

## 1   **Highlights**

- 2       •   *Ralstonia* sp. TCR112 and *Mitsuaria* sp. TWR114 were isolated from *Allium* plants.
- 3       •   Both isolates effectively suppressed bacterial wilt in pot and field experiments.
- 4       •   Both isolates effectively reduced the pathogen population in pot grown tomatoes.
- 5       •   Both isolates have stable rhizosphere and endophytic colonization capacities.
- 6       •   The first study of *Mitsuaria* as biocontrol agent against tomato bacterial wilt.

1 **Biocontrol potential of *Ralstonia* sp. TCR112 and *Mitsuaria* sp. TWR114 against tomato**  
2 **bacterial wilt**

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4 Malek Marian <sup>a</sup>, Tomoki Nishioka <sup>a</sup>, Hiroyuki Koyama <sup>a</sup>, Haruhisa Suga <sup>b</sup>,  
5 Masafumi Shimizu <sup>a, \*</sup>

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7 <sup>a</sup> The United Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, Japan.

8 <sup>b</sup> Life Science Research Center, Gifu University, Gifu 501-1193, Japan.

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10 \*Corresponding author

11 E-mail address: [shimizma@gifu-u.ac.jp](mailto:shimizma@gifu-u.ac.jp)

12 Telephone number: +81-58-293-2850

13 Fax number: +81-58-293-2850

## Abstract

In this study, we aimed to identify potential biocontrol agents capable of suppressing tomato bacterial wilt caused by *Ralstonia pseudosolanacearum*. In total, 441 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, 275 isolates were selected and further evaluated using a tomato seedling bioassay. Eighteen 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 10 isolates of *Mitsuaria* were assessed for their biocontrol effect in a series of pot experiments. Among the isolates, TCR112 (identified as *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. Furthermore, 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. Altogether, the results suggest that both isolates suppress tomato bacterial wilt by preventing pathogen multiplication and infection via direct antagonism and/or indirect effects such as competing for nutrients and inducing

38 resistance in tomato plants. Furthermore, this is the first study reporting the potential of  
39 *Mitsuaria* as a biocontrol agent against tomato bacterial wilt.

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41 **Keywords:** non-pathogenic *Ralstonia*; *Mitsuaria*; *Ralstonia pseudosolanacearum*; Tomato;  
42 rhizobacteria; biological control

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## 1. Introduction

Bacterial wilt is caused by *Ralstonia solanacearum* (Yabuuchi et al., 1995), *R. pseudosolanacearum*, and *R. syzygii* subsp. *indonesiensis* (formerly classified as *R. solanacearum*) (Safni et al., 2014), and is the second most destructive bacterial disease of plants worldwide (Mansfield et al., 2012). Bacterial wilt affects the yield of many solanaceous plants, such as tomato (*Solanum lycopersicum*), potato (*S. tubersum*), tobacco (*Nicotiana tabacum*), and eggplant (*S. melongena*) (Hayward, 1991). Five million hectares of tomatoes are estimated to be grown annually worldwide, producing >170 million tons (FAOSTAT, 2014). In Japan, tomatoes are grown on a total area of 12 thousand hectares, with an annual production of 740 thousand tons (FAOSTAT, 2014), and thus is 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 (Elphinstone, 2005). 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* (Saddler, 2005). 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 (Momma, 2008). However, grafting is expensive, requires more labor, and result in the production of fruits of inferior quality (taste, color, and sugar contents) (Lee et al., 2010). 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 (Nakaho et al., 2004). Moreover, for the RSD method, achieving sufficient disinfection in the deep soil layers where the pathogen might localize is difficult (Momma, 2008). 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 (Raaijmakers et al., 2009). 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 (Denny, 2007). 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. (Lemessa and Zeller, 2007), *Bacillus* spp. (Kurabachew and Hydra, 2013), and *Flavobacterium johnsoniae* and *Chryseobacterium daecheongensis* (Huang et al., 2013) 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 (Hiddink et al., 2010). 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 (Lai et al., 2011; Yu, 1999). Nishioka et al. (2016a) 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 (Hendrick and Sequeira, 1984) 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 (Ramesh et al., 2009). **A 70-μL aliquot of 24-h-old culture broth (approximately 10<sup>7</sup>–10<sup>8</sup> 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 (Aino et al., 1996), 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 to the above seeded tubes, followed by inoculation with 2 mL of pathogen suspension (ca.  $8 \times 10^5$  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 scored on a scale of 0–2, where 0 represents no symptoms, 1 indicates 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 10 isolates of *Mitsuaria* and 1 isolate of *Ralstonia* 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.  $3 \times 10^8$  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.  $1 \times 10^8$  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.  $4 \times 10^7$  CFU/mL) to obtain a final concentration of ca.  $1 \times 10^7$  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.  $9 \times 10^8$  CFU/mL) to obtain a final concentration of ca.  $3 \times 10^8$  CFU/g soil. Three days after the treatment, plants were challenged with 100 mL of VT0801 cell suspension (ca.  $2 \times 10^7$  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 (1983), 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 =  $\{[\text{total number of diseased plants (scale 1–4) in the treatment} / \text{total number of plants investigated}]\} \times 100$

Disease severity =  $[(\text{the number of diseased plants in each scale} \times \text{disease scale}) / (\text{total number of plants investigated} \times \text{the highest disease scale})] \times 100$ .

AUDPC was calculated on the basis of disease severity using the trapezoid integration of disease progress curve over time according to the following formula:  $\text{AUDPC} = \sum [0.5 (x_i + x_{i-1})] (t_i - t_{i-1})$ , 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) (French et al., 1995). 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. S1) 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. This experimental field was naturally infested with *R. pseudosolanacearum*, with an initial population of  $3.6 \pm 0.2$  (log CFU/g wet soil) and  $3.0 \pm 0.3$  (log CFU/g wet soil) in 2016 and 2017, respectively. 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<sub>2</sub>O<sub>5</sub>:K<sub>2</sub>O 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. Furthermore, limestone was added at a rate of 2.3 tons/ha (55.4% CaO, pH 9.5) (Shinko Kogyo Co., Ltd., Japan).

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.  $3 \times 10^8$  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.  $3 \times 10^8$  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

Bacterial isolates showing *in vitro* antibacterial activity were tentatively identified based on the partial sequence of 16S rRNA gene, according to a protocol described previously (Nishioka et al., 2016b). 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 (Nishioka et al., 2016b). The 16S rRNA gene sequences were compared with those of type strains in the EzBioCloud database (<https://www.ezbiocloud.net/>) (Yoon et al., 2017). 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 (Tamura et al., 2013). The 16S rRNA gene sequences of the isolates were deposited in GeneBank database under the accession numbers MG877646–MG877664.

