Evaluation of Biocontrol Agents Against Bacterial Wilt in Tomato Using Seedling Screening

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Abstract. Tomato is among the most cultivated vegetable crops worldwide, and bacterial wilt (BW) caused by the *Ralstonia solanacearum* species complex (RSSC) is the most devastating disease affecting tomato, impacting food and nutrition security in many areas. Pesticides used for controlling plant diseases are hazardous to producers, consumers, and the environment, whereas biological control is potentially a sustainable and environmentally safe alternative for disease management. To identify efficient biocontrol agents (BCAs), twenty-five potential BCA isolates were screened for control efficacy to BW on ten-day-old tomato seedlings of highly susceptible (L390) and moderately resistant (L180) cultivars previously inoculated with *R. solanacearum* strain PSS4 (=Asian origin, Race 1, Phylotype I; Biovar 3). After ten days incubation at 28 °C in the growth chamber, wilting (W%) and biocontrol efficacy (BE%) percent were evaluated. Of the 25 BCAs tested, four significantly reduced W%, with BE% ranging from 50% to 80% for both varieties. The four BCA isolates were identified as *Talaromyces* sp., *Trichoderma* sp., *Bacillus* sp., and *Variovorax* sp. The seedling method allows the rapid and cost-effective *in vivo* screening of many potential BCAs to reliably identify those with higher bacterial wilt control efficacy for further testing.

Keywords: Tomato · Biocontrol · Bacterial wilt · Seedling screening

1 Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most cultivated and economically important vegetable crops with an estimated global market value of USD 88 billion [1]. Tomato production faces constant challenges of pests and diseases impacting food and nutrition security worldwide. Bacterial wilt, caused by strains of *Ralstonia solanacearum* species complex (RSSC), is among the most important and challenging diseases to control in tomato production systems, with yield losses ranging from 10–100% worldwide [2]. The bacteria enter the plant roots from the soil and multiply in and gradually block the vascular system causing wilting within a few days of infection often associated with yellowing of the youngest leaves. Under favorable conditions, the infected plant suddenly wilts and dies [3]. There is no efficient method to control bacterial wilt in tomato due

A. G. Abdullah et al. (Eds.): SEAVEG 2021, ABSR 23, pp. 415–422, 2023.
https://doi.org/10.2991/978-94-6463-028-2_43

to the broad host range, complex diversity of the pathogen, long-term survival in the soil, and latent infection in the host [4]. Integrated Pest Management (IPM) uses a combination of disease management methods for the sustainable, ecological, and effective control of the disease or disease complex [5]. Biocontrol is a crucial component of IPM and an alternative to pesticides that are harmful to producers, consumers, and the environment. Biocontrol is where the plant, the pathogen, the biocontrol agent (BCA), and the environment interaction results in a reduction of the pathogen population and reduced infection [6]. However, the discovery and screening of BCAs are often focused on the interaction of the pathogen and BCAs in the lab, and few BCAs are screened *in vivo* due to the costs, time, and laborious process of mass screening *in vivo*. Recently, a screening technique to study bacterial wilt using seedlings was reported [7], and adaptation for screening BCAs in seedlings also were proposed [8]. The seedling method allows for rapid screening of many BCA isolates and different host genotypes within a short time and at a minimum cost. Evaluation of a maximal number of putative BCAs increases the chance of discovering efficient BCAs. Here we report the isolation of potential biocontrol agents from soil and the seedling screening method for a fast and cost-effective evaluation of BCAs to control bacterial wilt in tomato. The seedling method is an innovative and valuable tool that reduces the space, time, and cost of screening BCAs *in vivo* to control bacterial wilt in tomato for further assessments.

