole S agar method. (B) Protease production on skim milk agar medium. (C) Polygalacturonase activity on a tryptic soy agar medium supplemented with 0.7% polygalacturonic acid. (D) The amount of indole-3-acetic acid synthesized by the isolates on tryptic soy broth medium amended with 50 µg/ml of L-tryptophan.

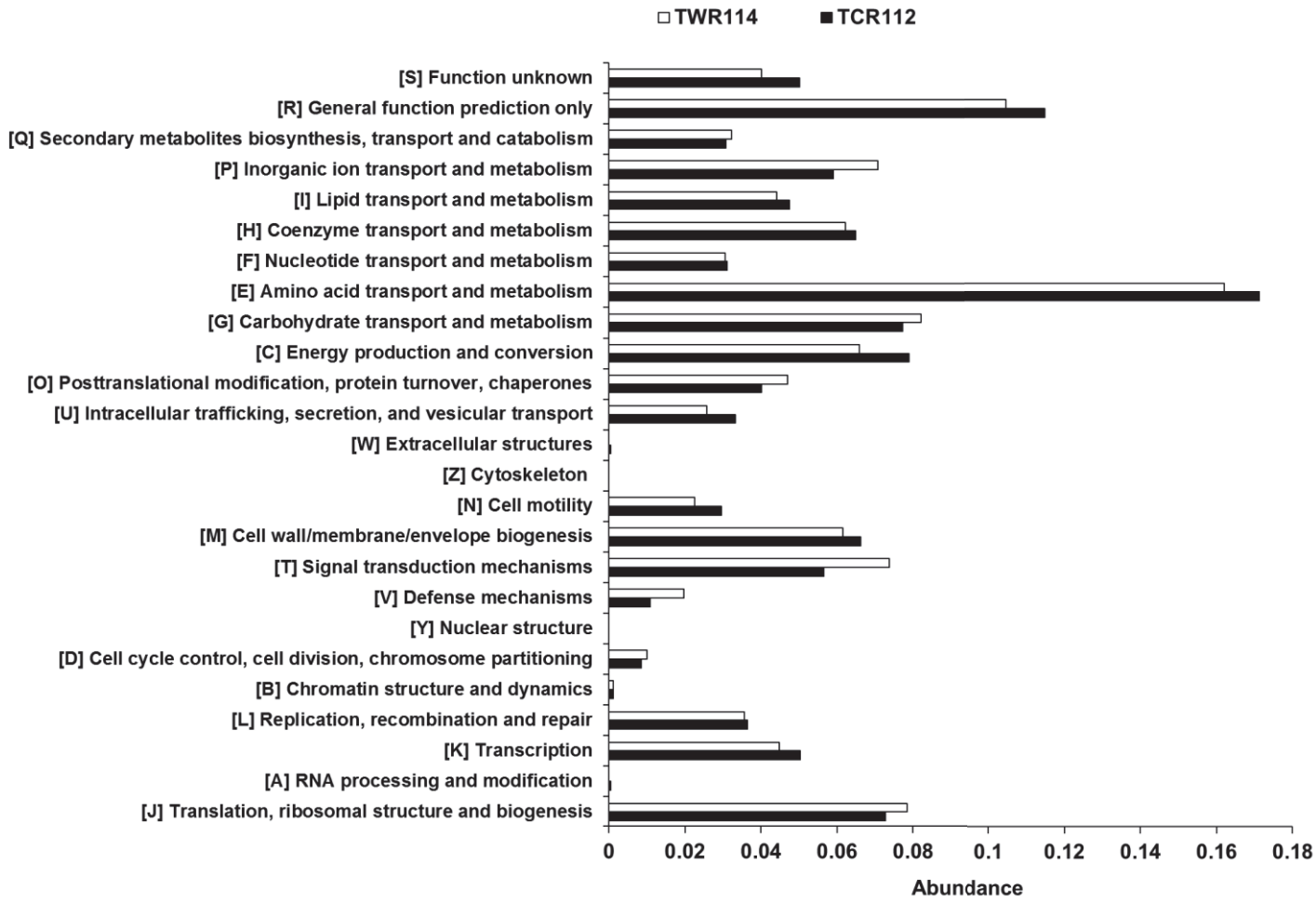


Figure 2. Distribution of genes with COG functional categories in *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112.

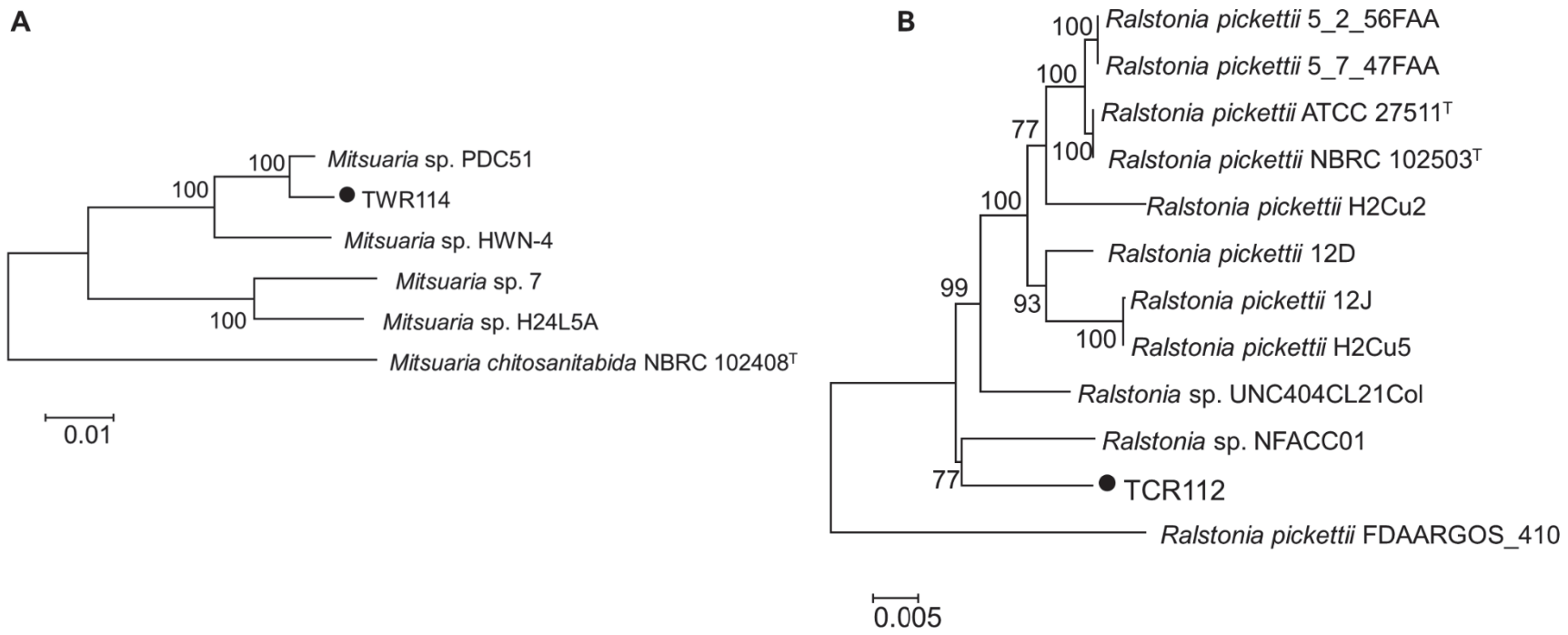


Figure 3. Phylogenetic position of *Mitsuaria* sp. TWR114 (A) and non-pathogenic *Ralstonia* TCR112 (B) based on the concatenated amino acid sequences of the genus *Mitsuaria* core-genome (2744 genes) and the genus *Ralstonia* core-genome (3120 genes), respectively. The tree was constructed by the maximum likelihood method using the MEGA software.

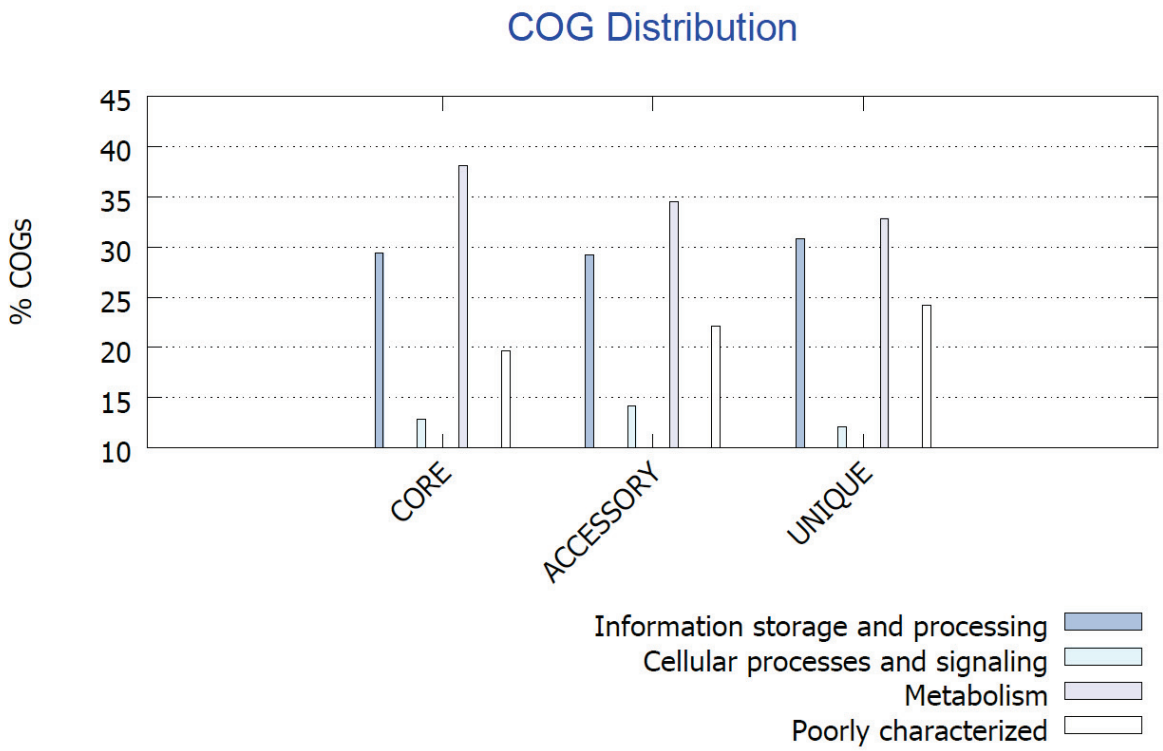


Figure 4. Differential distribution of COG functional categories in core, accessory, and unique genes of six isolates belonging to the genus *Mitsuaria*.

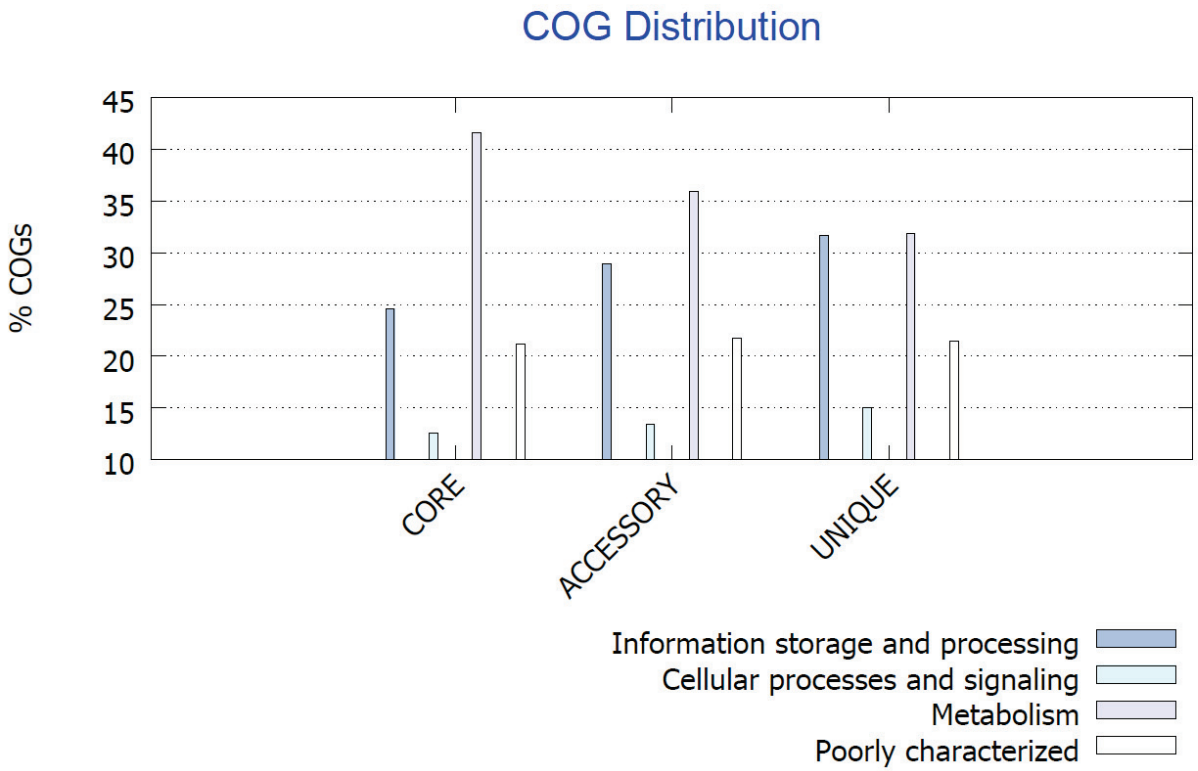


Figure 5. Differential distribution of COG functional categories in core, accessory, and unique genes of twelve isolates belonging to the genus *Ralstonia*.