#### 2.10. Production of siderophore, indole-3-acetic acid, hydrogen cyanide, protease, and polygalacturonase

The production of siderophore by TCR112 and TWR114 isolates was detected using the overlaid chrome azurol S agar (O-CAS) method (Pérez-Miranda et al., 2007). In brief, both isolates were cultured on nutrient agar (NA) at 30°C. After 3 days of incubation, 0.9% (w/v) agarose containing chrome azurol S was applied on the NA plates. Siderophore production was assessed based on a change in the color of the overlaid agarose from blue to orange.

The production of indole-3-acetic acid (IAA) was assayed with the colorimetric method using Salkowski reagent (Kurabachew and Hydra 2013). A 100-μL aliquot of cell suspension ( $OD_{600} = 0.5$ ) of each isolate was inoculated in 10 mL tryptic soy broth (Difco, Sparks, MD, USA), supplemented with 0.5 mg L-tryptophan and incubated at 28°C for 48 h with shaking at 200 rpm. After incubation, broth cultures were centrifuged and the supernatants were mixed with 2 mL of Salkowski reagent. IAA concentration was determined by measuring the absorbance at 535 nm.

Hydrogen cyanide (HCN) production was determined as described by Kurabachew and Hydra (2013).

Protease production was determined on skim milk agar according to the method of Smibert and Krieg (1994).

Polygalacturonase production was assayed as described previously (Suzuki et al., 1999).

### *2.11. 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, 441 bacteria were successfully isolated from rhizosphere soil samples and used as a pool for antibacterial screening. Of these isolates, 275 (62.4%) exhibited weak-to-strong antibacterial activity against *R. pseudosolanacearum* in the agar well diffusion assay (data not shown) 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*, *Acinetobacter*, *Mitsuaria*, *Arthrobacter*, *Achromobacter*, and *Ralstonia* (Table S1).

#### 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, 55 of 275 isolates reduced the disease severity in the treated plants compared with that in the untreated control (data not shown). Among the 55 isolates, 18 showed 50–100% reduction in disease severity (Table 1). Therefore, the disease suppressive effect of these 18 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). These isolates comprised two genera, namely *Ralstonia* (isolates TCR111, TCR112, TCR113, TCR123, TCR124, TCR133, TCF143, and TCF148) and *Mitsuaria* (isolates TCR103, 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 1). In trial 1 performed in a glasshouse with 10 *Mitsuaria* isolates, nine isolates reduced disease severity, which was expressed as AUDPC (Table 2). In particular, three isolates, TCR103, TCR159, and TWR114, showed the lowest AUDPC. Therefore, the biocontrol effect of these three isolates was again evaluated in trial 2. In this trial, the highest reduction of AUDPC was achieved using the isolate TWR114 (45.7%)(Table 2); 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 3 and Fig. 1).

### 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. 2). 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. 2A and 2C). 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. 2B). 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. 2D).

### 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. 3). 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. 3A). 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. 3B). 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 two consecutive years, the wilt incidence was considerably suppressed in plots drenched treated with the biocontrol isolates at weekly intervals (Fig. 4). 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. 4). 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. 4).

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. 5).

### 3.7. Classification of the biocontrol isolates TCR112 and TWR114

The analysis of full-length 16S rRNA sequence of biocontrol isolates indicated that TCR112 (approximately 1,230 bp) were 99.8% similar to those of *R. pickettii* (accession number: JOVL01000020), whereas TWR114 (approximately 1,200 bp) shared 99.3% similarity with *Mitsuaria chitosanitabida* (accession number: BCYP01000048). To clarify the phylogenetic position of both isolates, a phylogenetic tree was constructed on the basis of the complete 16S rRNA gene sequences (Fig. 6). As a result, the isolates TCR112 and

TWR114 showed a clear distinction from the known type strains of *R. pickettii* ATCC 27511<sup>T</sup> and *M. chitosanitabida* 3001<sup>T</sup>, respectively. Thus, TCR112 was identified as a *Ralstonia* sp., and TWR114 was identified as a *Mitsuaria* sp.

### 3.8. 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. 7A). Both isolates showed positive reaction for protease activity (Fig. 7B). Polygalacturonase was produced by TWR114 only (Fig. 7C), 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. 7D).

## 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. S2), which have been used as companion plants to suppress bacterial wilt (Pan et al., 1990; Yu, 1999). 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 18 isolates selected based on the disease suppressive effect in the seedling bioassays were classified as either non-pathogenic *Ralstonia* or *Mitsuaria* on the

443 basis of 16S rRNA gene sequence analysis (Table 1). To our knowledge, only few studies  
 444 have described the biocontrol effect of non-pathogenic *Ralstonia* spp. against soil-borne  
 445 diseases including tomato bacterial wilt (Chen et al., 1995; Doumbou et al., 1998; Wei et al.,  
 446 2013). *Mitsuaria* isolates have been recently reported to have biocontrol effect against  
 447 *Rhizoctonia solani* and *Pythium aphanidermatum* in tomato and soybean (Benítez and  
 448 Gardener, 2009), but there was no report on the suppressive effect of *Mitsuaria* species  
 449 against bacterial diseases. Therefore, this is the first study to describe the biocontrol capacity  
 450 of the genus *Mitsuaria* against bacterial wilt. In this study, two isolates TCR112 and  
 451 TWR114 were selected as potential biocontrol agent. According to the phylogenetic analysis  
 452 based on the full-length 16S rRNA sequence, the isolate TCR112 was found to be a species  
 453 closely-related to *R. pickettii* (Fig. 6A). However, it was reported that the application of 16S  
 454 rRNA gene as a phylogenetic marker is insufficient resolution at the genus level of *Ralstonia*  
 455 species (Glaeser and Kämpfer, 2015). Therefore, additional phylogenetic analysis based on  
 456 multiple protein coding genes will be necessary to characterize the isolate TCR112 at species  
 457 level. On the other hand, the *Mitsuaria* isolate TWR114 was clearly separated from the  
 458 known-type strains of *M. chitosanitabida* (Fig. 6B). Moreover, TWR114 had the ability to  
 459 produce  $\beta$ -glucosidase and use some sugars (sucrose, D-fructose, D-mannose, D-xylose, D-  
 460 galactose, and N-acetyl-D-glucosamine, and L-arabinose) (data not shown), which could not  
 461 be produced or used by the known-type strains of *M. chitosanitabida* 3001<sup>T</sup> (Amakata et al.,  
 462 2005) and *M. chitosanitabida* IAM 14711<sup>T</sup> (Gomila et al., 2007). To date, the genus  
 463 *Mitsuaria* comprises only one species of *M. chitosanitabida*; therefore, our isolate might  
 464 represent a new species of this genus. This is in agreement with that reported by Someya et al.  
 465 (2011) who found a clear distinction between several isolates of *Mitsuaria* and the known-  
 466 type strain of *M. chitosanitabida* 3001<sup>T</sup> 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. 6B).

