Applied Soil Ecology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

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

Malek Marian[a], Tomoki Nishioka[a], Hiroyuki Koyama[a], Haruhisa Suga[b], Masafumi Shimizu[a,*]

[a] The United Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, Japan
[b] Life Science Research Center, Gifu University, Gifu 501-1193, Japan

ARTICLE INFO

*Keywords:*
Non-pathogenic *Ralstonia*
*Mitsuaria*
*Ralstonia pseudosolanacearum*
Tomato
Rhizobacteria
Biological control

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 resistance in tomato plants. Furthermore, 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* (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

* Corresponding author.
*E-mail address:* shimizma@gifu-u.ac.jp (M. Shimizu).

https://doi.org/10.1016/j.apsoil.2018.04.005
Received 28 September 2017; Received in revised form 2 April 2018; Accepted 4 April 2018

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. 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 daecheongense* (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 (TSA) 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 $10^7$–$10^8$ 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 (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-old cultures 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. $8 \times 10^5$ 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 1–3). 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