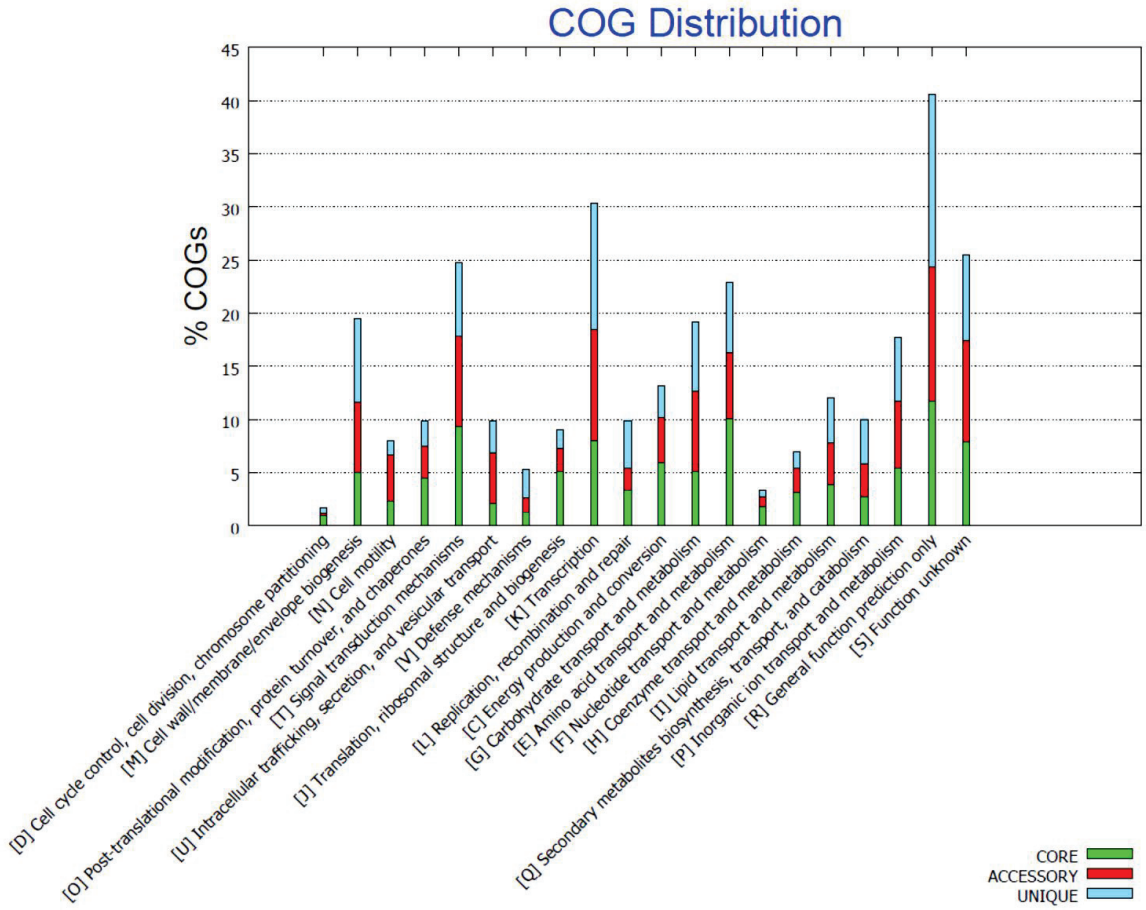


Figure 6. Differential distribution of COG functional categories in core and strain-specific genes of six isolates belonging to the genus *Mitsuaria*.

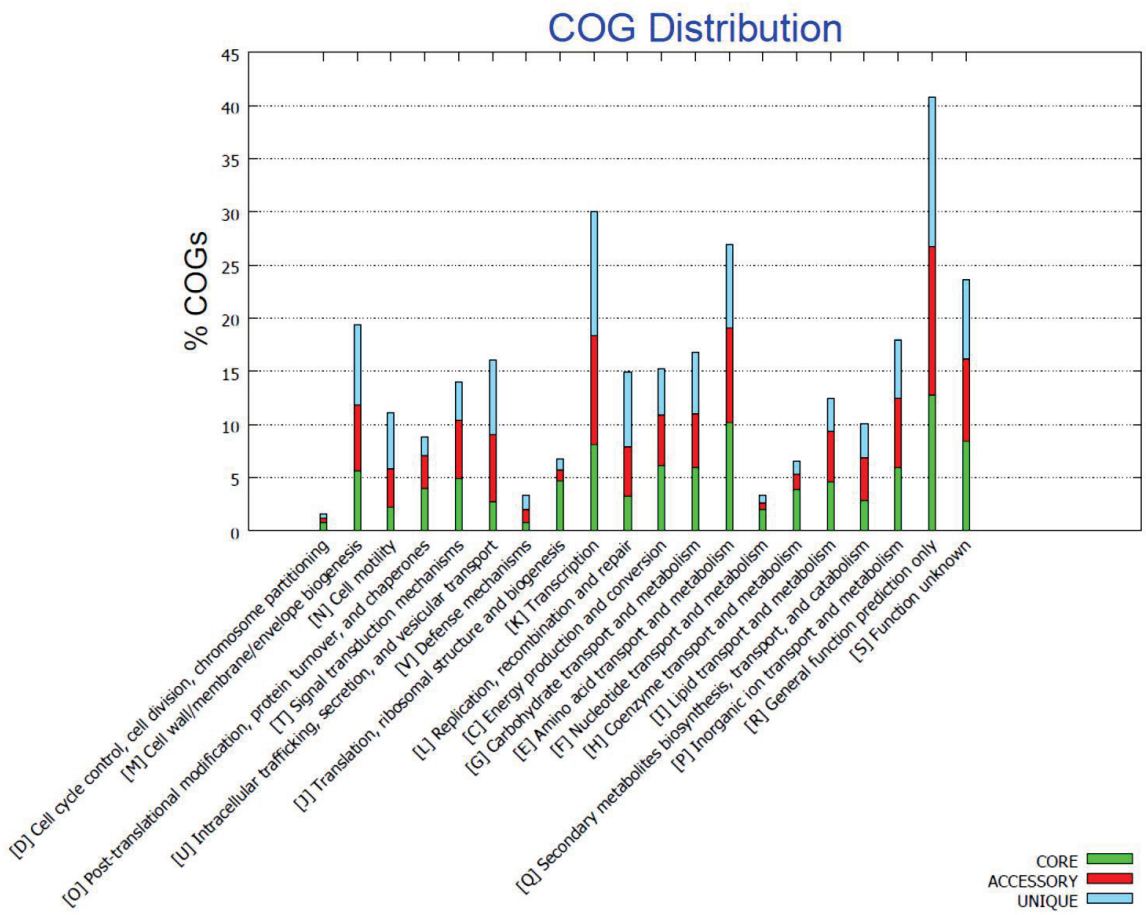


Figure 7. Differential distribution of COG functional categories in core and strain-specific genes of twelve isolates belonging to the genus *Ralstonia*.

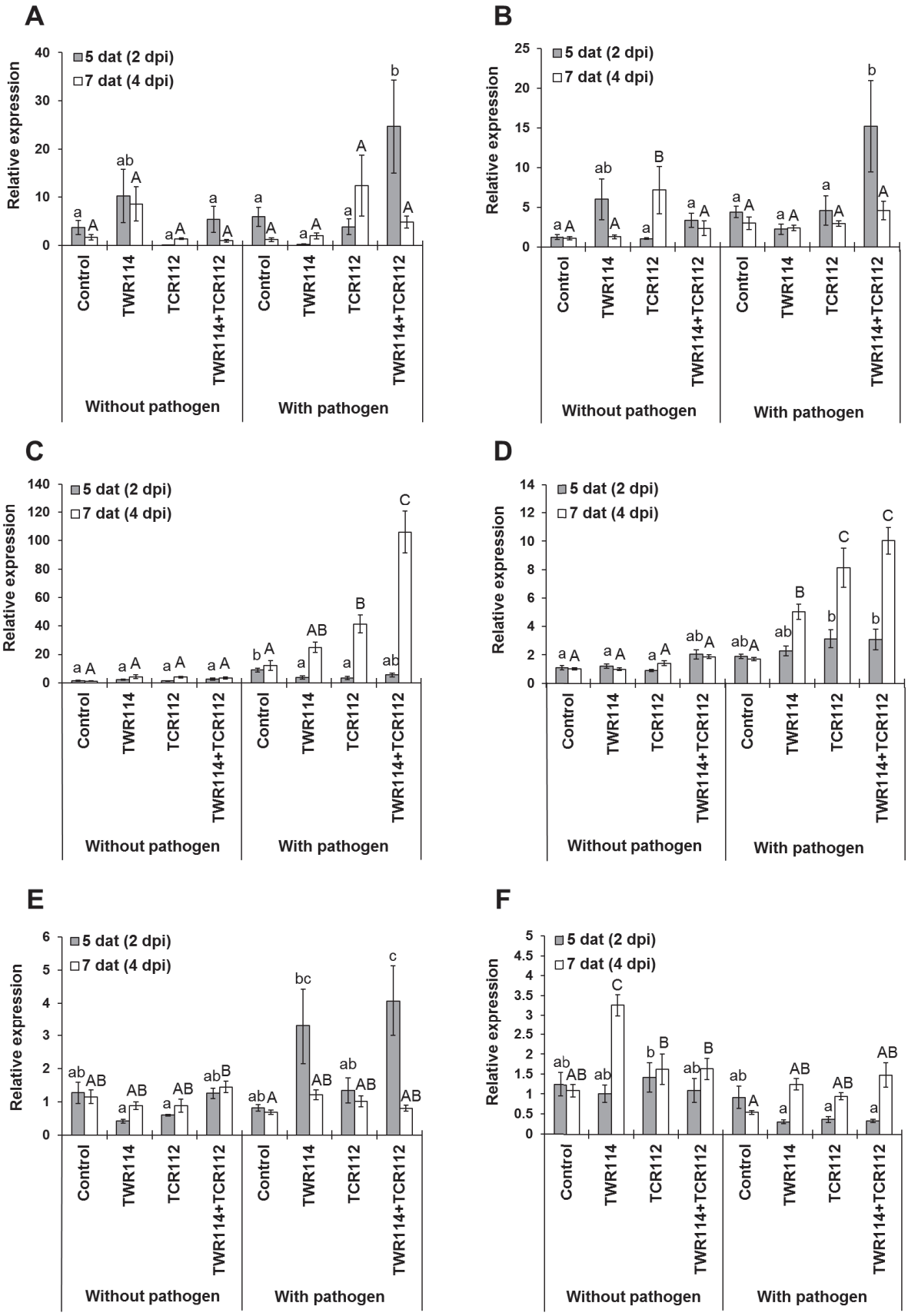

A
B
C
D
E
F
Relative expression
5 dat (2 dpi)
7 dat (4 dpi)
Control
TWR114
TCR112
TWR114+TCR112
Without pathogen
With pathogen

Figure 8. Expression pattern of defense-related genes in tomato plants grown in a chamber with a controlled environment and treated with the combination of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 and the individual isolates, or untreated control, and then inoculated without or with *Ralstonia pseudosolanacearum* at 5 and 7 days after treatment (dat) (2 and 4 days post-challenge inoculation [dpi], respectively). (A) *PR-1a*. (B) *GluA*. (C) *GluB*. (D) *Osmotin*-like. (E) *Le4*. (F) *LoxD*. The housekeeping gene β-tubulin was used for normalization. The expression level of the target genes in different samples was calculated using the following formula $2^{-\Delta\Delta Cq}$ (118), and the expression level of each gene was given as a values relative to the untreated control plants (not inoculated with the pathogen). Bars represent the mean ± standard error of three biological replicates per treatment with three technical repetitions for each sample. Different lowercase and uppercase letters indicate significant differences between treatments according to Tukey's test at $P<0.05$.

4. Discussion

In this chapter, we aimed to investigate the different mechanism confers by the biocontrol isolates *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 which are involved in the disease suppression of tomato bacterial wilt. Our results showed that TWR114 was able to produce extracellular polygalacturonase, whereas both isolates exhibit protease activity (Fig. 1B and 1C). In addition, the genomic analysis revealed that our isolates harbor genes encoding the lytic enzymes polygalacturonase and protease. We previously showed that the treatment with these isolates considerably reduced the pathogen population in the rhizosphere (Chapters 1 and 2). These results suggest that both isolates suppress the pathogen multiplication through at least antimicrobial enzyme mediated antagonism. It has been reported that the strong competitive ability of rhizobacteria to utilize pectin by producing extracellular pectinases plays a significant role in their rhizoplane competence, possibly resulting in suppressing the multiplication of *R. solanacearum* (86, 183). The biocontrol activity of *Stenotrophomonas maltophilia* against bacterial wilt was recently reported to occur because of the direct antagonism against *R. solanacearum* which depends on the proteolytic enzyme production (52).

In this study, the isolate TCR112 was capable of producing siderophores (Fig. 1A). In addition, we found two BGCs coding for putative siderophores in the genome of TCR112. These results suggest that TCR112 isolate might affect the pathogen multiplication via siderophore-mediated competition for iron. A previous study has indicated that the production of siderophores by *Pseudomonas* spp. contributes to the suppression of bacterial wilt, possibly by limiting iron availability to *R. solanacearum* (168).

Moreover, production of phythormones such as IAA by beneficial bacteria has been reported to induce resistance (156). As described earlier, the isolate TCR112 produces siderophore (Fig. 1A). Additionally, the isolates TCR112 and TWR114 produce IAA (Fig. 1D). Some of these compounds have already been reported to induce resistance against bacterial wilt (169).

Our results from qRT-PCR analysis revealed that, upon pathogen inoculation, the expression of several genes was induced by the TWR114+TCR112 treatment and the corresponding individual treatments. However, both types of treatments showed varying levels of expression, in which the expression of most of these genes was more strongly induced in tomato plants treated with TWR114+TCR112 than in those treated with the individual isolates, indicating an enhanced priming effect (Fig. 8). We previously found that the population of *R. pseudosolanacearum* in the above-ground regions, particularly in the mid-stem and upper stem was considerably decreased by the combined treatment of TWR114 and TCR112 compared with the levels upon their individual treatments. Interestingly, although the pathogen population in the above-ground regions of TWR114+TCR112-treated plants increased to 2.4–4.8 log CFU/g fresh tissue at 7 dpi, its population decreased to an undetectable level (<1.5 log CFU/g fresh tissue) at 28 dpi (Chaper 2). This may have been due to the enhanced defense responses upon the TWR114+TCR112 treatment. It was previously suggested that the priming of defense responses by treatment with the rhizobacterium *Pseudomonas putida* could reduce the population of *R. solanacearum* in root tissues of tomato plants (4). The importance of host defense priming in the suppression of several soil-borne diseases, including bacterial wilt, by the treatment with beneficial microbes or some chemical elements such as silicon has already been reported (5, 64, 94, 148). Additionally, the greater induction of

some defense-related enzymes has been suggested to be one of the mechanisms responsible for the enhanced biocontrol effect achieved by the combination of BCAs against bacterial wilt on tomato (90) and tobacco (237) plants. It has been well demonstrated that an SA-dependent signaling pathway is involved in the systemic acquired resistance, whereas JA- and ET-dependent signaling pathways are involved in the induction of systemic resistance (ISR) (158). However, recent evidence also suggested the partial involvement of an SA-dependent pathway during ISR in some cases (148). These signaling pathways do not work independently but instead influence one another through a complex network of synergistic and antagonistic interactions (66). Several studies have indicated that SA, JA, ET, and ABA signaling pathways are involved in the BCA-mediated ISR against bacterial wilt (34, 58, 72, 73, 200). In this study, the treatments of TWR114 or TCR112 alone resulted in priming for the enhanced expression of the ET-responsive marker genes *GluB* (coding a basic intracellular β-1,3-glucanase) and *Osmotin*-like, while only the TWR114-treatment boosted the expression of the ABA-regulated gene *Le4* (coding a desiccation protective protein), suggesting that these two isolates might activate different signaling pathways. Interestingly, the expression of ET- and ABA-responsive marker genes was significantly more pronounced in the TWR114+TCR112 treatment, and the expression of SA-regulated genes *PR-1a* and *GluA* (coding pathogenesis-related protein-1a and acidic extracellular β-1,3-glucanase, respectively) was also primed by this treatment only (Fig. 8). Based on these findings, we propose that the TWR114+TCR112-mediated ISR in tomato plants against the necrotrophic pathogen *R. pseudosolanacearum* may be due to the enhanced priming effect of SA-, ET-, and ABA-dependent defense responses. Recently, Alizadeh et al. (9) showed that the combined application of *Trichoderma*

harzianum and *Pseudomonas* sp. provided better disease suppression than their individual applications against Fusarium wilt of cucumber, and the enhanced effectiveness was mainly due to the priming effect of both SA- and JA-dependent defense responses upon pathogen inoculation.