*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 3). 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. 4). 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. (2015) 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. (2013) 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 (Nion and Toyota, 2008) may increase our biocontrol isolates to pathogen ratio and enhance the biocontrol efficacy. Considering these points, we are currently studying several approaches to maximize the biocontrol effect of our isolates.

The population of *R. pseudosolanacearum* in tomato rhizosphere was considerably decreased by the treatment with the isolates TCR112 and TWR114 (Fig. 2), 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. 3 and 5). We suggest that the colonization ability of these isolates might contribute to suppressing 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 (Raaijmakers et al., 2009), including bacterial wilt (Huang et al., 2013). Additionally, maintaining the BCA population in the rhizosphere at  $>10^6$  CFU/g dry soil is critical for controlling bacterial wilt (Yuan et al., 2014). 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* (data not shown). With regard to this, the antibacterial activity of the isolates TCR112 and TWR114 was noticeably weak (narrow and fuzzy inhibition zone)(data not shown), 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. Because the isolate TCR112 belongs to genus *Ralstonia*, its ecological and physiological characteristics may be similar to that of pathogenic *Ralstonia*, this suggests that TCR112 compete for the same nutrient sources and occupies the same niches as the pathogenic *Ralstonia*. Our results showed that the isolate TCR112 was capable of producing siderophores, whereas the isolate TWR114 was able to produce extracellular polygalacturonase (Figs. 7A and 7C). 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* (Ran et al., 2005a). 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* (Ikeda et al., 1998; Shiomi et al., 1999). Furthermore, we found that both isolates exhibit protease activity (Fig. 7B). 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 (Elhalag et al., 2016).

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 (Clough et al., 1997). 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 (Huang and Allen, 2000). 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.2 and 2D). Our results demonstrated that both isolates can colonize the inside crown tissues (Figs. 3 and 5). 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 including siderophores (Kloepper and Ryu, 2006). Moreover, production of phytohormones such as IAA by beneficial bacteria has been reported to induce resistance (Petti et al., 2012). As described earlier, the isolate TCR112 produces siderophore (Fig. 7A). Additionally, the isolates TCR112 and TWR114 produce IAA (Fig. 7D). Some of these compounds have already been reported to induce resistance against bacterial wilt (Ran et al., 2005b). Therefore, we will investigate whether our isolates can enhance the defense responses in tomato plants against bacterial wilt.

In conclusion, the findings from this study clearly demonstrate that *Ralstonia* sp. TCR112 and *Mitsuaria* sp. TWR114 are potential BCAs capable of suppressing tomato bacterial wilt. Studies are underway to develop practical application method of TCR112 and TWR114 for maximizing their biocontrol effect. Further research will be conducted to better understand the detailed mechanism of disease suppression of our isolates.

558    **Acknowledgements**

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560       This work was financially supported by JSPS KAKENHI (grant numbers JP24780317 and  
561    JP15KT0029).

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680 *syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. solanacearum* phylotype  
681 IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease  
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**Figure captions****Fig. 1.**

Suppression of bacterial wilt in tomato plants by treatment with *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*.

**Fig. 2.**

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 *Ralstonia* sp. TCR112 (upper graphs) and *Mitsuaria* sp. TWR114 (lower graphs). The initial density of the pathogen in the soil was approximately  $ca. 1 \times 10^7$  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).

**Fig. 3.**

Population dynamics of *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  $ca. 3 \times 10^8$  CFU/g soil.

**Fig. 4.**

Effect of weekly drenching of *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.  $3 \times 10^8$  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)]}  $\times$  100.

**Fig. 5.**

Populations of *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.

**Fig. 6.**

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  $\geq$  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.

**Fig. 7.**

Biocontrol traits of the isolates *Ralstonia* sp. TCR112 (left side photos) and *Mitsuaria* sp. TWR114 (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%

767 polygalacturonic acid (D) The amount of indole-3-acetic acid synthesized by the isolates on  
768 tryptic soy broth medium amended with 50 µg/mL of L-tryptophan.

769

770 **Supplementary Fig. S1**

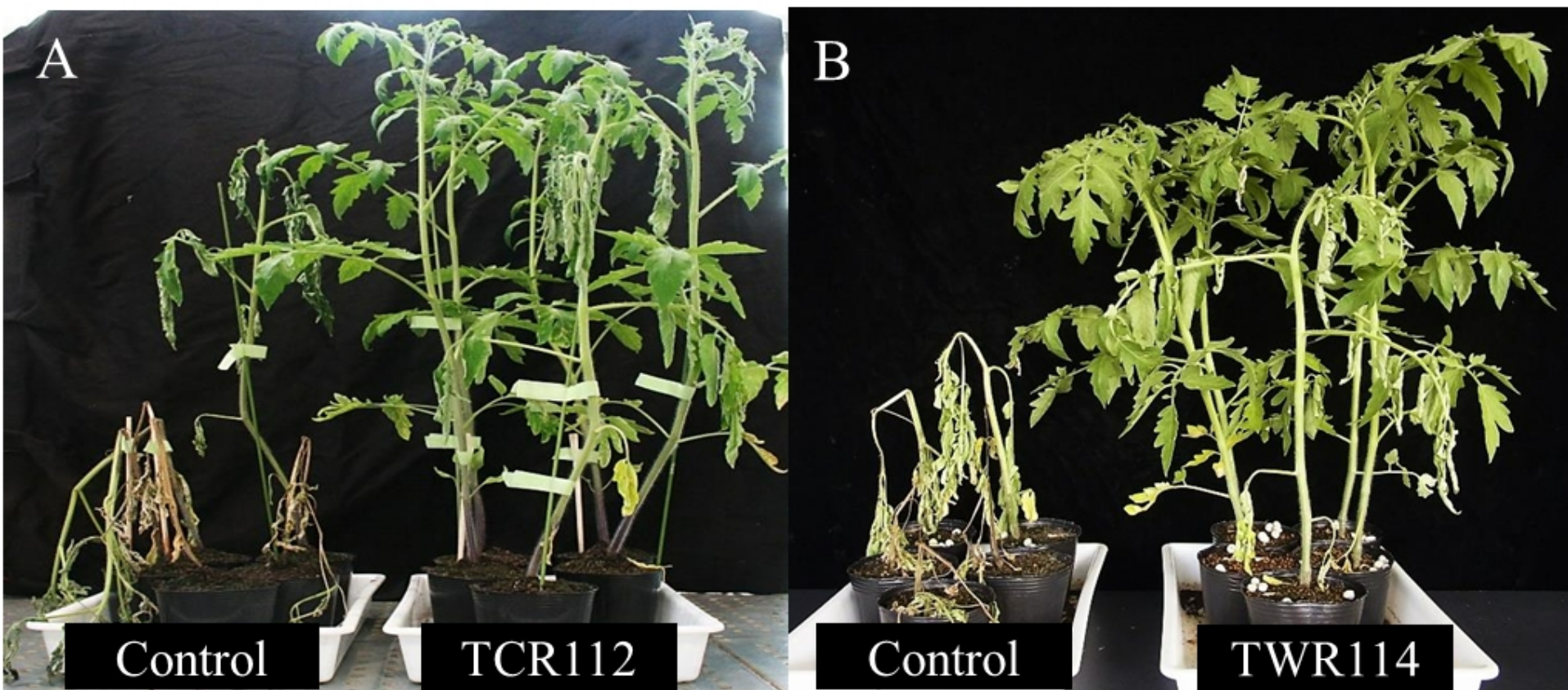
771 Typical colonies of *Ralstonia* sp. TCR112 (A) and *Mitsuaria* sp. TWR114 (B) after  
772 incubating for 48 h on 1/10 TSA supplemented with the selected antibiotics that are  
773 optimized for its enumeration.

