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Evaluation of *Bacillus*-fortified organic fertilizer for controlling tobacco bacterial wilt in greenhouse and field experiments

Saifei Yuan[a], Lili Wang[c], Kai Wu[a], Junxiong Shi[b], Maosheng Wang[b], Xingming Yang[a], Qirong Shen[a], Biao Shen[a,*]

[a] Jiangsu Key Laboratory for Organic Solid Waste Utilization, Nanjing Agricultural University, Nanjing 210095, China
[b] Institute of Tobacco Science Research, Guiyang 550081, China
[c] Ningbo Academy of Agricultural Science, Zhejiang 315040, China

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ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most serious tobacco diseases worldwide, and no effective control measures are available to date. Three *Bacillus* isolates (*Bacillus amyloliquefaciens* SQR-7 and SQR-101 and *Bacillus methylotrophicus* SQR-29) were obtained from the rhizosphere soil of tobacco. These bacilli exhibited strong inhibition against *R. solanacearum* and produced indole acetic acid and siderophores. The three antagonistic strains were used to fortify organic fertilizers to produce bioorganic fertilizers (BOFs named for each isolate) for the control of tobacco bacterial wilt. The application of BOFs delayed wilt development and effectively decreased the disease incidence under both greenhouse and field conditions. The tobacco bacterial wilt control efficacy was 44.3%, 70.5%, and 85.1% using BOF101, BOF29, and BOF7 in the greenhouse. Although the control efficacies in the field were lower, the application of BOF7 still achieved 58.0% and 56.2% control efficacies in two years field experiments. The application of bioorganic fertilizer significantly ($p < 0.001$) repressed the pathogen *R. solanacearum* in soil in both pot and field experiments, though the abundance of *R. solanacearum* increased as during the growth period of the tobacco plants. In general, the populations of the antagonistic bacterial strains declined after soil application and as the tobacco plants grew; however, the density of SQR-7 and SQR-29 in the rhizosphere soil remained at a high level ($\geq 10^6$ cfu/g) in the later growth stages. Additionally, the application of bioorganic fertilizers promoted tobacco growth and increased the leaf yield.

1. Introduction

The bacterial wilt of plants is a systemic vascular disease caused by *Ralstonia solanacearum* (Yabuuchi et al., 1995), a soil-borne bacterial pathogen notorious for its lethality, persistence, complex subspecies, wide host range, and broad geographic distribution (Elphinstone, 2005). Soil polarization, resistant cultivars, short rotation, and soil fumigation have been suggested as integrated control strategies for bacterial wilt (French, 1994; Schönfeld et al., 2003). However, traditional control methods do not always show positive effects, as most pathogens, such as *R. solanacearum*, can persist for a long time in the soil in association with infested plant debris. Thus, the effectiveness of management is limited once the disease occurs (King et al., 2008). Chemical bactericides have proven ineffective in controlling bacterial wilt, causing even more damage than the organisms originally targeted for control, and are also harmful to the environment (Gamliel et al., 2000; Yi et al., 2007). Furthermore, disease-resistant tobacco cultivars often produce low-quality tobacco (Peng et al., 2007). Therefore, environmentally friendly pesticide alternatives, such as organic amendments (Bailey and Lazarovits, 2003; Céline et al., 2007; Gamliel et al., 2000) and beneficial microorganisms (Wang et al., 2012; Wei et al., 2011; Yang et al., 2011; Zhao et al., 2011), have been developed as components of conventional methods for controlling soil-borne diseases.

Several beneficial microorganisms have been successfully implemented as biocontrol agents for suppressing *R. solanacearum* under laboratory and/or greenhouse conditions: *Pseudomonas putida*, *Pseudomonas fluorescens*, *Bacteriophages*, *Streptomyces* spp., *Acinetobacter* spp., *Enterobacter* spp., *Bacillus* spp., and *Paenibacillus macerans* (Ji et al., 2008; Ling et al., 2010; Liu et al., 2012; Ramesh et al., 2009; Vanitha et al., 2009; Xue et al., 2009; Zhang et al., 2008). In contrast, reports on the control efficacy of these beneficial microorganisms on *R. solanacearum* in the field are rare.

Root colonization by biocontrol agents is considered a prerequisite and is directly related to their efficacy in controlling soil-borne diseases (Ji et al., 2008). In addition, niches and nutrients also become important when competition occurs between

* Corresponding author. Tel.: +86 25 84395212; fax: +86 25 84395212.
E-mail addresses: shenbiao@njau.edu.cn, yuansaifei@126.com (B. Shen).

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pathogens and antagonists (Kamilova et al., 2005). Previous studies have reported inconsistent results, whereby many bacterial antagonists with strong inhibition against *R. solanacearum* in vitro failed to show biocontrol activity under field conditions (Kamilova et al., 2005). Wei et al. (2011) demonstrated that two *Bacillus amyloliquefaciens* strains strongly antagonistic to *R. solanacearum* in the laboratory showed little disease suppression in autumn crop seasons. This finding is likely due to poor colonization or inadequate delivery of the agent in the rhizosphere environment (Lugtenberg et al., 2001).