We predicted six and nine genomic islands in the genome of TWR114 and TCR112, respectively. One of the important mechanisms in the evolution of bacteria is horizontal gene transfer. Bacteria could get genes from other different species such as archaea, bacteriophage, and eukaryotes (103). Genomic Islands are evidence of horizontal acquisition (108). It was also suggested that genomic islands found in non-pathogenic species are important in the evolution of these bacteria, influencing traits such as antibiotic resistance, symbiosis and fitness, and adaptation in general (45). We predicted three CRISPR elements in TWR114 genome (Table 3), which could be of importance to the resistance of this isolate against exogenous DNA. It was suggested that CRISPRs can confer resistance to exogenous genetic elements such as phages and plasmids (21). We found that both of our isolates harbor the T3SS and T6SS gene clusters. The T3SS and T6SS might be important for these isolates particularly for their endophytic colonization and bacterial competition. Bacteria frequently use protein secretion systems to interact with their hosts (161). It was previously reported that T3SS genes are present in environmental isolates of *Burkholderia* and *Pseudomonas* (151). Although they are often regarded as pathogens, many isolates of these taxa can colonize plant roots to high levels, and some have beneficial effects on plant growth and disease resistance (24). T6SS delivers effectors into neighboring organisms, including bacteria and hosts, leading to cytotoxicity and cell death of targets (176). In addition to the presence of broad variety of antimicrobial BGCs and lytic enzymes encoding genes

within the genome of both isolates, several genes encoding phospholipases were also found. These enzymes have been suggested to be toxic to other organisms (211). Furthermore, several multidrug efflux pumps, acriflavin, and fusaric acid resistance proteins were present in the genome of these isolates, which might contribute to the survival ability of our isolates from natural environmental compounds.

In conclusion, the results of this study strongly suggest that *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 suppress tomato bacterial wilt by the combination of multiple biocontrol mechanisms of antibiosis, production of siderophore and enzymes, competition for nutrients, and induction of systemic resistance. Comparative genomic analyses suggest that TWR114 and TCR112 might be a representative of a new species. The genome sequences of both isolates may provide a foundation not only for plant disease control research but also for genomics and comparative genomics research.

General discussion

General discussion

The results from this study clearly demonstrate the effectiveness of the rhizobacteria *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 in controlling tomato bacterial wilt caused by *Ralstonia pseudosolanacearum*. Individual treatment with these isolates effectively suppressed bacterial wilt up to 2 weeks in tomato plants in the glasshouse and in a naturally infested field (Chapter 1). Moreover, their combination treatment could exert a synergistic suppressive effect on the biocontrol of wilt disease under glasshouse conditions. A single application of TWR114+TCR112 achieved a more intense and prolonged biocontrol effect (lasted for up to 4 weeks) than their individual treatments (Chapter 2). In a field experiment conducted in 2018, the combination treatment provided considerable protection to the tomato plants against bacterial wilt. However, we could not observe any significant differences between the application of TWR114+TCR112 in different week intervals (i.e., 2-weeks, 3-weeks, and 4-weeks). Tomato seedlings used in this experiment were ungrafted having moderate resistance against bacterial wilt. However, because of the high pathogen pressure in our field, it is very difficuly to evaluate the biocontrol effect of TWR114+TCR112 using ungrafted seedlings. Thus, in the future, we recommend using grafted seedlings having high resistance against bacterial wilt in order to better evaluate the effectiveness of the TWR114+TCR112.

The population of *R. pseudosolanacearum* in the rhizosphere was greatly reduced by the treatment of TWR114 and TCR112 (Chapters 1 and 2). In addition, both isolates exhibited *in vitro* antibacterial activity against the pathogen (Chapter 1), and they were capable of producing lytic enzymes such as polygalacturonase, protease,

and/or siderophore (Chapter 3). These results suggest that both isolates suppress the pathogen multiplication through antibiosis and antimicrobial enzyme mediated antagonism. The pathogen population in the above-ground regions was considerably decreased by the treatment of TWR114 and TCR112 (Chapters 1 and 2). Furthermore, our results from qRT-PCR analysis revealed that, upon pathogen inoculation, the expression of several tomato defense-related genes was induced by the TWR114 and TCR112 treatment, indicating priming effect (Chapter 3). These results suggest that these two isolates prevented the pathogen multiplication in the above-ground regions by priming of defence responses in tomato plant. Thus, in summary, the biocontrol mechanisms of TWR114 and TCR112 against tomato bacterial wilt consisted of antibiosis, siderophore and enzymes production, and induced systemic resistance. However, our results strongly suggest that other mechanisms, specifically nutrient competition may also play an important role in the suppression of the pathogen. Several lines of evidence may support this hypothesis. First, the TWR114 and TCR112 isolates greatly suppressed the pathogen multiplication in tomato plant, which can not be explained by their weak *in vitro* antibacterial activity (narrow and fuzzy inhibition zone) against *R. pseudosolanacearum* VT0801 (Chapter 1). Second, the compatibility test between our isolates showed that TWR114 has an *in vitro* antibacterial activity towards TCR112, and as mentioned earlier TWR114 has also an activity against VT0801, thus assumed that TWR114 produces some antibacterial compounds that suppress *Ralstonia* species. Yet, *in planta*, TWR114 suppressed the population density of the pathogen only, while it did not affect that of TCR112 (Chapter 2). Third, since the isolate TCR112 belongs to genus *Ralstonia*, its ecological and physiological

characteristics may be similar to that of pathogenic *Ralstonia*, suggesting that TCR112 compete for the same nutrient sources and occupies the same niches as the pathogenic *Ralstonia*. Recently, several studies have demonstrated the importance of competition for nutrients against *R. solanacearum* as one of the mechanisms responsible for the suppression of tomato bacterial wilt (82, 221). Morever, Yang et al. (231) showed that certain mixture of non-pathogenic *Ralstonia* species could constrain the relative density of *R. solanacearum* under microcosm conditions. This was attributed to the ability of these species to stongly compete against the pathogen for resources. Studies are underway to investigate whether our isolates can compete for nutrients against the pathogen.

We found that the TWR114 and TCR112 isolates were capable of solubilizing inorganic phosphate, producing ammonia, and fixing nitrogen under *in vitro* conditions (data not shown). In addition, the TCR112 isolate was also capable of producing siderophore (Chapter 3) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Furthermore, we found that the genome of TCR112 isolate harbor genes related to siderophore and ACC deaminase production (data not shown). These results suggest that our isolates might also be able to promote the growth of tomato plant. Rhizobacteria can either directly or indirectly facilitate the growth of plants. Direct stimulation may include providing plants with fixed nitrogen, phytohormones, iron that has been sequestered by bacterial siderophores, soluble phosphate, or the enzyme ACC deaminase that can lower plant ethylene levels (3). Several studies in the past have reported the ability of some *Mitsuaria* and *Ralstonia* species in promoting the growth of plants such as pea, wheat, corn, mung bean, and rape (84, 93, 109, 241).

The TWR114 isolate exhibited cellulase activity under *in vitro* conditions (data now shown). In addition, we found a wide array of genes encoding hydrolytic enzymes with antifungal activity such as chitinase, cellulases, glucanase, and chitosanase within the genome of TWR114 (Chapter 3). Thus, these results suggest that the TWR114 isolate might have biocontrol potential against fungal and oomycetes pathogens. Many bacteria produce lytic enzymes that can hydrolyze a wide variety of polymeric compounds including chitin, proteins, cellulose, lipids, glucan, and chitosan, some of the major components of fungal and/or oomycetes cell wall. Benítez et al. (23) reported that some *Mitsuaria* isolates exhibited strong disease suppression against damping off pathogens on soybean and tomato plants. The genome of one of these isolates *Mitsuaria* sp. H24L5A was further sequenced and analyzed and found to harbor genes related to the production of the enzymes chitinase, chitosanase, cellulases, and polygalacturonase (175).

The phylogenetic analyses based on the sequences of the 16S rRNA gene and/or protein-coding housekeeping genes revealed that TWR114 and TCR112 were clearly separated from their closest relative type strains (Chapter 1). Furthermore, the data of the comparative genomic analyses clearly show that TWR114 and TCR112 isolates are well separated from their relative type strains (Chapter 2), further indicating that these isolates may be a representative of a new species. Recently, many researchers have used similar bioinformatics approach that led to the successful identification of several novel bacterial species (29, 121, 124) including *Mitsuaria noduli* sp. nov., (55).

Conclusion and perspectives

We successfully developed a practical biocontrol strategy for tomato bacterial wilt using two biocontrol bacteria *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112. These findings offer great opportunities for the future development and commercialization of new and practical plant protection products based on our biocontrol isolates for controlling tomato bacterial wilt.

The isolates TWR114 and TCR112 exhibited *in vitro* antibacterial activity towards the pathogen (Chapter 1). Therefore, developing mutant isolates lacking antibacterial activity would still be necessary to further support the role of antibiosis as a biocontrol mechanism. Moreover, additional analysis and characterization of the antimicrobial compounds produced by these isolates might also provide a better understanding of the role of antibiosis in the suppression of tomato bacterial wilt.

The biocontrol isolates TWR114 and TCR112 were capable of colonizing the inside stem tissues of tomato plants (Chapters 1 and 2). Thus, the colonization pattern in tomatoes can be better explored and visualized by developing green fluorescent protein-labeled TWR114 and TCR112 that can be examined using confocal laser scanning microscopy.

Based on the analysis of defense-related genes studies, the treatment with TWR114 and TCR112 isolates might activate different signaling pathways resulting in the induction of resistance in tomato plant (Chapter 3). However, more comprehensive studies are still required to better understand the involvement of the SA-, ET- and ABA-dependent signaling pathways in the TWR114- and TCR112-mediated ISR by using tomato mutant lines impaired in phytohormone biosynthesis.

In addition, we propose that future studies investigate changes in the global gene expression profile using transcriptomic approach such as microarray analysis.

We used MiSeq system to sequence the whole genome of our isolates, and then performed *de novo* assembly using velvet (Chapter 3). However, in order to obtain a complete genome, a hybrid sequencing strategy should be considered. For instance, the short reads obtained from MiSeq can be used in conjunction with a third generation sequencing such as PacBio sequencing technology to obtain long reads (172).

References

1. Abo-Elyousr, K. A., Ibrahim, Y. E., and Balabel, N. M. (2012). Induction of Disease Defensive Enzymes in Response to Treatment with acibenzolar-S-methyl (ASM) and *Pseudomonas fluorescens* Pf2 and Inoculation with *Ralstonia solanacearum* race 3, biovar 2 (phylotype II). J. Phytopathol., 160, 382–389.
2. Adhikari, T. B., and Basnyat, R. C. (1998). Effect of crop rotation and cultivar resistance on bacterial wilt of tomato in Nepal. Can. J. Plant Pathol., 20, 283–287.
3. Ahemad, M., and Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. J. King Saud Uni. Sci., 26, 1–20.
4. Ahn, I. P., Lee, S. W., Kim, M. G., Park, S. R., Hwang, D. J., and Bae, S. C., (2011). Priming by rhizobacterium protects tomato plants from biotrophic and necrotrophic pathogen infections through multiple defense mechanisms. Mol. Cells, 32, 7–14.
5. Aimé, S., Alabouvette, C., Steinberg, C., and Olivain, C. (2013). The endophytic strain *Fusarium oxysporum* Fo47: a good candidate for priming the defense responses in tomato roots. Mol. Plant-Microbe Interact., 26, 918–926.
6. Aino, M., Maekawa, Y., and Mayama, S. (1996). Selection of biocontrol agent against tomato bacterial wilt from fluorescent pseudomonads. Ann. Phytopathol. Soc. Jpn., 62, 315 (in Japanese).
7. Akiew, S., and Trevorrow P. (1999). Biofumigation of bacterial wilt. *In*: Magarey, R.C., [eds.,], Proceedings of the First Australasian Soilborne Disease Symposium. Brisbane, Australia: Bureau of Sugar Experiment Stations, 207–8.
8. Aliye, N., Fininsa, C., and Hiskias, Y. (2008). Evaluation of rhizosphere bacterial antagonists for their potential to bioprotect potato (*Solanum tuberosum*) against bacterial wilt (*Ralstonia solanacearum*). Biol. Control, 47, 282-288.