774

775 **Supplementary Fig. S2**

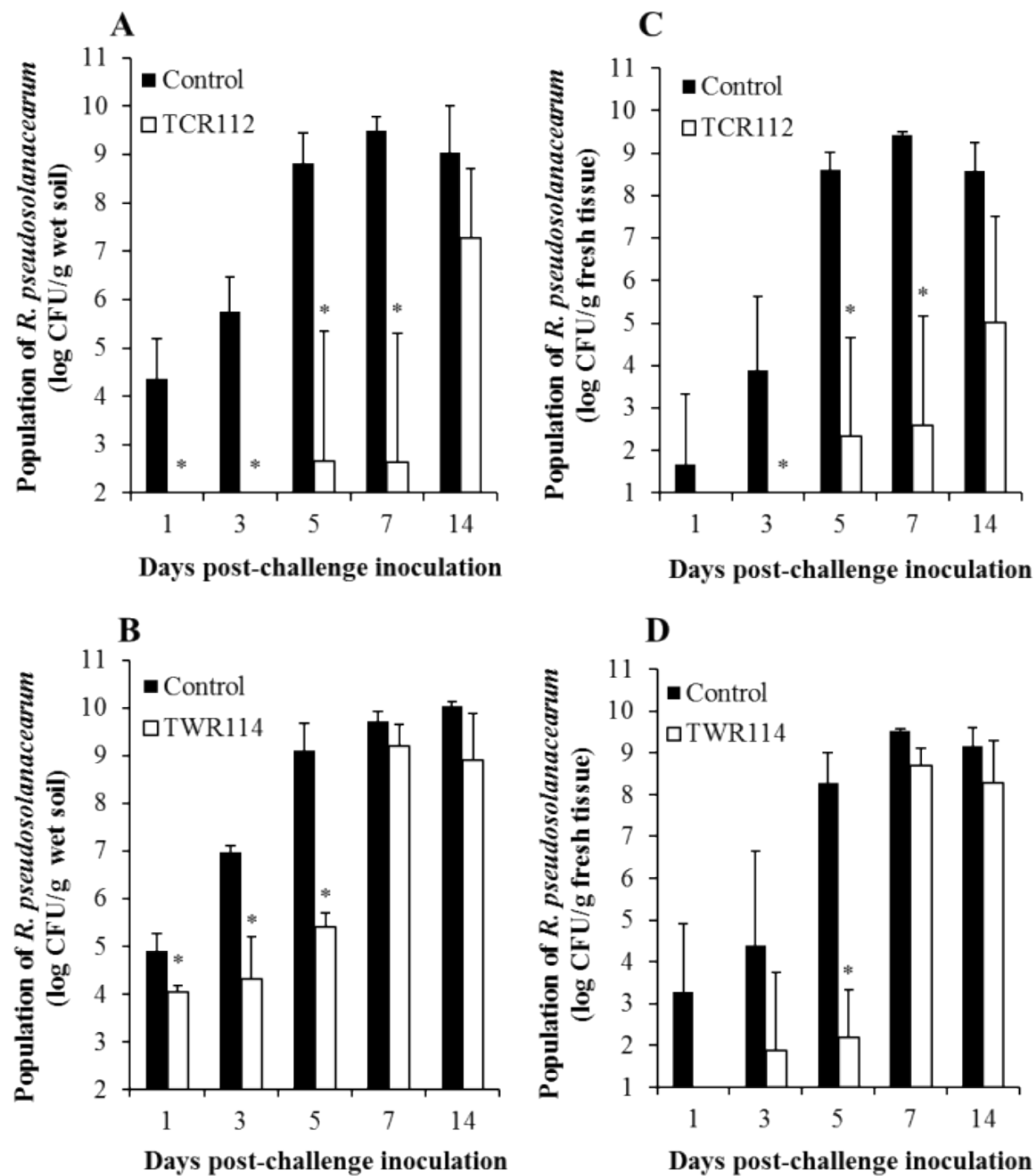
776 Suppressive effect of rhizobacterial isolates from tomato, Chinese chive, and Welsh onion  
777 plants grown in fields against tomato bacterial wilt using seedling bioassay.