2 Materials and Methods

2.1 Biocontrol Agent Isolation, Selection, and Inoculum Preparation

Soil samples were collected from surrounding the roots of tomato and pepper plants, and from an uncultivated organic, and a bacterial wilt sick field in the World Vegetable Center Headquarter campus in Taiwan (23°06'51.1"N, 120°17'45.6"E). Putative BCAs were isolated from the soil by serial dilution and culture plating on five different media Nutrient Agar (NA, Difco), Potato Dextrose agar (PDA, Merck), Semi-selective media (SM-1, [9]), modified Soil extract media (SEM, [10]), and *Trichoderma* selective media (TSM, [11]). Fungal and bacterial colonies were randomly selected for purification on NA for bacteria and PDA for fungi. Purified isolates were pre-selected based on gram stain test for bacteria and microscopic morphological observation for fungi. Gram-negative bacteria and fungi with morphological characteristics similar to *Fusarium, Colletotrichum,* and *Botrytis* were excluded to reduce the risk of selection of plant pathogenic isolates. Bacterial candidates were grown on 523 media [12], harvested using glass slides, suspended in water, and the concentration adjusted to about 10^8 cfu/ml ($O.D._{600} = 0.3$) for inoculum. For inoculum preparation fungal candidates were grown on PDA, harvested with glass slides, suspended in water with 0.01% TWEEN 20, and the concentration adjusted to 10^6 spores/ml with the aid of a hemocytometer.

2.2 Ralstonia Pseudosolanacearum Isolate and Inoculum Preparation

A virulent *R. pseudosolanacearum* (race 1, biovar 3, phylotype 1) strain (PSS4) isolated from bacterial wilt symptomatic tomato in Taiwan [13] was obtained from the World Vegetable Center Bacteriology Lab culture collection. To prepare inoculum, the stored culture was streaked on triphenyl tetrazolium chloride (TTC) plates and incubated at 30 °C

for 48 h. It was then transferred to 523 medium plates, incubated at 30 ºC for 24 h, harvested using glass slides, suspended in water, and the concentration adjusted to about 10^8 cfu/ml (O.D. $_{600} = 0.3$).

2.3 Seedling Inoculation

Seeds of the highly susceptible (L390) and the moderately resistant (L180) tomato cultivars were sterilized and placed on a sterile wet filter paper in a petri dish and incubated in a growth chamber at 28 ºC in the dark for 48 h. After germination, the growth chamber was set to a 12 h dark and 12 h light cycle. Sterile water was added when required to sustain the growth of seedlings. The inoculation process was slightly modified from the methods described by Singh et al. and Agarwal et al. [7, 8]. Briefly, ten-day-old seedlings were inoculated by root-dipping for 1 min in a 10^8 cfu/ml suspension of the virulent (PSS4) strain of *R. pseudosolanacearum.* After inoculation with the pathogen, seedlings were dried for 5 min and root dipped for 5 min in a suspension of 10^6 spores/ml for fungal or 10^8 cfu/ml for a bacterial candidate BCA. After inoculation with BCAs, seedlings were let to dry again for 5 min and then placed in a 2 ml microcentrifuge tube with water and incubated in a growth chamber at 28 ºC with 12 h:12 h light:dark intervals (Fig. 1). Ten days after inoculations, the BCA treatments presenting reduced wilting percent (W%) and higher biocontrol efficacy percent (BE%) were selected for evaluation in the greenhouse. Twenty-eight treatments were arranged in a randomized complete block design with four replicates and five plants per replicate. The positive control consisted of the commercial biopesticide product PMB01 with active ingredient based on *Bacillus amyloliquefaciens,* and the negative control consisted of seedling only inoculated with the PSS4 and no biocontrol. A check consisting of seedlings without inoculation of both PSS4 and biocontrol agents was also included.

2.4 Wilting and Biocontrol Efficacy Percent

Wilting and biocontrol efficacy were calculated using the following formulas:

$$\text{Wilting percent (W\%)} = (\text{Nw}/\text{Nt}) \times 100\% \quad (1)$$

Where Nw = number of wilted plants, and Nt = total number of plants.

$$\text{Biocontrol Efficacy percent (BE \%)} = [(\text{WUT} - \text{WT})/\text{WUT}] \times 100\% \quad (2)$$

Where WUT = Percent wilting in untreated plants (control), and WT = Percent wilting in biocontrol treated plants.