9. Alizadeh, H., Behboudi, K., Ahmadzadeh, M., Javan-Nikkhah, M., Zamioudis, C., Pieterse, C. M. J., and Bakker, P. A. H. M. (2013). Induced systemic resistance in cucumber and *Arabidopsis thaliana* by the combination of *Trichoderma harzianum* Tr6 and *Pseudomonas* sp. Ps14. Biol. Control, 65, 14–23.
10. Álvarez, B., Biosca, E. G., and López, M. M. (2010). On the life of *Ralstonia solanacearum*, a destructive bacterial plant pathogen. *In*: Mendez-Vilas, A., [eds.], Current research, technology and education topics in applied microbiology and microbial biotechnology, 1, pp. 267–279. Formatex, Badajoz, Spain.
11. Alves, A. O., Santos, M. M. B., Santos, T. C. G., Souza, E. B., and Mariano, R. L. R. (2014). Biofumigation with essential oils for managing bacterial wilt of sweet peppers. J. Plant Pathol., 96, 363–367.
12. Amakata, D., Matsuo, Y., Shimono, K., Park, J. K., Yun, C. S., Matsuda, H., Yokota, A., and Kawamukai, M. (2005). *Mitsuaria chitosanitabida* gen. nov., sp. nov., and aerobic, chitosanase-producing member of the “Betaproteobacteria”. Int. J. Syst. Evol. Microbiol., 55, 1927–1932.
13. Anith, K. N., Momol, M. T., Kloepper, J. W., Marois, J. J., Olson, S. M., and Jones, J. B. (2004). Efficacy of plant growth-promoting rhizobacteria, acibenzolar-S-methyl, and soil amendment for integrated management of bacterial wilt on tomato. Plant Dis., 88, 669–673.
14. Atherton, J., and Rudich, J. [eds.]. (2012). The tomato crop: a scientific basis for improvement. Springer Science & Business Media.
15. Atlas, R. M., Brown, A. E., and Parks, L. C. (1995). Experimental microbiology: Laboratory manual. St. Louis, USA, Mosby.

16. Auch, A. F., Jan, M., Klenk, H. P., and Göker, M. (2010). Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. Standards in Genomic Sciences, 2, 117.
17. Ayana, G., Fininsa, C., Ahmed, S., and Wydra, K. (2011). Effects of soil amendment on bacterial wilt caused by *Ralstonia solanacerum* and tomato yields in Ethiopia. J. Plant Prot. Res., 51, 72–76.
18. Bakker, P. A., Van Peer, R. O. N., and Schippers, B. (1991). Suppression of soil-borne plant pathogens by fluorescent pseudomonads: mechanisms and prospects. *In*: Beemster, A. B. R., Bollen, G. J., Gerlagh, M., Ruissen, M. A., Schippers B., and Tempel, A. [eds.], Biotic Interactions and Soil-borne Diseases - Proceedings of the First Conference of the European Foundation for Plant Pathology (Developments in Agricultural and Managed forest Ecology 23), pp. 217–230. Elsevier, Amsterdam, Netherlands.
19. Bakker, P. A., Pieterse, C. M., and Van Loon, L. C. (2007). Induced systemic resistance by fluorescent *Pseudomonas* spp. Phytopathol., 97, 239–243.
20. Bardas, G. A., Lagopodi, A. L., Kadoglidou, K., and Tzavella-Klonari, K. (2009). Biological control of three *Colletotrichum lindemuthianum* races using *Pseudomonas chlororaphis* PCL1391 and *Pseudomonas fluorescens* WCS365. Biol. Control., 49, 139–145
21. Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A., and Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. Science, 315, 1709–1712.

22. Barretti, P. B., de Souza, R. M., Pozza, E. A., and de Souza, J. T. (2012). Combination of endophytic bacteria and resistant cultivars improves control of Ralstonia wilt of tomato. Australasian Plant Pathol., 41, 189–195.
23. Benítez, M. S., and McSpadden Gardener, B. B. (2009). Linking sequence to function in soil bacteria: Sequence-directed isolation of novel bacteria contributing to soilborne plant disease suppression. Appl. Environ. Microbiol., 75, 915–924.
24. Berg, G., Eberl, L., and Hartmann, A. (2005). The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. Environ. Microbiol., 7, 1673–1685.
25. Bocsanczy, A. M., Huguet-Tapia, J. C., and Norman, D. J. (2017). Comparative Genomics of *Ralstonia solanacearum* Identifies Candidate Genes Associated with Cool Virulence. Front. Plant Science, 8, 1565.
26. Boukaew, S., Chuenchit, S., and Petcharat, V. (2011). Evaluation of Streptomyces spp. for biological control of *Sclerotium* root and stem rot and *Ralstonia* wilt of chili pepper. BioControl, 56, 365–374.
27. Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T. L. (2009). BLAST+: architecture and applications. BMC bioinformatics, 10, 421.
28. Cao, Y., Pi, H., Chandrangsu, P., Li, Y., Wang, Y., Zhou, H., Xiong, H., Helmann, J. D., and Cai, Y. (2018). Antagonism of Two Plant-Growth Promoting *Bacillus velezensis* Isolates Against *Ralstonia solanacearum* and *Fusarium oxysporum*. Sci. rep., 8, 4360.
29. Castejon, M., Menéndez, M. C., Comas, I., Vicente, A., and Garcia, M. J. (2018). Whole-genome sequence analysis of the *Mycobacterium avium* complex and

proposal of the transfer of *Mycobacterium yongonense* to *Mycobacterium intracellulare* subsp. *yongonense* subsp. nov. Int. J. Syst. Evol. Microbiol.

30. Castillo, J. A., and Greenberg, J. T. (2007). Evolutionary dynamics of *Ralstonia solanacearum*. Appl. Environ. Microbiol., 73, 1225–1238.
31. Chaudhari, N. M., Gupta, V. K., and Dutta, C. (2016). BPGA-an ultra-fast pan-genome analysis pipeline. Sci. Rep., 6, 24373.
32. Chen, C., Bauske, E. M., Musson, G., Rodriguezkabana, R., and Kloepper, J. W. (1995). Biological Control of Fusarium Wilt on Cotton by Use of Endophytic Bacteria. Biol. Control, 5, 83–91.
33. Chen, D., Liu, X., Li, C., Tian, W., Shen, Q., and Shen, B. (2014). Isolation of *Bacillus amyloliquefaciens* S20 and its application in control of eggplant bacterial wilt. J. Environ. Manage., 137, 120–127.
34. Chen, Y. Y., Lin, Y. M., Chao, T. C., Wang, J. F., Liu, A. C., Ho, F. I., and Cheng, C. P. (2009). Virus-induced gene silencing reveals the involvement of ethylene-, salicylic acid-and mitogen-activated protein kinase-related defense pathways in the resistance of tomato to bacterial wilt. Physiologia Plantarum, 136, 324–335.
35. Christensen, W. B. (1946). Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. J. Bacteriol., 52, 461.
36. Chun, B. H., Kim, K. H., Jeon, H. H., Lee, S. H., and Jeon, C. O. (2017). Pan-genomic and transcriptomic analyses of *Leuconostoc mesenteroides* provide insights into its genomic and metabolic features and roles in kimchi fermentation. Sci. Rep., 7, 11504.

37. Chunyu, L., Weicong, H., Bin, P., Yan, L., Saifei, Y., Yuanyuan, D., Rong, L., Xinyan, Z., Biao, S., and Qirong, S., (2017). Rhizobacterium *Bacillus amyloliquefaciens* strain SQRT3-mediated induced systemic resistance controls bacterial wilt of tomato. Pedosphere, 27, 1135–1146.
38. Clough, S. J., Flavier, A. B., Schell, M. A., and Denny, T. P. (1997). Differential expression of virulence genes and motility in *Ralstonia (Pseudomonas) solanacearum* during exponential growth. Appl. Environ. Microbiol., 63, 844–850.
39. Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl. Environ. Microbiol., 71, 4951–4959.
40. Conrath, U., Beckers, G. J., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M. A., Pieterse, C. M., Poinssot, B., Pozo, M. J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L., and Mauch-Mani, B. (2006). Priming: getting ready for battle. MPMI, 19, 1062–1071.
41. Delcher, A. L., Harmon, D., Kasif, S., White, O., and Salzberg, S. L. (1999). Improved microbial gene identification with GLIMMER. Nucleic Acids Res., 27, 4636–4641.
42. Denny, T. (2007). Plant pathogenic *Ralstonia* species. *In*: Gnanamanickam, S. S. [ed.], Plant-Associated Bacteria, pp. 573–644. Springer, Netherlands.
43. Dhillon, B. K., Laird, M. R., Shay, J. A., Winsor, G. L., Lo, R., Nizam, F., Pereira, S. K., Waglechner, N., McArthur, A. G., Langille, M. G. and Brinkman, F. S. (2015). IslandViewer 3: more flexible, interactive genomic island discovery, visualization and analysis. Nucleic Acids Res., 43, W104–W108.

44. Ding, C., Shen, Q., Zhang, R., and Chen, W. (2013). Evaluation of rhizosphere bacteria and derived bio-organic fertilizers as potential biocontrol agents against bacterial wilt (*Ralstonia solanacearum*) of potato. Plant Soil, 366, 453-–466.
45. Dobrindt, U., Hochhut, B., Hentschel, U., and Hacker, J. (2004). Genomic islands in pathogenic and environmental microorganisms. Nature Rev. Microbiol., 2, 414.
46. Domenech, J., Reddy, M. S., Kloepper, J. W., Ramos, B., and Gutierrez-Manero, J. (2006). Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato. BioControl, 51, 245–258.
47. Doumbou, C. L., Akimov, V., and Beaulieu, C. (1998). Selection and Characterization of Microorganisms Utilizing Thaxtomin A, a Phytotoxin Produced by *Streptomyces scabies*. Appl. Environ. Microbiol. 64, 4313–4316.
48. Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., Mukherjee, P. K., Zeilinger, S., Grigoriev, I. V. and Kubicek, C. P. (2011). Trichoderma: the genomics of opportunistic success. Nature Rev. Microbiol., 9, 749.
49. Dunlap, C. A., Schisler, D. A., Bowman, M. J., and Rooney, A. P. (2015). Genomic analysis of *Bacillus subtilis* OH 131.1 and co-culturing with *Cryptococcus flavescens* for control of Fusarium head blight. Plant Gene, 2, 1–9.
50. Dunne, C., Moënne-Loccoz, Y., McCarthy, J., Higgins, P., Powell, J., Dowling, D. N., and O'gara, F. (1998). Combining proteolytic and phloroglucinol-producing bacteria for improved biocontrol of *Pythium*-mediated damping-off of sugar beet. Plant Pathol., 47, 299–307.

51. Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. Bioinformatics, 26, 2460–2461.
52. Elhalag, K. M., Messiha, N. A. S., Emara, H. M., and Abdallah, S. A., (2016). Evaluation of antibacterial activity of *Stenotrophomonas maltophilia* against *Ralstonia solanacearum* under different application conditions. J. Appl. Microbiol., 120, 1629–1645.
53. Elphinstone J.G. (2005). The current bacterial wilt situation: a global overview. *In*: Allen C., Piror P., and Hayward A. C. [eds.,], Bacterial wilt disease and the *Ralstonia solanacearum* complex, pp. 9–28. APS press, Minnesota USA.
54. Etchebar, C., Trigalet-Demery, D., van Gijsegem, F., Vasse, J., and Trigalet, A. (1998). Xylem colonization by an HrcV mutant of *Ralstonia solanacearum* is a key factor for the efficient biological control of tomato bacterial wilt. MPMI, 11, 869–877.
55. Fan, M. C., Nan, L. J., Zhu, Y. M., Chen, W. M., Wei, G. H., and Lin, Y. B. (2017). *Mitsuaria noduli* sp. nov., isolated from the root nodules of *Robinia pseudoacacia* in a lead–zinc mine. Int. J. Syst. Evol. Microbiol., 68, 87–92.
56. FAOSTAT (2016). FAOSTAT. Food Agric. Organ. United Nations. http://www.fao.org/faostat/en/#data/QC (accessed 30 June 2017).
57. Fegan, M., and Prior, P. (2005). How complex is the *Ralstonia solanacearum* species complex. *In*: Allen, C., Prior, P., and Hayward, A. C., [eds.], Bacterial wilt disease and the *Ralstonia solanacearum* species complex, pp. 449–461. APS press, St. Paul, Minnesota USA.
58. Feng, D. X., Tasset, C., Hanemian, M., Barlet, X., Hu, J., Trémousaygue, D., Deslandes, L., and Marco, Y. (2012). Biological control of bacterial wilt in

Arabidopsis thaliana involves abscissic acid signalling. New Phytol., 194, 1035–1045.

59. Fock, I., Collonnier, C., Purwito, A., Luisetti, J., Souvannavong, V., Vedel, F., Servaes, A., Ambroise, A., Kodja, H., Ducreux, G., and Sihachakr, D. (2000). Resistance to bacterial wilt in somatic hybrids between *Solanum tuberosum* and *Solanum phureja*. Plant Sci., 160, 165-176.
60. Fravel, D. R. (1988). Role of antibiosis in the biocontrol of plant diseases. Ann. Rev. Phytopathol., 26, 75–91.
61. French, E. R., Gutarra, L., Aley, P., and Elphinstone, J. (1995). Culture media for *Pseudomonas solanacearum* isolation, identification and maintenance. Fitopatologia, 30, 126–130.
62. Garbeva, P., Van Elsas, J. D., and De Boer, W. (2012). Draft genome sequence of the antagonistic rhizosphere bacterium *Serratia plymuthica* strain PRI-2C. J. Bacteriol., 194, 4119–4120.
63. Garrity, G. M. (2016). A new genomics-driven taxonomy of bacteria and archaea: are we there yet?. J. Clin. Microbiol., 54, 1956–1963.
64. Ghareeb, H., Bozsó, Z., Ott, P. G., Repenning, C., Stahl, F., and Wydra, K. (2011). Transcriptome of silicon-induced resistance against *Ralstonia solanacearum* in the silicon non-accumulator tomato implicates priming effect. Physiol. Mol. Plant Pathol., 75, 83–89.
65. Glaeser, S.P., and Kämpfer, P., (2015). Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. Syst. Appl. Microbiol., 38, 237–245.
66. Glazebrook, J., (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol., 43, 205–227.

67. Gomila, M., Bowien, B., Falsen, E., Moore, E. R. B., and Lalucat, J. (2007). Description of *Pelomonas aquatica* sp. nov. and *Pelomonas puraquae* sp. nov., isolated from industrial and haemodialysis water. Int. J. Syst. Evol. Microbiol., 57, 2629–2635.

68. Grissa, I., Vergnaud, G., and Pourcel, C. (2007). CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. Nucleic Acids Res., 35, W52–W57.

69. Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol., 59, 307–321.

70. Haas, D., and Keel, C. (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Ann. Rev. Phytopathol., 41, 117–153.

71. Harvás, A., Landa, B., and Jiménez-Díaz, R. M. (1997). Influence of chickpea genotype and *Bacillus* sp. on protection from Fusarium wilt by seed treatment with nonpathogenic *Fusarium oxysporum*. Eur. J. Plant Pathol., 103, 631–642.