**Fig. 1.**





**Fig. 2.**



**Fig. 3.**

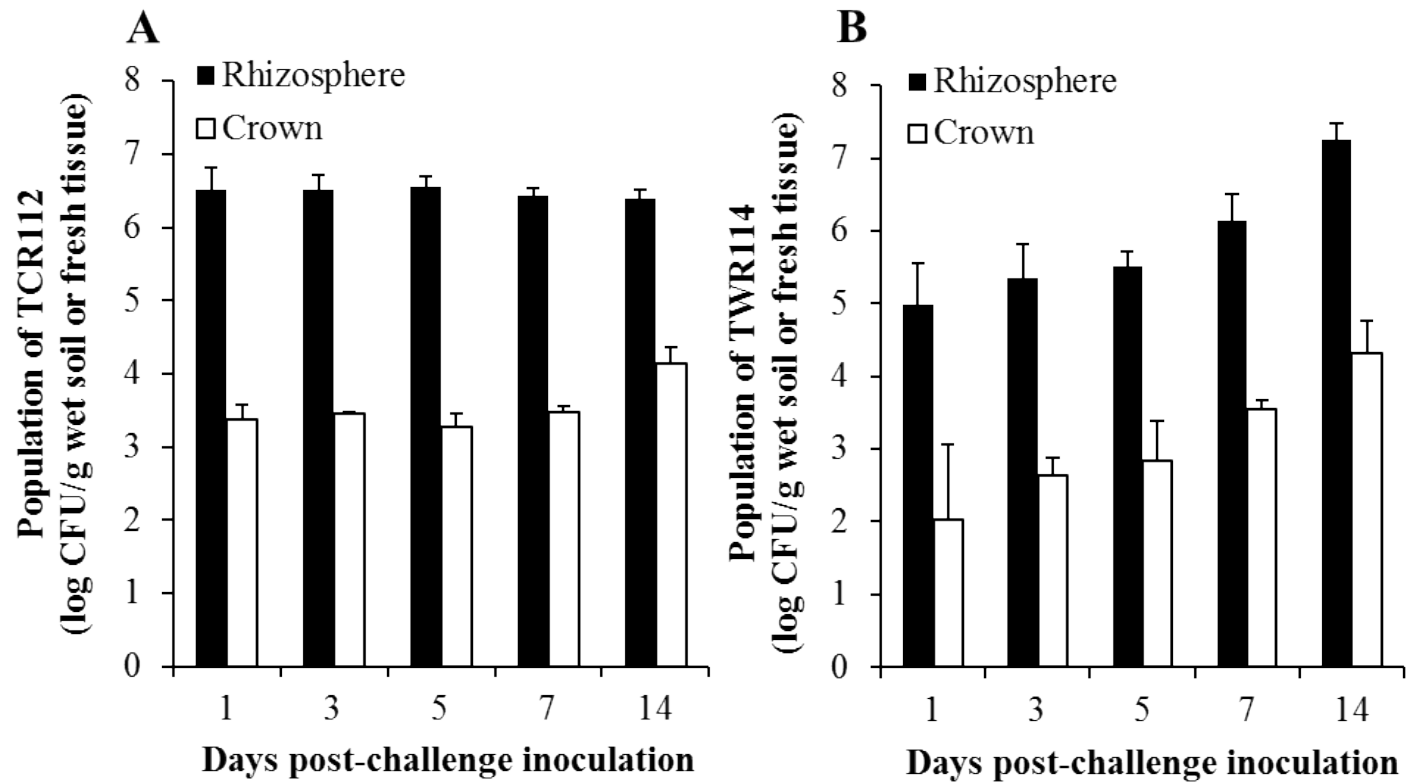
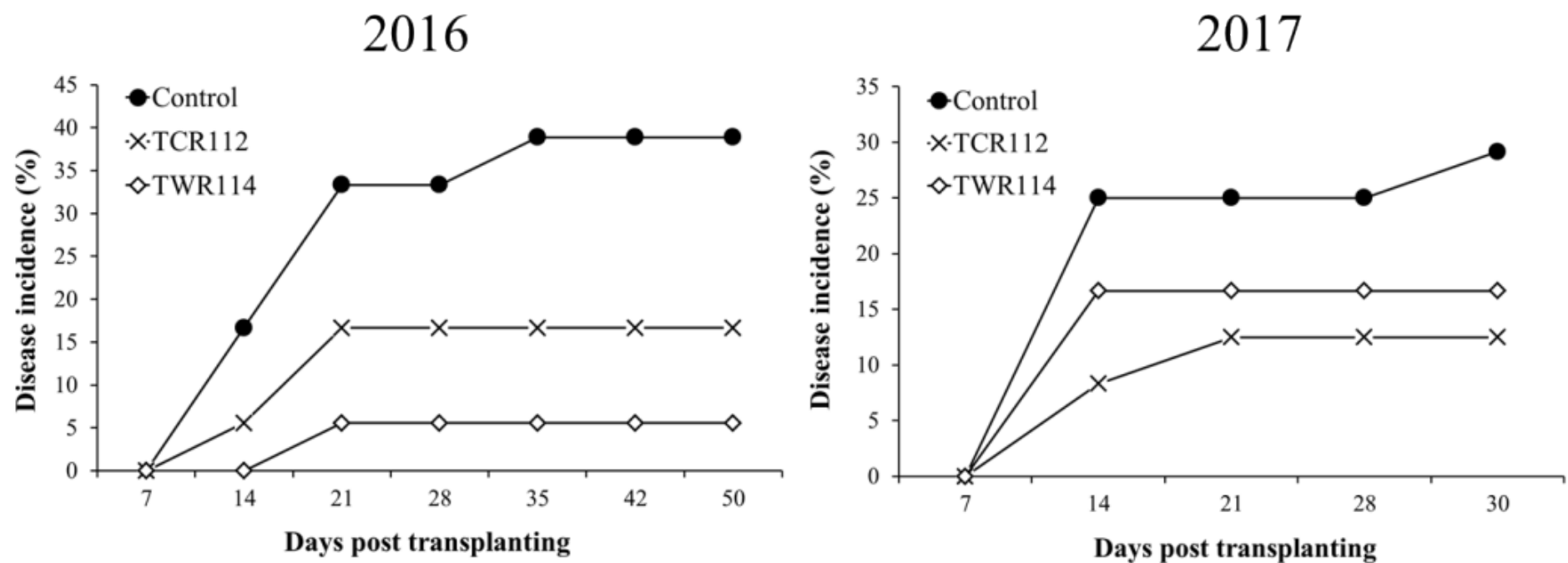
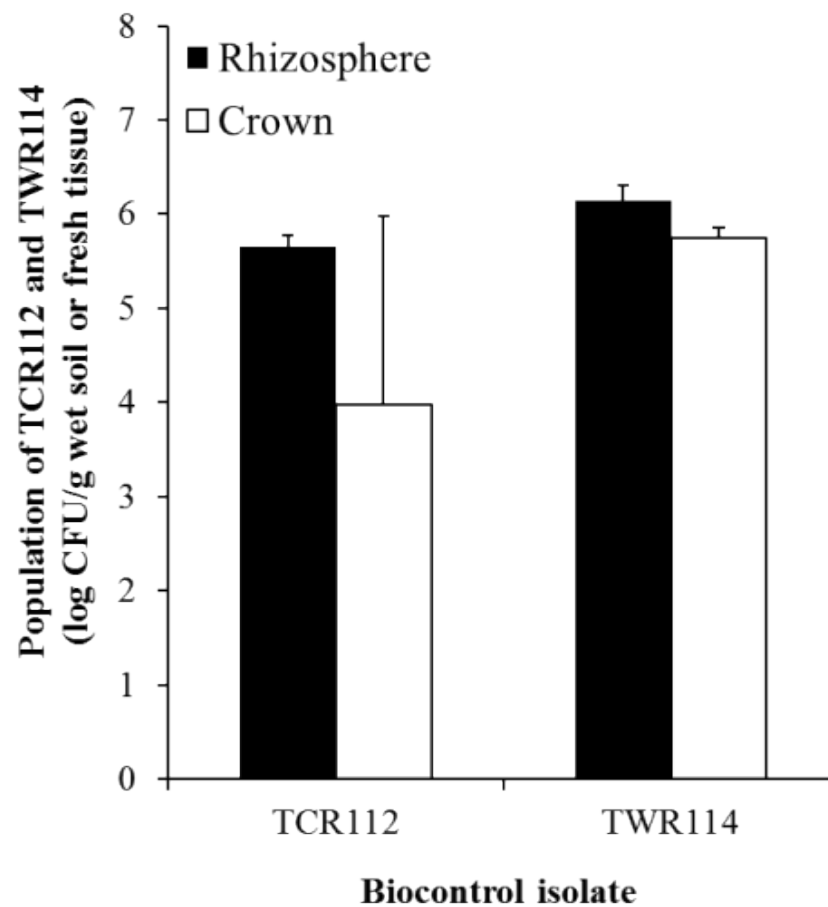


Fig. 4.