2.5 Biocontrol Identification

Biocontrol agent candidates with reduced wilting percent (W%) and higher biocontrol efficacy percent (BE%) were selected for identification by morphological and DNA methods. Isolates were characterized based on colony macroscopic characteristics, and fungal isolates spores and mycelium were observed under the microscope. Fungal DNA

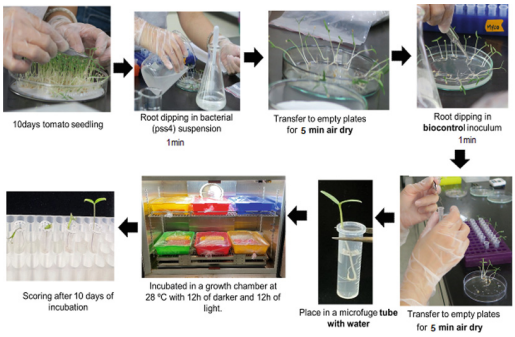

Fig. 1. Step of the seedling inoculation method.

were isolated as described by Burlakoti et al. [14] using FTA™ (Flinder Technology Associates, Whatman™) cards, and PCR amplified the gene region of ITS4 and ITS5. For bacteria isolates, bacterial suspension was directly processed for PCR amplification of the 16S-23S rDNA region. After sequencing, identification was confirmed based on DNA sequence similarity (98%) matched by BLASTn searching the National Center for Biotechnology Information (NCBI) GenBank database.

2.6 Data Analysis

For data analysis, wilting (W%) and biocontrol efficacy percent (BE%) were *arcsine* and square-root transformed and differences in W% and BE% between treatments were tested by analysis of variance (ANOVA), and means were compared by Tukey's HSD test at ($p \leq 0.05$) JMP 11. 1.1 (SAS Institute, Cary, NC).

3 Results and Discussion

Twenty-five of 125 potential biocontrol agents isolated from the soil surrounding the roots of tomato and bacterial wilt sick field were randomly selected and screened on ten-day-old tomato seedlings to evaluate the efficacy to control bacterial wilt. This screening was fast and cost-effective taking a total of 20 days to complete the experiment compared to the conventional *in vivo* screening that can take more than 60 days [15] and has the high cost of maintaining the plants, uses more space, greater amount of inoculum, and low feasibility to assess many BCA candidates at once. Eighteen out of twenty-five isolates reduced wilting significantly on both varieties L390 (highly susceptible) and L180 (moderated resistant) compared to the negative control (Table 1). Previous studies have reported different fungal and bacterial isolates that were able to control bacterial wilt in tomato [16–18]. Wilting percent on L390 was higher than on the moderately resistant cultivar L180 treated with biocontrol. This is not unexpected since the cultivars differ in reaction to infection by *R. pseudosolanacearum* where L390 is highly susceptible, and L180 is moderately resistant with wilting percent ranging from 90–100% and 40–60%, respectively [15, 19, 20]. This study found that wilting percent reduced up to 50% for L390 and 10% for L180 when BCA was applied to seedlings (Table 1), indicating that the

combination of resistance and BCA can better control BW than either alone. Of the 25 BCAs evaluated, four significantly reduced the W% with higher BE% ranging from 50% to 80% for both varieties (Table 1). The positive control, the commercial biopesticide product PMB01 (*B. amyloliquefaciens*) also showed higher BE%, and was among the five most efficient BCA candidates evaluated. Although all five BCAs significantly reduced W%, there was a difference among isolates efficacy ($P < 0.001$). This is expected as the top four isolates are different microorganisms, with two bacteria and two fungi. Further, DNA sequencing identified distinct species: isolate 52–86 was a *Talaromyces* sp., 26–81 was a *Trichoderma* sp., 106–85 was a *Bacillus* sp., and 106–86 was a *Variovorax* sp. Distinct species of BCA might differ in the mechanism of action yielding differences in biocontrol efficacy [6], and genetic variation within the same species of BCA that might cause variability in control efficacy [16, 21]. Higher W% was observed in some biocontrol treatments compared to the negative control suggesting an interaction between the biocontrol pathogen and the plant [6, 22]. Ten of the biocontrol treatments (isolates 80-45, 55-45, 48-86, 38-53, 32-86, 20-45, 110-81, 97-81, and 115-53) showed an increase of W% ranging from 17% to 5% compared to negative control. Two biocontrol treatments (isolates 115-53 and 97-81) showed contrasting interaction between the different tomato genotypes suggesting synergetic interaction between BCA with pathogen and BCA with host genotype. BCA mechanism of action may vary from antagonism to induction of plant resistance [6]. Identifying the mechanism of action of each biocontrol isolate was beyond the scope of this study; however, *Trichoderma* and *Bacillus,* two of the biocontrol candidates with high BE%, have been reported to promote plant growth, induce resistance, and antagonists of *RSSC* strains [6, 16, 23–25]. Species of *Variovorax* and *Taloromyces* have been reported to have antagonistic metabolites, compete with plant pathogenic microbes, and promote plant growth [23, 25, 26]. Using the seedling technique, this study demonstrated a fast and cost-effective screening of several BCA isolates with possible different mechanisms of action that can be selected for potential biocontrol application. Most studies select BCAs conducting *in vitro* analysis such as pair culturing to assess the BCA's antagonism effect on the pathogen [24, 25, 27]. Strains with no *in vitro* effect are often neglected and not tested *in vivo*. The selection of only BCA with antagonistic effect *in vitro* reduces the potential selection of BCA with mechanisms of action that directly interact with the host genotype, such as plant immunity inducers or growth promoters. The variation of the biocontrol treatments on different genotypes indicates that through the seedling method, screening *in vivo* biocontrol isolates allows identification of high BE% isolates that uses different mechanisms of action to control bacterial wilt.