72. Hase, S., Shimizu, A., Nakaho, K., Takenaka, S., and Takahashi, H. (2006). Induction of transient ethylene and reduction in severity of tomato bacterial wilt by *Pythium oligandrum*. Plant Pathol., 55, 537–543.

73. Hase, S., Takahashi, S., Takenaka, S., Nakaho, K., Arie, T., Seo, S., Ohashi, Y., and Takahashi, H. (2008). Involvement of jasmonic acid signalling in bacterial wilt disease resistance induced by biocontrol agent *Pythium oligandrum* in tomato. Plant Pathol., 57, 870–876.

74. Hassan, M. A. E., and Buchenauer, H. (2008). Enhanced control of bacterial wilt of tomato by DL-3-aminobutyric acid and the fluorescent *Pseudomonas* isolate CW2. J. Plant Dis. Prot., 115, 199–207.

75. Hayward, A.C. (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol., 29, 65–87.

76. Hendrick, C. A., and Sequeira, L. (1984). Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. Appl. Environ. Microbiol., 48, 94–101.

77. Hiddink, G. A., Termorshuizen, A. J., and van Bruggen, A. H. (2010). Mixed cropping and suppression of soilborne diseases. *In*: Lichtfouse, E. [ed.], Genetic Engineering, Biofertilisation, Soil Quality and Organic Farming, pp. 119–146. Springer, Dordrecht.

78. Hu, H. Q., Li, X. S., and He, H. (2010). Characterization of an antimicrobial material from a newly isolated *Bacillus amyloliquefaciens* from mangrove for biocontrol of Capsicum bacterial wilt. Biol. control, 54, 359–365.

79. Huang, C. N., Lin, C. P., Hsieh, F. C., Lee, S. K., Cheng, K. C., and Liu, C. T. (2016). Characterization and evaluation of *Bacillus amyloliquefaciens* strain WF02 regarding its biocontrol activities and genetic responses against bacterial wilt in two different resistant tomato cultivars. World J. Microbiol. Biotechnol., 32, 183.

80. Huang, J., Wei, Z., Tan, S., Mei, X., Yin, S., Shen, Q., and Xu, Y. (2013). The rhizosphere soil of diseased tomato plants as a source for novel microorganisms to control bacterial wilt. Appl. Soil Ecol., 72, 79–84.

81. Huang, J., Wei, Z., Tan, S., Mei, X., Shen, Q., and Xu, Y. (2014). Suppression of bacterial wilt of tomato by bioorganic fertilizer made from the antibacterial compound producing strain *Bacillus amyloliquefaciens* HR62. J. Agric. Food Chem., 62, 10708–10716.
82. Huang, J., Wei, Z., Hu, J., Yang, C., Gu, Y. A., Mei, X., Shen, Q., Xu, Y., and Riaz, K. (2017). *Chryseobacterium nankingense* sp. nov. WR21 effectively suppresses *Ralstonia solanacearum* growth via intensive root exudates competition. BioControl, 62, 567–577.
83. Huang, Q., and Allen, C. (2000). Polygalacturonases are required for rapid colonization and full virulence of *Ralstonia solanacearum* on tomato plants. Physiol. Mol. Plant Pathol., 57, 77–83.
84. Huang, X. F., Zhou, D., Lapsansky, E. R., Reardon, K. F., Guo, J., Andales, M. J., Vivanco, J. M., and Manter, D. K. (2017). *Mitsuaria* sp. and *Burkholderia* sp. from Arabidopsis rhizosphere enhance drought tolerance in *Arabidopsis thaliana* and maize (*Zea mays* L.). Plant Soil, 419, 523–539.
85. Hyakumachi, M., Nishimura, M., Arakawa, T., Asano, S., Yoshida, S., Tsushima, S., and Takahashi, H. (2013). *Bacillus thuringiensis* suppresses bacterial wilt disease caused by *Ralstonia solanacearum* with systemic induction of defense-related gene expression in tomato. Microbes Environ., 28, 128–134.
86. Ikeda, K., Toyota, K., and Kimura, M. (1998). Role of extracellular pectinases in the rhizoplane competence of a rhizobacterium *Burkholderia pickettii* MSP3Rif. Soil Biol. Biochem., 30, 323–329.

87. Ishikawa, R., Fujimori, K., and Matsuura, K. (1996). Antibacterial Activity of Validamycin A against *Pseudomonas solanacearum* and Its Efficacy against Tomato Bacterial Wilt. Japanese J. Phytopathol., 62, 478–482.

88. Jetiyanon, K., and Kloepper, J. W. (2002). Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. Biol. control, 24, 285–291.

89. Jetiyanon, K., Fowler, W. D., and Kloepper, J. W. (2003). Broad-spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. Plant Dis., 87, 1390–1394.

90. Jetiyanon, K. (2007). Defensive-related enzyme response in plants treated with a mixture of *Bacillus* strains (IN937a and IN937b) against different pathogens. Biol. control, 42, 178–185.

91. Ji, P., Momol, M. T., Olson, S. M., Pradhanang, P. M., and Jones, J. B. (2005). Evaluation of thymol as biofumigant for control of bacterial wilt of tomato under field conditions. Plant Dis., 89, 497–500.

92. Ji, X., Lu, G., Gai, Y., Zheng, C., and Mu, Z. (2008). Biological control against bacterial wilt and colonization of mulberry by an endophytic *Bacillus subtilis* strain. FEMS Microbiol. Ecol., 65, 565–573.

93. Jimtha, J. C., Smitha, P. V., Anisha, C., Deepthi, T., Meekha, G., Radhakrishnan, E. K., Gayatri, G. P., and Remakanthan, A. (2014). Isolation of endophytic bacteria from embryogenic suspension culture of banana and assessment of their plant growth promoting properties. Plant Cell Tissue Organ Cult., 118, 57–66.

94. Jogaiah, S., Abdelrahman, M., Tran, L. S. P., and Shin-ichi, I. (2013). Characterization of rhizosphere fungi that mediate resistance in tomato against bacterial wilt disease. J. Exp. Bot., 64, 3829-3842.

95. Katafiire, M., Adipala, E., Lemaga, B., Olanya, M., El-Bedewy, R., and Ewell, P. (2005). Management of bacterial wilt of potato using one-season rotation crops in southwestern Uganda. *In:* Allen, C., Prior, P., and Hayward, A. C. [eds.], Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex, pp. 197–204. APS Press, St. Paul, MN, USA.

96. Kempe, J., and Sequeira, L. (1983). Biological control of bacterial wilt of potatoes: attempts to induce resistance by treating tubers with bacteria. Plant Dis., 67, 499–503.

97. Kheirandish, Z., and Harighi, B. (2015). Evaluation of bacterial antagonists of *Ralstonia solanacearum*, causal agent of bacterial wilt of potato. Biol. Control, 86, 14–19.

98. Kim, Y., Koh, I., Lim, M. Y., Chung, W. H., and Rho, M. (2017). Pan-genome analysis of Bacillus for microbiome profiling. Sci. Rep., 7, 10984.

99. Kloepper, J. W., Ryu, C. M., and Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathol., 94, 1259–1266.

100. Kloepper, J. W., and Ryu, C. M. (2006). Bacterial endophytes as elicitors of induced systemic resistance. *In*: Schulz, B. J. E., Boyle, C. J. C., Sieber, T. N. [eds.], Microbial root endophytes, pp. 33–52. Springer, Verlag Berlin, Heidelberg, Germany.

101. Konstantinidis, K. T., and Tiedje, J. M. (2005). Genomic insights that advance the species definition for prokaryotes. Proc. Nat. Acad. Sci. U.S.A., 102, 2567–2572.

102. Konstantinidis, K. T., Ramette, A., and Tiedje, J. M. (2006). Toward a more robust assessment of intraspecies diversity, using fewer genetic markers. Appl. Environ. Microbiol., 72, 7286–7293.

103. Koonin, E. V., Makarova, K. S., and Aravind, L. (2001). Horizontal gene transfer in prokaryotes: quantification and classification. Annu. Rev. Microbiol., 55, 709–742.

104. Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol., 33, 1870–1874.

105. Kurabachew, H., and Wydra, K. (2013). Characterization of plant growth promoting rhizobacteria and their potential as bioprotectant against tomato bacterial wilt caused by *Ralstonia solanacearum*. Biol. Control, 67, 75–83.

106. Lai, R., You, M., Jiang, L., Lai, B., Chen, S., Zeng, W., and Jiang, D. (2011). Evaluation of garlic intercropping for enhancing the biological control of *Ralstonia solanacearum* in flue-cured tobacco fields. Biocontrol Sci. Technol., 21, 755–764.

107. Lane, D. J. (1991). 16S/23S rRNA sequencing. *In*: Stackebrandt, E., Goodfellow, M. [eds.], Nucleic acid techniques in bacterial systematics, pp. 115–175. John Wiley Sons, New York, USA.

108. Langille, M. G., Hsiao, W. W., and Brinkman, F. S. (2010). Detecting genomic islands using bioinformatics approaches. Nature Rev. Microbiol., 8, 373.

109. Laslo, É., György, É., Ábrahám, B., and Mara, G. (2017). Bacterial Strains with Nutrient Mobilisation Ability from Ciuc Mountains (Transylvania Region, Romania). *In*: Singh, D. P., Singh, H. B., and Prabha, R. [eds.], Plant-Microbe Interactions in Agro-Ecological Perspectives, 1, pp. 531–548. Springer, Singapore.

110. Lee, J. M., Kubota, C., Tsao, S. J., Bie, Z., Echevarria, P. H., Morra, L., and Oda, M. (2010). Current status of vegetable grafting: Diffusion, grafting techniques, automation. Sci. Hortic., 127, 93–105.

111. Lefort, F., Calmin, G., Pelleteret, P., Farinelli, L., Osteras, M., and Crovadore, J. (2014). Whole-genome shotgun sequence of Bacillus amyloliquefaciens strain UASWS BA1, a bacterium antagonistic to plant pathogenic fungi. Genome announcements, 2, e00016-14.

112. Lemessa, F., and Zeller, W. (2007). Screening rhizobacteria for biological control of *Ralstonia solanacearum* in Ethiopia. Biol. Control, 42, 336–344.

113. Li, J. G., and Dong, Y. H. (2013). Effect of a rock dust amendment on disease severity of tomato bacterial wilt. Antonie van Leeuwenhoek, 103, 11–22.

114. Lin, W. C., Lu, C. F., Wu, J. W., Cheng, M. L., Lin, Y. M., Yang, N. S., Black, L., Green, S. K., Wang, J. F., and Cheng, C. P. (2004). Transgenic tomato plants expressing the Arabidopsis NPR1 gene display enhanced resistance to a spectrum of fungal and bacterial diseases. Transgenic Res., 13, 567-581.

115. Lin, Y., He, Z., Rosskopf, E. N., Conn, K. L., Powell, C. A., and Lazarovits, G. (2010). A nylon membrane bag assay for determination of the effect of chemicals on soilborne plant pathogens in soil. Plant Dis., 94, 201–206.

116. Liu, H. X., Li, S. M., Luo, Y. M., Luo, L. X., Li, J. Q., and Guo, J. H. (2014). Biological control of Ralstonia wilt, Phytophthora blight, Meloidogyne root-knot on bell pepper by the combination of *Bacillus subtilis* AR12, *Bacillus subtilis* SM21 and *Chryseobacterium* sp. R89. European J. Plant Pathol., 139, 107–116.

117. Liu, Y., Shi, J., Feng, Y., Yang, X., Li, X., and Shen, Q. (2013). Tobacco bacterial wilt can be biologically controlled by the application of antagonistic strains in combination with organic fertilizer. Biol. Fert. Soils., 49, 447–464.

118. Livak, K. J., and Schmittgen, T., D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods, 25, 402–408.

119. Loper, J. E., Kobayashi, D. Y., and Paulsen, I. T. (2007). The genomic sequence of *Pseudomonas fluorescens* Pf-5: insights into biological control. Phytopathol., 97, 233–238.

120. Loper, J. E., Hassan, K. A., Mavrodi, D. V., Davis II, E. W., Lim, C. K., Shaffer, B. T., Elbourne, L. D., Stockwell, V. O., Hartney, S. L., Breakwell, K. and Henkels, M. D. (2012). Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. PLoS Genetics, 8, e1002784.

121. López, M. M., Lopez-Soriano, P., Garita-Cambronero, J., Beltrán, C., Taghouti, G., Portier, P., Cubero, J., Fischer-Le Saux, M. and Marco-Noales, E. (2018). *Xanthomonas prunicola* sp. nov., a novel pathogen that affects nectarine (*Prunus persica* var. *nectarina*) trees. Int. J. Syst. Evol. Microbiol.

122. Lucas, J. A., Solano, B. R., Montes, F., Ojeda, J., Megias, M., and Mañero, F. G. (2009). Use of two PGPR strains in the integrated management of blast disease in rice (*Oryza sativa*) in Southern Spain. Field Crop. Res., 114, 404–410.

123. Ma, L., Zhang, H. Y., Zhou, X. K., Yang, C. G., Zheng, S. C., Duo, J. L., and Mo, M. H. (2018). Biological control tobacco bacterial wilt and black shank and root colonization by bio-organic fertilizer containing bacterium *Pseudomonas aeruginosa* NXHG29. Appl. Soil Ecol., 129, 136–144.

124. Machado, R. A., Wüthrich, D., Kuhnert, P., Arce, C. C., Thönen, L., Ruiz, C., Zhang, X., Robert, C. A., Karimi, J., Kamali, S. and Ma, J. (2018). Whole-genome-based revisit of *Photorhabdus* phylogeny: proposal for the elevation of most *Photorhabdus* subspecies to the species level and description of one novel species *Photorhabdus bodei* sp. nov., and one novel subspecies *Photorhabdus laumondii* subsp. *clarkei* subsp. nov. Int. J. Syst. Evol. Microbiol.

125. MAFF (2015). Ministry of Agriculture, Forestry and Fisheries of Japan. http://www.maff.go.jp/e/data/stat/index.html (accessed 30 June 2018)

126. Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S. V, Machado, M. A., Toth, I., Salmond, G., and Foster, G. D. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. Mol. Plant Pathol., 13, 614–629.

127. Mao, L., Wang, Q., Yan, D., Ma, T., Liu, P., Shen, J., Li, Y., Ouyang, C., Guo, M., and Cao, A. (2014). Evaluation of chloropicrin as a soil fumigant against *Ralstonia solanacarum* in ginger (*Zingiber officinale* Rosc.) production in China. PloS one, 9, e91767.