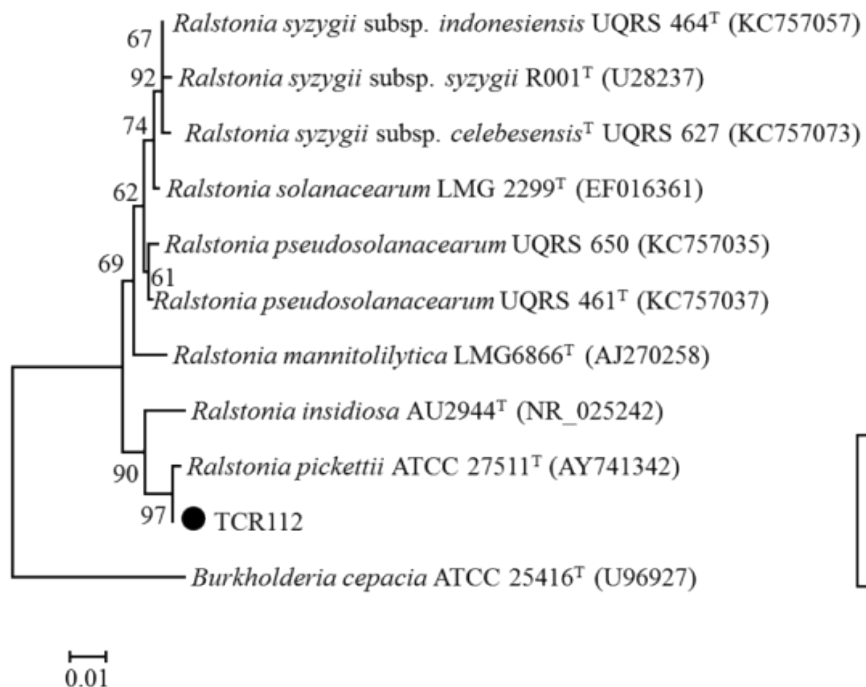


**Fig. 5.**

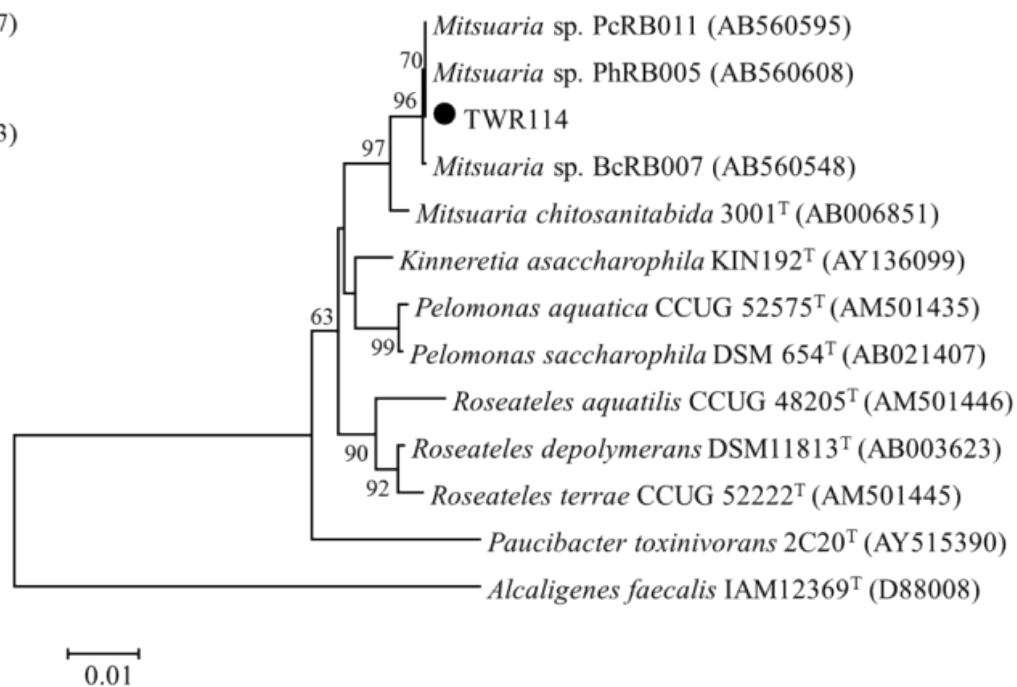


**Fig. 6.**

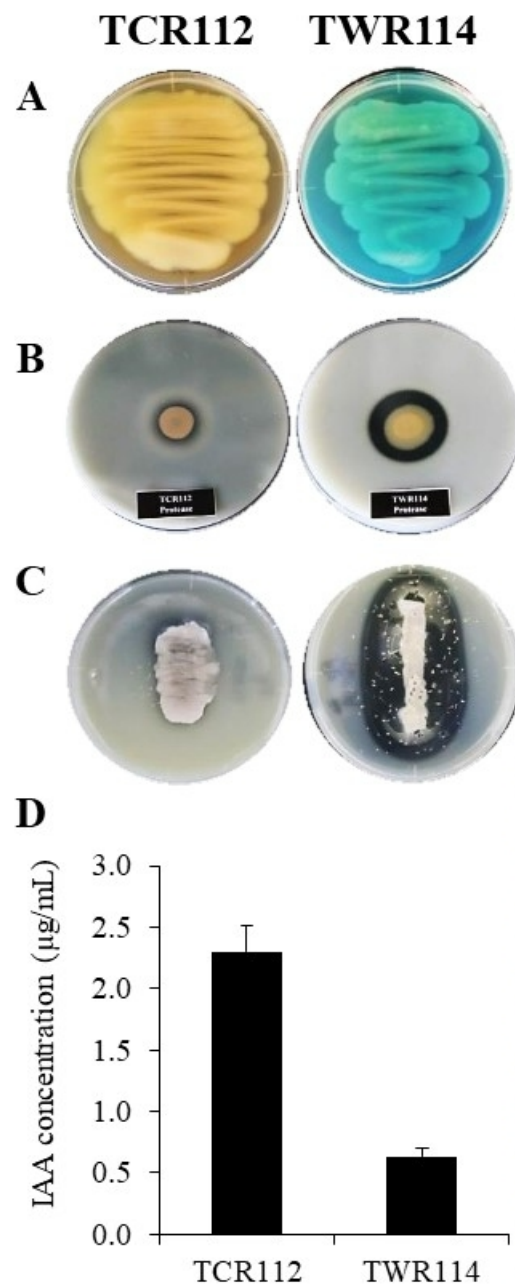
**A**



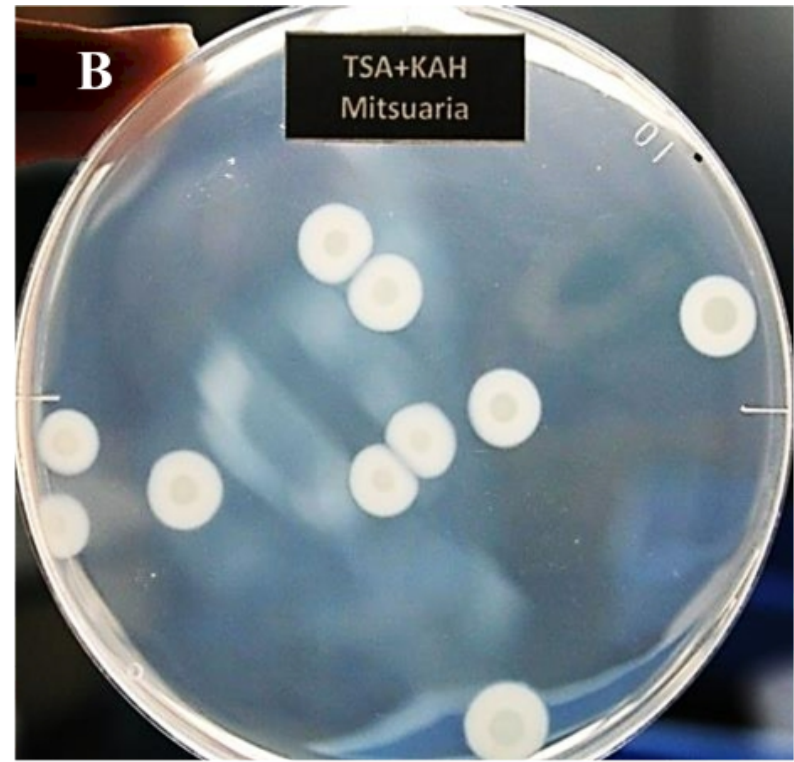
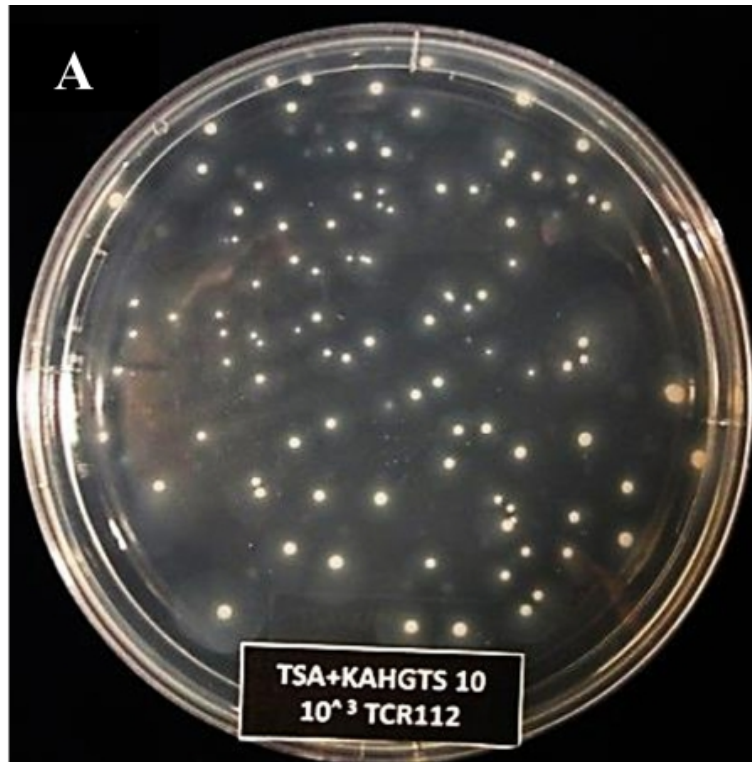