4 Conclusion

Biocontrol candidates can be isolated from the soil of tomato fields, and inoculations of both BCAs and pathogen on young tomato seedlings allows the rapid and cheap *in vivo* screening to select BCAs with higher BW control efficacy for further testing reliably. Combining BCAs with the moderately resistant cultivar L180 significantly reduced W% compared to L180 alone. Isolates of *Talaromyces* sp., *Trichoderma* sp., *Bacillus* sp., and *Variovorax* sp. can reduce W% and are thus biocontrol candidates suitable for further testing for the control of bacterial wilt of tomato.

Table 1. Biocontrol efficiency (be%) and wilting (w%) percent of candidate BCAs against Bacterial Wilt in highly susceptible and moderately resistant tomato cultivars

Biocontrol Agents	L390 (BW susceptible)		L180 (BW Moderately resistant)	
	BE%	W%	BE%	W%
26-81	50^{b}	45^{DE}	56^{b}	20^{DC}
106-86	50b	45^{DE}	56^{b}	20^{DC}
PMB01	63^{a}	33^{E}	56^{b}	20^{CD}
52-86	61^{a}	35^{E}	89^{a}	5^{E}
120-81	56^{ab}	40^{E}	78^{a}	10^{D}
115-53	50^{b}	45^{DE}	-33^{g}	60^{A}
11-81	48^{bc}	47^{DE}	68^{b}	29^{C}
102-81	44^{c}	50^{D}	33^{c}	30^{C}
6-45	44^{c}	50^{D}	44^{bc}	25^{C}
26-81	44^{c}	50^{D}	78^{a}	10^{D}
44-45	44^{c}	50^{D}	0^{e}	45^{B}
28-45	33^{d}	60^{C}	0^{e}	45^{B}
127-81	22^{de}	70^{B}	44^{bc}	25^{C}
35-81	17^{e}	75^{B}	0^{e}	45^{B}
97-81	17^{e}	75^{B}	-38^{h}	62^{A}
27-81	8^{f}	83^{AB}	0^{e}	45^{B}
31-86	8^{f}	83^{AB}	29^{d}	32^{C}
116-86	0^{fg}	90^{A}	0^{e}	45^{B}
150-86	0^{fg}	90^{A}	0^{e}	45^{B}
39-53	0^{fg}	90^{A}	0^{e}	45^{B}
75-53	-6^{h}	95^{A}	0^{e}	45^{B}
110-81	-11^{h}	100^{A}	0^{e}	45^{B}
20-45	-11^{h}	100^{A}	-11^{f}	50^{AB}
32-86	-11^{h}	100^{A}	0^{e}	45^{B}
38-53	-11^{h}	100^{A}	0^{e}	45^{B}
48-86	-11^{h}	100^{A}	0^{e}	45^{B}
55-45	-11^{h}	100^{A}	0^{e}	45^{B}
80-45	-11^{h}	100^{A}	0^{e}	45^{B}
No bio	0^{fg}	90^{A}	0^{e}	45^{B}

Means were compared using Tukey's-HSD at the 95% confidence level. Means sharing the same letter(s) do not differ significantly.

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