128. Mao, L., Jiang, H., Wang, Q., Yan, D., and Cao, A. (2017). Efficacy of soil fumigation with dazomet for controlling ginger bacterial wilt (*Ralstonia solanacearum*) in China. Crop Prot., 100, 111-116.

129. Martínez-Medina, A., Fernández, I., Sánchez-Guzmán, M. J., Jung, S. C., Pascual, J. A., and Pozo, M. J. (2013). Deciphering the hormonal signalling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato. . Front Plant Sci., 4, 206.

130. Mauch-Mani, B., Baccelli, I., Luna, E., and Flors, V. (2017). Defense priming: an adaptive part of induced resistance. Ann. Rev. Plant Biol., 68, 485–512.

131. McLaughlin, R. J., and Sequeira, L. (1988). Evaluation of an avirulent strain of *Pseudomonas solanacearum* for biological control of bacterial wilt of potato. Am. Potato J., 65, 255–268.

132. Medini, D., Donati, C., Tettelin, H., Masignani, V., and Rappuoli, R. (2005). The microbial pan-genome. Curr. Opin. Genetics Dev., 15, 589–594.

133. Meier-Kolthoff, J. P., Auch, A. F., Klenk, H. P., and Göker, M. (2013). Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics, 14, 60.

134. Michel, V. V., Hartman, G. L., and Midmore, D. J. (1996). Effect of previous crop on soil populations of *Burkholderia solanacearum*, bacterial wilt, and yield of tomatoes in Taiwan. Plant Dis., 80, 1367-1372.

135. Michel, V. V., Wang, J. F., Midmore, D. J., and Hartman, G. L. (1997). Effects of intercropping and soil amendment with urea and calcium oxide on the incidence of bacterial wilt of tomato and survival of soil-borne *Pseudomonas solanacearum* in Taiwan. Plant Pathol., 46, 600–610.

136. Milling, A., Babujee, L., and Allen, C. (2011). *Ralstonia solanacearum* extracellular polysaccharide is a specific elicitor of defense responses in wilt-resistant tomato plants. PLoS One, 6, e15853.

137. Momma, N. (2008). Biological soil desinfestation (BSD) of soilborne pathogens and its possible mechanisms. Japan Agric. Res., Q. 42, 7–12.

138. Mulya, K., Watanabe, M., Goto, M., Takikawa, Y., and Tsuyumu, S. (1996). Suppression of Bacterial Wilt Disease of Tomato by Root-dipping with *Pseudomonas fluorescens* PfG32. Japanese J. Phytopathol., 62, 134–140.

139. Myresiotis, C. K., Karaoglanidis, G. S., Vryzas, Z., and Papadopoulou-Mourkidou, E. (2012). Evaluation of plant-growth-promoting rhizobacteria, acibenzolar-S-methyl and hymexazol for integrated control of Fusarium crown and root rot on tomato. Pest Manage. Sci., 68, 404–411.

140. Nakaho, K., Inoue, H., and Takayama, T. (2004). Distribution and multiplication of *Ralstonia solanacearum* in tomato plants with resistance derived from different origins. J. Gen. Plant Pathol., 70, 115–119.

141. Nakaho, K., and Allen, C. (2009). A Pectinase-deficient *Ralstonia solanacearum* Strain Induces Reduced and Delayed Structural Defences in Tomato Xylem. J. Phytopathol. 157, 228–234.

142. Neilands, J. B. (1981). Microbial iron compounds. Annu. Rev. Biochem., 50, 715–731.

143. Neilands, J. B., Konopa, K., Schwyn, B., Coy, M., Francis, R. T., Paw, B. H., and Bagg, A. (1987). Comparative biochemistry of microbial iron assimilation. *In*: Winkelman, G., van der Helm, D., and Neilands, J. B. [eds], Iron transport in microbes, plants and animals, pp. 3–33. VCH Publishers, Weinheim, Germany.

144. Neupane, S., Finlay, R. D., Kyrpides, N. C., Goodwin, L., Alström, S., Lucas, S., Land, M., Han, J., Lapidus, A., Cheng, J. F. and Bruce, D. (2012). Complete genome sequence of the plant-associated *Serratia plymuthica* strain AS13. Stand. Genomic Sci., 7, 22.

145. Nion, Y. A., and Toyota, K. (2008). Suppression of bacterial wilt and Fusarium wilt by a *Burkholderia nodosa* strain isolated from Kalimantan soils, Indonesia. Microbes Environ., 23, 134–141.

146. Nishioka T., Suzuki Y., Suga H., Kobayashi I., Kobayashi Y., Hyakumachi M., and Shimizu M. (2016). Metagenomic analysis of bacterial flora in rhizospheres of *Allium* plants and suppressive activity of rhizopseudomonas isolates against Fusarium wilt of cucumber. IOBC-WPRS Bull., 115, 107–111.

147. Nishioka, T., Elsharkawy, M. M., Suga, H., Kageyama, K., Hyakumachi, M., and Shimizu, M. (2016). Development of culture medium for the Isolation of *Flavobacterium* and *Chryseobacterium* from rhizosphere soil. Microbes Environ., 31, 104–110.

148. Niu, D. D., Wang, C. J., Guo, Y. H., Jiang, C. H., Zhang, W. Z., Wang, Y. P., and Guo, J. H. (2012). The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces resistance in tomato with induction and priming of defence response. Biocontrol Sci. Technol., *22*, 991–1004.

149. Noguchi, H., Taniguchi, T., and Itoh, T. (2008). MetaGeneAnnotator: detecting species-specific patterns of ribosomal binding site for precise gene prediction in anonymous prokaryotic and phage genomes. DNA research, 15, 387-396.

150. Pal, K. K., and Gardener, B. M. (2006). Biological control of plant pathogens. The Plant Health Instructor, 2, 1117-1142.

151. Pallen, M. J., Beatson, S. A., and Bailey, C. M. (2005). Bioinformatics, genomics and evolution of non-flagellar type-III secretion systems: a Darwinian perpective. FEMS Microbiol. Rev., 29, 201–229.

152. Pan, L.Y. (1990). Effect of Welsh Onion on Bacterial Wilt of Tomato and Survival of *Pseudomonas solanacearum* in Soils. (Master's thesis, National Chung Hsing University, Taichung, Taiwan).

153. Paret, M. L., Cabos, R., Kratky, B. A., and Alvarez, A. M. (2010). Effect of plant essential oils on *Ralstonia solanacearum* race 4 and bacterial wilt of edible ginger. Plant Dis., 94, 521–527.

154. Peng, D., Luo, K., Jiang, H., Deng, Y., Bai, L., and Zhou, X. (2017). Combined use of Bacillus subtilis strain B-001 and bactericide for the control of tomato bacterial wilt. Pest Manage. Sci., 73, 1253–1257.

155. Pérez-Miranda, S., Cabirol, N., George-Téllez, R., Zamudio-Rivera, L. S., and Fernández, F. J. (2007). O-CAS, a fast and universal method for siderophore detection. J. Microbiol. Methods, 70, 127–131.

156. Petti, C., Reiber, K., Ali, S. S, and Berney, M. (2012). Auxin as a player in the biocontrol of Fusarium head blight disease of barley and its potential as a disease control agent. BMC Plant Biol., 12, 224.

157. Pierson, E. A., and Weller, D. M. (1994). Use of mixtures of fluorescent *pseudomonads* to Suppress Take-all and Improve the Growth of Wheat. Phytopathol., 84, 940–947.

158. Pieterse, C. M., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. (2009). Networking by small-molecule hormones in plant immunity. Nature Chem. Biol., 5, 308.

159. Pieterse, C. M., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C., and Bakker, P. A. (2014). Induced systemic resistance by beneficial microbes. Ann. Rev. Phytopathol., 52, 347–375.

160. Piombo, E., Sela, N., Wisniewski, M., Hoffmann, M., Gullino, M. L., Allard, M. W., Levin, E., Spadaro, D., and Droby, S. (2018). Genome sequence, assembly and characterization of two Metschnikowia fructicola strains used as biocontrol agents of postharvest diseases. Front. Microbiol., 9, 593.

161. Preston, G. M. (2007). Metropolitan microbes: type III secretion in multihost symbionts. Cell Host Microbe, 2, 291–294.

162. Prior, P., Ailloud, F., Dalsing, B. L., Remenant, B., Sanchez, B., and Allen, C. (2016). Genomic and proteomic evidence supporting the division of the plant pathogen *Ralstonia solanacearum* into three species. BMC Genomics, 17, 90.

163. Quimby, P. C., King, L. R., and Grey, W. E. (2002). Biological control as a means of enhancing the sustainability of crop/land management systems. Agric. Ecosyst. Environ., 88, 147–152.

164. Raaijmakers, J. M., Vlami, M., and De Souza, J. T. (2002). Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek, 81, 537.

165. Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C., and Moënne-Loccoz, Y. (2009). The rhizosphere: A playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil, 321, 341–361.

166. Ramesh, R., Joshi, A. A., and Ghanekar, M. P. (2009). Pseudomonads: Major antagonistic endophytic bacteria to suppress bacterial wilt pathogen, *Ralstonia solanacearum* in the eggplant (*Solanum melongena* L.). World J. Microbiol. Biotechnol., 25, 47–55.

167. Ramesh, R., and Phadke, G. S. (2012). Rhizosphere and endophytic bacteria for the suppression of eggplant wilt caused by *Ralstonia solanacearum*. Crop Prot., 37, 35–41.

168. Ran, L. X., Liu, C. Y., Wu, G. J., Van Loon, L. C., Bakker, P. A. H. M. (2005). Suppression of bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. in China. Biol. Control, 32, 111–120.

169. Ran, L. X, Li, Z. N, Wu, G. J, Van Loon, L. C., and Bakker, P. A. H. M. (2005). Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. Eur. J. Plant Pathol., 113, 59–70.

170. Raupach, G. S., and Kloepper, J. W. (1998). Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathol., 88, 1158–1164.

171. Remenant, B., Coupat-Goutaland, B., Guidot, A., Cellier, G., Wicker, E., Allen, C., Fegan, M., Pruvost, O., Elbaz, M., Calteau, A. and Salvignol, G. (2010). Genomes of three tomato pathogens within the *Ralstonia solanacearum* species complex reveal significant evolutionary divergence. BMC Genomics, 11, 379.

172. Rhoads, A., and Au, K. F. (2015). PacBio sequencing and its applications. Genomics, Proteomics Bioinformatics, 13, 278–289.

173. Richter, M., Rosselló-Móra, R., Oliver Glöckner, F., and Peplies, J. (2015). JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics, 32, 929–931.

174. Roberts, D. P., Lohrke, S. M., Meyer, S. L., Buyer, J. S., Bowers, J. H., Baker, C. J., Li W, de Souza, J. T., Lewis, J. A., and Chung, S. (2005). Biocontrol agents

applied individually and in combination for suppression of soilborne diseases of cucumber. Crop Prot., 24, 141–155.

175. Rong, X., Gurel, F. B., Meulia, T., and Gardener, B. B. M. (2012). Draft genome sequences of the biocontrol bacterium *Mitsuaria* sp. strain H24L5A. J. Bacteriol., 194, 734–735.

176. Russell, A. B., Peterson, S. B., and Mougous, J. D. (2014). Type VI secretion system effectors: poisons with a purpose. Nature Rev. Microbiol., 12, 137.

177. Saddler, G. S., 2005. Management of bacterial wilt disease, *In*: Allen, C., Piror, P., and Hayward, A. C. [eds.,], Bacterial wilt disease and the *Ralstonia solanacearum* complex, pp. 121–132. APS press, Minnesota USA.

178. Safni, I., Cleenwerck, I., De Vos, P., Fegan, M., Sly, L., and Kappler, U. (2014). Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: proposal to emend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify current *R. syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum.* Int. J. Syst. Evol. Microbiol. 64, 3087–3103.

179. Santiago, C. D., Yagi, S., Ijima, M., Nashimoto, T., Sawada, M., Ikeda, S., Asano, K., Orikasa, Y., and Ohwada, T. (2017). Bacterial compatibility in combined inoculations enhances the growth of potato seedlings. Microbes Environ., 32, 14–23.

180. Santiago, T. R., Grabowski, C., Rossato, M., Romeiro, R. S., and Mizubuti, E. S. (2015). Biological control of eucalyptus bacterial wilt with rhizobacteria. Biol. Control, 80, 14–22.

181. Sarma, B. K., Yadav, S. K., Singh, S., and Singh, H. B. (2015). Microbial consortium-mediated plant defense against phytopathogens: readdressing for enhancing efficacy. Soil Biol. Biochem., 87, 25-33.

182. Shen, M., Xia, D., Yin, Z., Zhao, Q., and Kang, Y. (2018). *Bacillus pumilus* WP8 exhibits biocontrol efficacy against tomato bacterial wilt via attenuation of the virulence of the pathogenic bacterium. Acta AGR. SCAND. B Soil Plant Sci., 68, 379–387.

183. Shiomi, Y., Nishiyama, M., Onizuka, T., and Marumoto, T. (1999). Comparison of bacterial community structures in the rhizoplane of tomato plants grown in soils suppressive and conducive towards bacterial wilt. Appl. Environ. Microbiol. 65, 3996–4001

184. Singh, P. P., Shin, Y. C., Park, C. S., and Chung, Y. R. (1999). Biological control of Fusarium wilt of cucumber by chitinolytic bacteria. Phytopathol., 89, 92–99.

185. Singh, R., Kalra, A., Ravish, B. S., Divya, S., Parameswaran, T. N., Srinivas, K. V. N. S., and Bagyaraj, D. J. (2012). Effect of potential bioinoculants and organic manures on root-rot and wilt, growth, yield and quality of organically grown Coleus forskohlii in a semiarid tropical region of Bangalore (India). Plant Pathol., 61, 700–708.

186. Singh, R., Soni, S. K., and Kalra, A. (2013). Synergy between *Glomus fasciculatum* and a beneficial *Pseudomonas* in reducing root diseases and

improving yield and forskolin content in Coleus forskohlii Briq. under organic field conditions. Mycorrhiza, 23, 35–44.

187. Smibert, R. M., and Krieg, N. R. (1994). Phenotypic characterization. *In*: Gerhardt, P, Murray, R. G. E., Wood, W. A., and Krieg, N. R., [eds.,], Methods for general and molecular bacteriology, pp. 607–654. ASM, Washington, DC, USA.