**B**



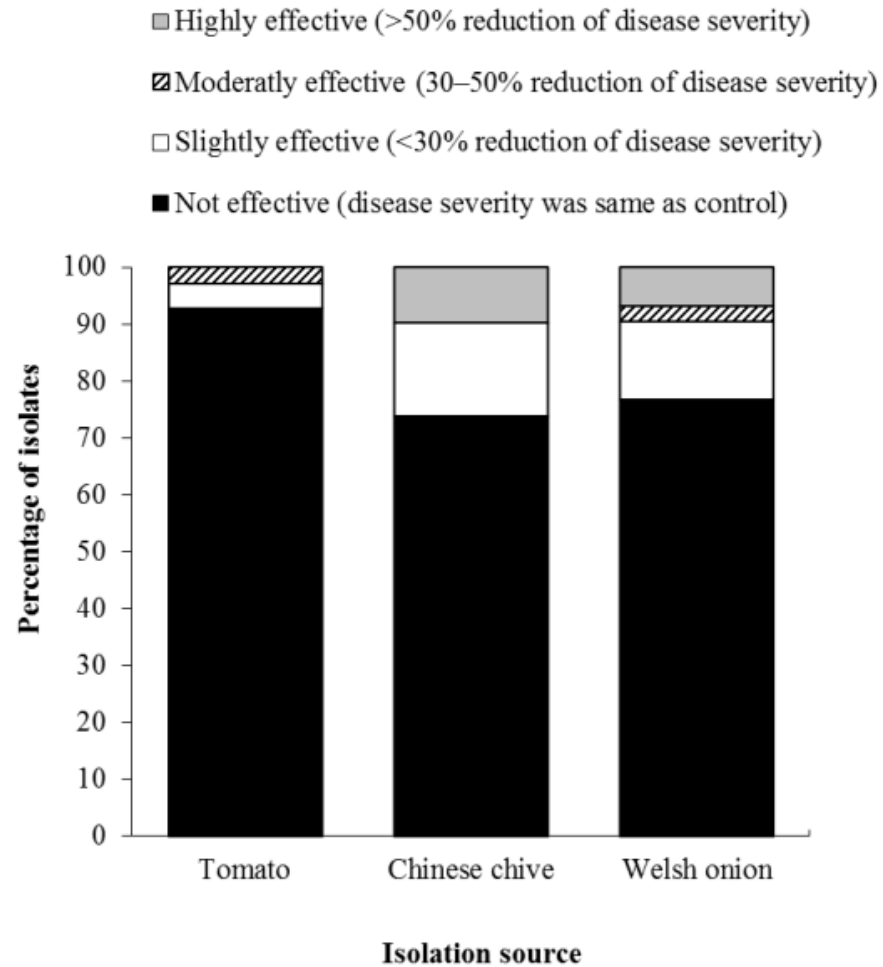
**Fig. 7.**



## Supplementary Fig. S1



## Supplementary Fig. S2





1 **Table 1. Eighteen** bacterial isolates used for the seedling bioassay and their disease  
2 suppressive effect against bacterial wilt in tomato seedlings

Isolate	Source	Reduction in disease severity		Closest hit ( <b>accession number</b> )	Identity (%)
		(%) <sup>†</sup>			
		Trial 1 <sup>‡</sup>	Trial 2 <sup>§</sup>		
TCR111	Chinese chive	63.3	73.3 ± 6.9 <sup>#</sup>	<i>Ralstonia pickettii</i> (JOVL01000020)	100
TCR112	Chinese chive	100.0	90.0 ± 5.8	<i>Ralstonia pickettii</i> (JOVL01000020)	99.8
TCR113	Chinese chive	90.0	85.6 ± 3.0	<i>Ralstonia pickettii</i> (JOVL01000020)	99.8
TCR123	Chinese chive	86.7	83.9 ± 4.6	<i>Ralstonia pickettii</i> (JOVL01000020)	100
TCR124	Chinese chive	83.3	82.7 ± 2.5	<i>Ralstonia pickettii</i> (JOVL01000020)	100
TCR133	Chinese chive	60.0	72.2 ± 6.2	<i>Ralstonia pickettii</i> (JOVL01000020)	99.4
TCF143	Chinese chive	96.7	79.0 ± 6.2	<i>Ralstonia mannitolilytica</i> (AJ270258)	99.5
TCF148	Chinese chive	76.7	79.0 ± 3.3	<i>Ralstonia mannitolilytica</i> (AJ270258)	99.5
TCR103	Chinese chive	76.7	94.8 ± 7.6	<i>Mitsuaria chitosanitabida</i> (BCYP01000048)	99.1
TCR156	Chinese chive	96.7	94.8 ± 1.0	<i>Mitsuaria chitosanitabida</i> (BCYP01000048)	99.2
TCR158	Chinese chive	76.7	95.9 ± 0.8	<i>Mitsuaria chitosanitabida</i> (BCYP01000048)	99.3
TCR159	Chinese chive	83.3	95.9 ± 6.6	<i>Mitsuaria chitosanitabida</i> (BCYP01000048)	99.3
TCR167	Chinese chive	93.3	87.8 ± 4.0	<i>Mitsuaria chitosanitabida</i> (BCYP01000048)	99.3
TWR114	Welsh onion	50.0	68.5± 11.6	<i>Mitsuaria chitosanitabida</i> (BCYP01000048)	99.3
TWR120	Welsh onion	66.7	81.7 ± 7.5	<i>Mitsuaria chitosanitabida</i> (BCYP01000048)	99.3
TWR137	Welsh onion	90.0	86.2 ± 3.1	<i>Mitsuaria chitosanitabida</i> (BCYP01000048)	99.3
TWR165	Welsh onion	80.0	86.2 ± 3.1	<i>Mitsuaria chitosanitabida</i> (BCYP01000048)	99.3
TWR167	Welsh onion	70.0	79.0 ± 5.9	<i>Mitsuaria chitosanitabida</i> (BCYP01000048)	99.3

3 <sup>†</sup>Reduction in disease severity was calculated using the following formula: reduction of  
4 disease severity (%) = [(mean disease severity of control treatment) – (mean disease severity  
5 of bacterial treatment)/(mean disease severity of control treatment)] × 100%.

6 <sup>‡</sup>Trial 1 included three tubes for each treatment, and the experiment was conducted once.

7 <sup>§</sup>Trial 2 included three tubes for each treatment and the experiment was conducted thrice.

8 <sup>#</sup>Each value represents a mean ± standard error.

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

Isolate	Reduction of AUDPC (%) <sup>†</sup>	
	Trial 1 <sup>‡</sup>	Trial 2 <sup>§</sup>
TCR103	81.6	-7.2
TCR156	6.9	NT <sup>#</sup>
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

<sup>†</sup>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

<sup>‡</sup>Trial 1 included five tomato plants in each treatment.

<sup>§</sup>Trial 2 had three replicates, and each replicate included nine tomato plants.

<sup>#</sup>NT: not tested.

**Table 3.** Biocontrol effect of *Ralstonia* sp. TCR112 and *Mitsuaria* sp. TWR114 against bacterial wilt in tomato plants grown under glasshouse conditions

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

<sup>†</sup>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.**  $3 \times 10^8$  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.

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

<sup>§</sup>Reduction of **AUDPC** (%) = [(mean **AUDPC** of the control treatment – mean **AUDPC** of bacterial treatment)/mean **AUDPC** of the control] × 100

<sup>#</sup>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).

- 1 **Supplementary Table S1.** Bacterial genera of rhizobacterial isolates exhibiting antibacterial  
 2 activity against *Ralstonia pseudosolanacearum*

Chinese chive	Number of isolates	Welsh onion	Number of isolates	Tomato	Number of isolates
<i>Burkholderia</i>	59	<i>Burkholderia</i>	46	<i>Burkholderia</i>	48
<i>Pseudomonas</i>	15	<i>Acinetobacter</i>	11	<i>Pseudomonas</i>	6
<i>Achromobacter</i>	8	<i>Mitsuaria</i>	5	<i>Cupriavidus</i>	4
<i>Ralstonia</i>	8	<i>Arthrobacter</i>	3	<i>Streptomyces</i>	2
<i>Mitsuaria</i>	5	<i>Ochrobactrum</i>	3	<i>Paenibacillus</i>	2
<i>Arthrobacter</i>	6	<i>Agrobacterium</i>	3	<i>Arthrobacter</i>	1
<i>Paracoccus</i>	4	<i>Streptomyces</i>	1	<i>Flavobacterium</i>	1
<i>Delftia</i>	4	<i>Pseudomonas</i>	1	<i>Staphylococcus</i>	1
<i>Bacillus</i>	4			<i>Bacillus</i>	1
<i>Sphingomonas</i>	2				
<i>Agrobacterium</i>	2				
<i>Pseudoxanthomonas</i>	2				
<i>Streptomyces</i>	1				
<i>Novosphingobium</i>	1				
<i>Pantoea</i>	1				
<i>Mycobacterium</i>	1				
<i>Staphylococcus</i>	1				
<i>Acidovorax</i>	1				
<i>Cupriavidus</i>	1				
<i>Microbacterium</i>	1				