188. Smith E. F. (1896). A bacterial disease of the tomato, eggplant and Irish potato (*Bacillus solanacearum* n. sp.). Bull. Div. Veg. Physiol. Pathol. U S Dep Agric., 12, 1–28.

189. Smits, T. H., Rezzonico, F., Kamber, T., Goesmann, A., Ishimaru, C. A., Stockwell, V. O., Frey, J. E., and Duffy, B. (2010). Genome sequence of the biocontrol agent Pantoea vagans strain C9-1. J. Bacteriol., 192, 6486–6487.

190. Someya, N., Ikeda, S., Morohoshi, T., Tsujimoto, M. N., Yoshida, T., Sawada, H., Ikeda, T., and Tsuchiya, K. (2011). Diversity of culturable chitinolytic bacteria from rhizospheres of agronomic plants in Japan. Microbes Environ., 26, 7–14.

191. Spadaro, D., and Gullino, M. L., (2005). Improving the efficacy of biocontrol agents against soilborne pathogens. Crop Prot., 24, 601–613.

192. Srivastava, R., Khalid, A., Singh, U. S., and Sharma, A. K. (2010). Evaluation of arbuscular mycorrhizal fungus, fluorescent *Pseudomonas* and *Trichoderma harzianum* formulation against *Fusarium oxysporum* f. sp. *lycopersici* for the management of tomato wilt. Biol. control, 53, 24–31.

193. Sugawara, H., Ohyama, A., Mori, H., and Kurokawa, K. (2009). Microbial Genome Annotation Pipeline (MiGAP) for diverse users. Abstr. 20th Int. Conf. Genome Inf., Abstr., S001-1-2.

194. Sun, D., Zhuo, T., Hu, X., Fan, X., and Zou, H. (2017). Identification of a *Pseudomonas putida* as biocontrol agent for tomato bacterial wilt disease. Biol. Control, 114, 45–50.

195. Sun, S., Wei, A.M., Wu, H.X., and Wang, J. (2004). Advances in research on chemical and biological control of plant bacterial wilt. Jiangxi Plant Prot. 27, 157–162.

196. Sundaramoorthy, S., Raguchander, T., Ragupathi, N., and Samiyappan, R. (2012). Combinatorial effect of endophytic and plant growth promoting rhizobacteria against wilt disease of *Capsicum annum* L. caused by *Fusarium solani*. Biol Control, 60, 59–67.

197. Suzuki, S., Komiya, Y., Mitsui, T., Tsuyumu, S., and Kunoh, H. (1999). Activity of pectinases in conidia and germlings of *Blumeria graminis* and the expression of genes encoding pectinases. Ann. Phytopathol. Soc. Jpn., 65, 131–139.

198. Suzuki, Y., Hibino, T., Kawazu, T., Wada, T., Kihara, T., and Koyama, H. (2003). Extraction of total RNA from leaves of *Eucalyptus* and other woody and herbaceous plants using sodium isoascorbate. Biotechniques, 34, 988–993.

199. Tahir, H. A. S., Gu, Q., Wu, H., Niu, Y., Huo, R., and Gao, X. (2017). Bacillus volatiles adversely affect the physiology and ultra-structure of Ralstonia solanacearum and induce systemic resistance in tobacco against bacterial wilt. Sci. Rep., 7, 40481.

200. Takahashi, H., Nakaho, K., Ishihara, T., Ando, S., Wada, T., Kanayama, Y., Asano, S., Yoshida, S., Tsushima, S., and Hyakumachi, M. (2014). Transcriptional profile of tomato roots exhibiting *Bacillus thuringiensis*-induced resistance to *Ralstonia solanacearum*. Plant Cell Rep., 33, 99–110.

201. Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

202. Tan, H., Zhou, S., Deng, Z., He, M., and Cao, L. (2011). Ribosomal-sequence-directed selection for endophytic streptomycete strains antagonistic to *Ralstonia solanacearum* to control tomato bacterial wilt. Biol. Control, 59, 245–254.

203. Tan, S., Dong, Y., Liao, H., Huang, J., Song, S., Xu, Y., and Shen, Q. (2013). Antagonistic bacterium *Bacillus amyloliquefaciens* induces resistance and controls the bacterial wilt of tomato. Pest Manage. Sci., 69, 1245–1252.

204. Tan, S., Gu, Y., Yang, C., Dong, Y., Mei, X., Shen, Q., and Xu, Y. (2016). *Bacillus amyloliquefaciens* T-5 may prevent *Ralstonia solanacearum* infection through competitive exclusion. Biol. Fert. Soils, 52, 341–351.

205. Tatusov, R. L., Fedorova, N. D., Jackson, J. D., Jacobs, A. R., Kiryutin, B., Koonin, E. V., Krylov, D.M., Mazumder, R., Mekhedov, S.L., Nikolskaya, A.N., and Rao, B. S. (2003). The COG database: an updated version includes eukaryotes. BMC Bioinformatics, 4, 41.

206. Tayeb, L. A., Lefevre, M., Passet, V., Diancourt, L., Brisse, S., and Grimont, P. A. (2008). Comparative phylogenies of *Burkholderia*, *Ralstonia*, *Comamonas*, *Brevundimonas* and related organisms derived from *rpoB*, *gyrB* and *rrs* gene sequences. Res. Microbiol., 159, 169–177.

207. Tettelin, H., Masignani, V., Cieslewicz, M. J., Donati, C., Medini, D., Ward, N. L., Angiuoli, S. V., Crabtree, J., Jones, A. L., Durkin, A. S., and DeBoy, R. T. (2005). Genome analysis of multiple pathogenic isolates of *Streptococcus*

agalactiae: implications for the microbial “pan-genome”. Proc. Natl. Acad. Sci. USA., 102, 13950–13955.

208. Tettelin, H., Riley, D., Cattuto, C., and Medini, D. (2008). Comparative genomics: the bacterial pan-genome. Curr. Opin. Microbiol., 11, 472–477.

209. Thanh, D. T., Tarn, L. T. T., Hanh, N. T., Tuyen, N. H., Srinivasan, B., Lee, S. Y., and Park, K. S. (2009). Biological control of soilborne diseases on tomato, potato and black pepper by selected PGPR in the greenhouse and field in Vietnam. Plant Pathol. J., 25, 263–269.

210. Thompson, C. C., Chimetto, L., Edwards, R. A., Swings, J., Stackebrandt, E., and Thompson, F. L. (2013). Microbial genomic taxonomy. BMC genomics, 14, 913.

211. Titball, R. W. (1998). Bacterial phospholipases. J. Applied Microbiol., 84, 127S–137S.

212. Tsuchiya, K. (2014). Genetic diversity of *Ralstonia solanacearum* and disease management strategy. J. Gen. Plant Pathol., 80, 504–509.

213. Vallad, G. E., and Goodman, R. M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. Crop Sci., 44, 1920–1934.

214. Wang, X., and Liang, G. (2014). Control efficacy of an endophytic *Bacillus amyloliquefaciens* strain BZ6-1 against peanut bacterial wilt, *Ralstonia solanacearum*. BioMed Res. Int.

215. Weber, T., Blin, K., Duddela, S., Krug, D., Kim, H. U., Bruccoleri, R., Lee, S. Y., Fischbach, M. A., Müller, R., Wohlleben, W., and Breitling, R. (2015).

antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res., 43, W237–W243.

216. Wei, Z., Yang, X., Yin, S., Shen, Q., Ran, W., and Xu, Y. (2011). Efficacy of *Bacillus*-fortified organic fertiliser in controlling bacterial wilt of tomato in the field. Appl. Soil Ecol., 48, 152–159.

217. Wei, Z., Huang, J., Tan, S., Mei, X., Shen, Q., and Xu, Y. (2013). The congeneric strain *Ralstonia pickettii* QL-A6 of *Ralstonia solanacearum* as an effective biocontrol agent for bacterial wilt of tomato. Biol. Control, 65, 278–285.

218. Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot., 52, 487–511.

219. Whipps, J. M., and Gerhardson, B. (2007). Biological pesticides for control of seed- and soil-borne plant pathogens. *In*: van Elsas, J. D., Jansson, J. D., and Trevors, J. T. [eds.], Modern Soil Microbiology Edition, 2nd edn, pp. 479–501. CRC Press, Boca Raton, Florida, USA.

220. Wicker, E., Lefeuvre, P., De Cambiaire, J. C., Lemaire, C., Poussier, S., and Prior, P. (2012). Contrasting recombination patterns and demographic histories of the plant pathogen *Ralstonia solanacearum* inferred from MLSA. ISME J., 6, 961.

221. Wu, K., Su, L., Fang, Z., Yuan, S., Wang, L., Shen, B., and Shen, Q. (2017). Competitive use of root exudates by *Bacillus amyloliquefaciens* with *Ralstonia solanacearum* decreases the pathogenic population density and effectively controls tomato bacterial wilt. Sci. Hort., 218, 132–138.

222. Wu, S., Zhu, Z., Fu, L., Niu, B., and Li, W. (2011). WebMGA: a customizable web server for fast metagenomic sequence analysis. BMC Genomics, 12, 444.

223. Xiong, H., Li, Y., Cai, Y., Cao, Y., and Wang, Y. (2015). Isolation of *Bacillus amyloliquefaciens* JK6 and identification of its lipopeptides surfactin for suppressing tomato bacterial wilt. RSC Adv., 5, 82042–82049.

224. Xu, X. M., Jeffries, P., Pautasso, M., and Jeger, M. J. (2011). Combined use of biocontrol agents to manage plant diseases in theory and practice. Phytopathol., 101, 1024–1031.

225. Xue, Q. Y., Chen, Y., Li, S. M., Chen, L. F., Ding, G. C., Guo, D. W., and Guo, J. H. (2009). Evaluation of the strains of *Acinetobacter* and *Enterobacter* as potential biocontrol agents against Ralstonia wilt of tomato. Biol. Control, 48, 252–258.

226. Xue, Q. Y., Ding, G. C., Li, S. M., Yang, Y., Lan, C. Z., Guo, J. H., and Smalla, K. (2013). Rhizocompetence and antagonistic activity towards genetically diverse *Ralstonia solanacearum* strains–an improved strategy for selecting biocontrol agents. Applied Microbiol. Biotechnol., 97, 1361–1371.

227. Yabuuchi E., Kosako Y., Oyaizu H., Yano I., Hotta H., Hashimoto Y., Ezaki T., and Arakawa M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol., 36, 1251–1275.

228. Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H., and Nishiuchi, Y. (1995). Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* Gen. Nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) Comb. Nov., *Ralstonia solanacearum* (Smith 1896) Comb. Nov. and *Ralstonia eutropha* (Davis 1969) Comb. Nov. Microbiol. Immunol., 39, 897–904.

229. Yamamoto, S., Shiraishi, S., Kawagoe, Y., Mochizuki, M., and Suzuki, S. (2015). Impact of *Bacillus amyloliquefaciens* S13-3 on control of bacterial wilt and powdery mildew in tomato. Pest Manag. Sci*.,* 71, 722–727.

230. Yamazaki, H., and Hoshina, T. (1995). Calcium nutrition affects resistance of tomato seedlings to bacterial wilt. Hortscience 30:91–93.

231. Yang, T., Wei, Z., Friman, V. P., Xu, Y., Shen, Q., Kowalchuk, G. A., and Jousset, A. (2017). Resource availability modulates biodiversity-invasion relationships by altering competitive interactions. Environ. Microbial., 19, 2984-2991.

232. Yang, W., Xu, Q., Liu, H. X., Wang, Y. P., Wang, Y. M., Yang, H. T., and Guo, J. H. (2012). Evaluation of biological control agents against Ralstonia wilt on ginger. Biol. control, 62, 144–151.

233. Yano, K., Kawada, Y., Horita, M., Hikichi, Y., and Tsuchiya, K. (2011). Phylogenetic discrimination and host ranges of *Ralstonia solanacearum* isolates from Zingiberaceae plants. Japanese J. Phytopathol., 77, 88–95.

234. Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H., and Chun, J. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int. J. Syst. Evol. Microbiol., 67, 1613–1617.

235. Yu, J. Q. (1999). Allelopathic suppression of *Pseudomonas solanacearum* infection of tomato (*Lycopersicon esculentum*) in a tomato-Chinese chive (*Allium tuberosum*) intercropping system. J. Chem. Ecol., 25, 2409–2417.

236. Yuan, S., Wang, L., Wu, K., Shi, J., Wang, M., Yang, X., Shen, Q., and Shen, B. (2014). Evaluation of *Bacillus*-fortified organic fertilizer for controlling tobacco bacterial wilt in greenhouse and field experiments. Appl. Soil Ecol., 75, 86–94.

237. Yuan, S., Li, M., Fang, Z., Liu, Y., Shi, W., Pan, B., Wu, K., Shi, J., Shen, B., and Shen, Q. (2016). Biological control of tobacco bacterial wilt using *Trichoderma harzianum* amended bioorganic fertilizer and the arbuscular mycorrhizal fungi *Glomus mosseae*. Biol. Control, 92, 164–171.

238. Yuliar, Nion, Y.A., and Toyota, K. (2015). Recent trends in control methods for bacterial wilt diseases caused by *Ralstonia solanacearum*. Microbes Environ., 30, 1–11.

239. Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F. M., and Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. J Antimicrob. Chemother., 67, 2640–2644.

240. Zerbino, D. R., and Birney, E. (2008). Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res., 18, 821–829.

241. Zhang, Y. F., He, L. Y., Chen, Z. J., Wang, Q. Y., Qian, M., and Sheng, X. F. (2011). Characterization of ACC deaminase-producing endophytic bacteria isolated from copper-tolerant plants and their potential in promoting the growth and copper accumulation of *Brassica napus*. Chemosphere, 83, 57–62.

242. Zhou, T., Chen, D., Li, C., Sun, Q., Li, L., Liu, F., Shen, Q., and Shen, B. (2012). Isolation and characterization of *Pseudomonas brassicacearum* J12 as an antagonist against *Ralstonia solanacearum* and identification of its antimicrobial components. Microbiol. Res., 167, 388–394.

Summary

Bacterial wilt caused by *Ralstonia solanacearum*, *R. pseudosolanacearum*, and *R. syzygii* subsp. *indonesiensis* poses a serious threat to the worldwide production of many important solanaceous crops such as tomato. The current countermeasures for controlling tomato bacterial wilt consist of chemical controls and cultural practices. However, chemical controls using soil fumigants such as chloropicrin are potentially harmful to the environment and have not been efficient in eradicating the pathogen. Cultural practices through commercially grafted seedlings (grafting resistant rootstock with susceptible scion) restrict pathogen multiplication and movement in the rootstock, thereby suppressing the infection and wilting in the scion. However, grafting is expensive, requires more labor, and result in the production of fruits of inferior quality (taste, color, and sugar contents). In addition, new virulent races of the pathogen might overcome the resistance, resulting in colonization and migration of the pathogen into susceptible scions and causing wilt symptoms. The biological control method of using beneficial bacteria has been proposed as an effective, safe, and sustainable approach. The main objective of this study is to develop a practical biocontrol strategy against tomato bacterial wilt.

Chapter 1: **Screening of biocontrol bacteria for controlling tomato bacterial wilt**

We first isolated bacteria from the rhizosphere soil of tomato, Chinese chive, and Welsh onion plants, and then screened their biocontrol potential against tomato bacterial wilt. As a result, a total number of 442 bacteria were successfully isolated and tested for their antibacterial activity against *R. pseudosolanacearum*. Among the isolates, 276 exhibited an antibacterial activity, thus they were identified by analyzing

the partial sequence of the 16S rRNA gene. The isolates were assigned to 24 genera, including *Burkholderia*, *Pseudomonas*, *Mitsuaria, Acinetobacter, Arthrobacter*, *Achromobacter*, and *Ralstonia.* The suppressive effect of the 276 isolates against tomato bacterial wilt was further examined using the seedling bioassay. Nineteen isolates that belonged to that the genera *Ralstonia* and *Mitsuaria* exhibited a relatively higher disease suppression (>50% reduction in disease severity) than the other isolates. The isolate TCR112 of *Ralstonia* and 11 isolates of *Mitsuaria* were assessed for their biocontrol effect in a series of pot experiments. Among the isolates, TCR112 (identified as non-pathogenic *Ralstonia* sp.) and TWR114 (identified as *Mitsuaria* sp.), which showed a consistent disease suppression in pot experiments, were selected as final candidates for further evaluation under field conditions. Results showed that soil drenching at weekly intervals with TCR112 and TWR114 isolates reduced the wilt incidence in two consecutive years, the first year by 57.2% and 85.8% and the second year by 57.2% and 35.3%, respectively. The population of the pathogen was quantified in the rhizosphere and crown regions of TCR112- and TWR114-treated plants. As a result, the isolates effectively reduced the pathogen population in both regions of pot grown tomatoes. Monitoring the population dynamics of the biocontrol isolates revealed that both isolates have stable rhizosphere and endophytic colonization capacities.

Chapter 2: **Establishment of an effective application method of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112**

To establish a cost-effective method for applying our isolates in order to maximize their biocontrol effect against tomato bacterial wilt, we evaluated whether their

combined application TWR114+TCR112 can enhance the biocontrol effect against tomato bacterial wilt. In the first pot experiment, the effect of different inoculum ratios of TWR114+TCR112 was tested under glasshouse conditions. All the tested ratios (i.e., 1:1, 1:2, and 2:1) of the TWR114+TCR112 treatment significantly suppressed the incidence of tomato bacterial wilt, even at 28 days post-challenge inoculation (dpi) (13–47% wilt incidence), while the incidence of tomato plants treated with the individual isolates reached more than 60% within 10–12 dpi. Among the three ratios, that of 2:1 was associated with the greatest reduction of wilt incidence, which was expressed as AUDPC (93% reduction). Therefore, this ratio was selected for further evaluation. In the second pot experiment, the effect of different inoculum concentrations (i.e., original concentration, 2-fold diluted, and 10-fold diluted) of the TWR114+TCR112 treatment at a ratio of 2:1 was evaluated. All the tested inoculum concentrations significantly reduced the disease incidence, with the original concentration (ca. 9×10^8 CFU/ml) being proven to be the most effective, as demonstrated by it achieving the highest reduction of AUDPC (100%). The population of *R. pseudosolanacearum* in several regions of tomato plant was quantified. As a result, the pathogen population was considerably decreased by the TWR114+TCR112 treatment compared with that in the individual treatments. Moreover, the pathogen population in the above-ground regions (crown, mid-stem, and upper stem) of TWR114+TCR112-treated plants decreased to an undetectable level at 28 dpi. The population of the biocontrol isolates TWR114 and TCR112 was also investigated. Results showed that both isolates in the TWR114+TCR112 treatment and individual treatments were not significantly affected by each other, establishing almost similar population densities in both treatments. The results of this

study clearly indicate that combination of TWR114 and TCR112 at a ratio of 2:1 can exert a synergistic suppressive effect, resulting in enhanced biocontrol efficacy against tomato bacterial wilt.

Chapter 3: **Biocontrol mechanisms of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112**

The *in vitro* assay for siderophore, indole-3-acetic acid, protease, and polygalacturonase revealed that TWR114 produces the latter three substances whereas TCR112 produces the former three substances. Whole genome sequencing of TWR114 and TCR112 using MiSeq revealed that both isolates, in addition to the above compounds, they also harbor biosynthetic gene clusters coding for putative secondary metabolites such as bacteriocin and phenazine. The effect of combined TWR114 and TCR112 and the treatment with each of these individually on the expression of *PR-1a* and *GluA*, *GluB* and *Osmotin*-like, *Le4,* and *LoxD*, which are related to the salicylic acid (SA), ethylene (ET), abscisic acid (ABA) and jasmonic acid (JA) signalling pathways, was examined in the roots of tomato plants. The expression levels of these genes were determined by qRT-PCR in pathogen-uninoculated and -inoculated plants. Results revealed that upon pathogen inoculation, the expression of SA-, ET-, and ABA-responsive genes were more strongly induced in the TWR114+TCR112-treated plants than in those treated with the individual isolates. Comparative genomic analyses between TWR114 and TCR112 and other isolates belong to the same genera showed that these isolates have a clear distinction from their closest relative type strains of *M. chitosanitabida* and *R. pickettii*, respectively. Altogether, the results suggest that both isolates

suppress tomato bacterial wilt by the combination of multiple biocontrol mechanisms such as antibiosis, production of siderophore and enzymes, competition for nutrients, and induced resistance. In addition, the isolates TWR114 and TCR112 might represent a novel species of the genus *Mitsuaria* and *Ralstonia*, respectively.

Summary in Japanese

Ralstonia pseudosolanacearum による青枯病は，トマトに甚大な被害をもたらす最重要病害の一つである。生産現場では，土壌くん蒸消毒や抵抗性台木への接木が主要な青枯病対策となっている。しかし，いずれも防除効果が不安定である上に，土壌くん蒸剤は人体や環境に有害であることから，これらに代わる防除技術の開発が必要である。そこで本研究では，有用根圏細菌を用いたトマト青枯病の生物防除*法*の確立を目指した。

1. トマト青枯病菌に対する生物防除細菌の分離

ニラ，ネギおよびトマトから根圏細菌を 442 株分離し，青枯病菌に対する抗菌活性を検定した結果，276 細菌株に活性が認められた。次に，抗菌性菌株のトマト青枯病防除効果をシードリングアッセイ法で検定した結果，19 菌株（*Mitsuaria* 属菌 11 株，*Ralstonia* 属菌 8 株）が高い防除効果を示した。これら 19 菌株の防除効果をポット試験で評価した結果，*Mitsuaria* sp. TWR114 株と *Ralstonia* sp. TCR112 株が安定した効果を示したことから，候補株として選抜した。そこで次に， TWR114 と TCR112 の圃場条件下での防除効果を検討した。2016 年の圃場試験では，TCR112 処理区（1 週間おきに土壌潅注）および TWR114 処理区（同じく 1 週間おきに潅注）で，定植 50 日後の発病株率が対照区に比べて，それぞれ 57.2%および 86%減少した。2017 年の試験でも同様に両菌株の処理区で発病が顕著に抑制された。トマト苗の根圏およびクラウン組織内での青枯病菌の増殖に対する TCR112 処理および TWR114 処理の影響を調べたところ，いずれの菌株も両部位における青枯病菌の増殖を著しく抑制していた。さらに，TCR112 および TWR114 の定着についても調査した結果，両菌株ともトマト苗の根圏およびクラウン組織内に安定的に定着する能力を持つことが明らかになった。

2. TWR114 および TCR112 の効果的処理方法の検討

TWR114 と TCR112 の防除効果は 2 週間以内で消失する。そこで，両菌株を混用することで防除効果の持続が向上するかを検討した。TWR114（9×10^8 cfu/ml）と TCR112（9×10^8 cfu/ml）を 1:1，1:2 および 2:1 の比率で混用したときの防除効果を比較した。そ

の結果，単用区では病原菌接種 10～12 日後には 60%以上の苗が発病したが，混用区では病原菌接種 28 日後でも発病株率が 13～47%にとどまった。中でも，2:1（TWR114:TCR112）区の防除効果が最も高かった。単用区および 2:1 混用区における病原菌増殖量を比較した結果，根圏および茎内のいずれにおいても混用区の方が単用区よりも病原菌密度が顕著に低かった。一方，TWR114 と TCR112 の定着密度は，単用区と混用区で大きな差はなかった。以上の結果から，TWR114 と TCR112 を 2:1 の比率で混用することで，防除効果の持続性を大幅に向上できることが明らかとなった。

3. TWR114 および TCR112 の生物防除機構

生物防除効果に直接的・間接的に関与するシデロフォア，インドール-3-酢酸，プロテアーゼ，ポリガラクツロナーゼの生産能を *in vitro* 検定した。その結果，TWR114 は後者 3 物質，TCR112 は前者 3 物質を生産することが明らかとなった。さらに，MiSeq を用いて両菌株のゲノムシーケンスを解析したところ，上記物質に加えて，抗菌物質であるバクテリオシンやフェナジンの生合成遺伝子クラスターを有していることが判明した。次に，両菌株の単用区および混用区のトマト苗における防御関連遺伝子群の発現変動を qRT-PCR 解析した結果，いずれにおいても病原菌接種後の遺伝子発現量が対照区よりも顕著に増加することがわかった。発現する遺伝子のパターンは処理区により異なり，TWR114 単用区ではアブシジン酸（ABA）およびエチレン（ET）応答性の遺伝子，TCR112 単用区では ET 応答性の遺伝子が発現するが，混用区では ABA, ET 応答性の遺伝子に加えてサリチル酸（SA）応答性の遺伝子の発現量が増加した。以上のことから，両菌株の生物防除機構には，抗菌作用や養分競合，抵抗性誘導などが複合的に関与すると考えられた。

まとめ

本研究では，拮抗性根圏細菌 *Mitsuaria* sp. TWR114 および *Ralstonia* sp. TCR112 を用いたトマト青枯病の効果的な生物防除法の確立に成功した。さらに，その生物防除機構には，抗菌作用や抵抗性誘導などが複合的に関与することが明らかとなった。

Acknowledgements

First and foremost, I want to express my sincere gratitude to my primary academic supervisor, Assoc. Prof. Dr. **Masafumi Shimizu**, Laboratory of Plant Pathology, Faculty of Applied Biological Sciences, Gifu University. He has been supportive since the first days I began working on the development of innovative approaches for controlling tomato bacterial wilt. Even since, Shimizu sensei has supported me not only by providing a research assistantship over almost 6 and half years, but also academically and emotionally through the rough road to finish this thesis. Thanks to him I had the opportunity to develop practical biocontrol strategy against tomato bacterial wilt. He helped me come up with the thesis topic and gave me many constructive comments, useful advices, and clarifications that led to the successful completion of this study. I could not have imagined having a better advisor and mentor for my Ph.D. study.

My co-academic supervisors guided me over the course of three years. Thank you to Assoc. Prof. Dr. **Haruhisa Suga**, Life Science Research Center, Gifu University, and Proffesor Dr. **Akio Morita**, Faculty of Agriculture, Shizuoka University for being my co-supervisors, for providing me with your academic guidance, encouragement, and insightful comments.

Special thanks to Proffesor Dr. **Tohru Suzuki**, Faculty of Applied Biological Sciences, Gifu University, and the members of his laboratory of genome microbiology for their hard work of sequencing and analyzing the whole genome of my bacterial isolates.

My former and contemporary fellow lab mates at the Laboratory of plant pathology. Thank you all for your tremendous assistance and help in various ways and for your continuous support in every step of my research. A special acknowledgement goes to my friend **Tomoki Nishioka**, who has been very helpful translating many important documents and providing valuable comments concerning my papers.

The Ministry of Education, Culture, Sports, Science, and Technology (Monbukagakusho), Government of Japan; for giving me the opportunity to pursue my graduate studies in Japan by providing me with the such great full scholarship.

The staff of The United Graduate School of Agricultural Science and the International Student Center, Gifu University, for their valuable assistance to foreign students.

Special thanks to my girlfriend **Ayumi Sakaguchi**, for her tremendous love, support, and patience throughout my whole Ph.D. study.

Last but not the least; I finish with Jordan, where the most basic source of my life energy resides: My family, including of course, my cousins. Their support, prayers, and love has been unconditional all these years; they have given up many things for me to be at Gifu University; they have cherished with me every great moment and supported me whenever I needed it.

Publications

Marian, M., Morita, A., Koyama, H., Suga, H., and Shimizu, M. (2018). Enhanced biocontrol effect using the combined application of *Mitsuaria* sp. TWR114 and non-pathogenic *Ralstonia* sp. TCR112 for controlling tomato bacterial wilt. J. Gen. Plant Pathol. (In press).

Marian, M., Nishioka, T., Koyama, H., Suga, H., and Shimizu, M. (2018). Biocontrol potential of *Ralstonia* sp. TCR112 and *Mitsuaria* sp. TWR114 against tomato bacterial wilt. Appl. Soil Ecol., 128, 71–80.

Marian, M., Nishioka, T., Koyama, H., Suga, H., and Shimizu, M. (2018). Novel isolates of *Ralstonia* and *Mitsuaria* species as biocontrol agents for controlling tomato bacterial wilt. IOBC-WPRS Bulletin 133, pp 9–10.

Nishioka, T., Tanaka, C., **Marian, M.**, Igarashi, Y., Suga, H., and Shimizu, M. (2018). Role of Burkholderia-derived pyochelin in the Fusarium wilt suppression of *Allium*-cultivated soil. IOBC-WPRS Bulletin 133, pp 130–131.

Marian, M., Ogawa, K., Yoshikawa, Y., Takasaki, S., Usami, A., Shimizu, M., Miyazawa, M., and Hyakumachi, M. (2014). Agroecosystem Development of Industrial Fermentation Waste-Suppressive Effects of Beer Yeast Cell Wall Extract on Plant Diseases. Current Environ. Eng., 1(3), 207–211.