Some studies have shown that *Bacillus* spp., *Trichoderma* spp., and *Klebsiella* spp. better colonized plant roots if they were applied to the soil with such preparations as organic fertilizer or chicken manure (Hao et al., 2009; Huang et al., 2011; Yang et al., 2011). Indeed, a decrease in the *R. solanacearum* population was observed in soil amended with compost (Schönfeld et al., 2003). Gorissen et al. (2004) also reported that the application of pig slurry significantly reduced the *R. solanacearum* population, possibly by inducing changes in the structure of the soil microbial community. Importantly, the combination of organic fertilizers and biocontrol microbes meets the requirements for sustainable agriculture by minimizing organic wastes and reducing chemical fertilizer and fungicide use in crop production (Bailey and Lazarovits, 2003; Céline et al., 2007). Nonetheless, reports on the effect of a combination of biocontrol microbes with organic amendments (e.g., compost, manure, plant solid waste) on the suppression of tobacco bacterial wilt (TBW) in a continuous cropping tobacco field are rare.

Thus, the main objectives of this study were to screen potential antagonistic bacteria from the rhizosphere soil of tobacco plants and evaluate the effect of *Bacillus*-fortified organic fertilizers (organic fertilizers augmented with three selected *Bacillus* isolates) on tobacco bacterial wilt under greenhouse and continuous field conditions.

2. Materials and methods

2.1. Isolation of the pathogen responsible for TBW and antagonistic bacteria

The TBW pathogen was isolated from the tissue of diseased tobacco plants suffering from bacterial wilt collected from the field in Fuquan, Guizhou Province, China, according to the method of French et al. (1995). A colony showing the typical morphology of *R. solanacearum* was chosen and named FQY-4; identification as *R. solanacearum* was based on the analysis of the 16S rRNA gene sequence (GenBank accession number KC888020). Strain FQY-4 was ultimately confirmed as the pathogen responsible for TBW based on Koch's postulates.

Rhizosphere soil was sampled from healthy tobacco plant roots. After serial dilution, the soil suspension was spread onto plates containing nutrient broth agar (NA) (Lemessa and Zeller, 2007). The plates were incubated at 30 °C for 48 h. The colonies were purified and screened for their antagonism to *R. solanacearum* FQY-4 using a spot-spraying method (Nishiyama et al., 1999). Briefly, the isolates were spotted using sterilized toothpicks onto NA plates. After a 24 h incubation at 30 °C, the plates were sprayed evenly with an FQY-4 suspension (10^7 cfu/ml) (≈0.2 ml) and subsequently incubated at 30 °C for an additional 24 h to observe the inhibition zones. The isolates with inhibition zones (≥15 mm) were selected and purified three times. The strains were also evaluated for growth in soil extract. To produce the soil extract, 500 g soil from a Fuquan field was added to 1000 ml water, boiled for 30 min, and filtered. The isolates were inoculated and incubated at 30 °C and 170 rpm for 48 h, and the OD_{600} values were determined using a spectrophotometer. Three isolates (SQR-7, SQR-29, and SQR-101) that showed strong antagonism and enhanced growth in the soil extract were selected as the experimental strains.

2.2. Elucidation of the functional traits of the isolates

Functional activity related to the production of siderophores was determined according to the method of Schwyn and Neilands (1987). After incubation at 28 °C for 3 days on plates, siderophores production was estimated by a change with the color from the blue to orange.

Salkowski reagent was used to examine indole acetic acid (IAA) (Maor et al., 2004). Briefly, isolates were grown in 100 ml Landy medium which was contained Tryptophan (Trp) at a final concentration of 20 μg/ml. After 60 h incubation with 170 rpm and 28 °C, cell-free supernatants were collected and 1.5 ml of supernatants was added into 1.5 ml of Salkowski reagent (12 g of ferric chloride per liter in 7.9 M sulfuric acid), and then the mixture was left in the dark for 30 min at room temperature for color variation. As the control, the same volume of Landy medium was added into Salkowski reagent.

2.3. Preparation of bioorganic fertilizers (BOFs)

The preparation of BOFs followed the procedure described by Zhang et al. (2008). The organic fertilizer (OF) consisted of a compost of cattle manure and rice straw and an amino acid organic fertilizer (1:1, w/w). The compost of cattle manure and rice straw contained 35% organic matter, 2.51% N, 2.4% P_2O_5, 1.13% K_2O, and 22.3% water (Jiangsu Tianniang Agri-technology Ltd., Jiangsu, China). The fertilizer contained a biologically hydrolyzed rapeseed cake with 44.2% organic matter, 12.93% total amino acids, 4.4% N, 3.5% P_2O_5, 0.67% K_2O, and 28.5% H_2O and was provided by Jiangsu Xintiandi Ltd. (Jiangyin, Jiangsu Province, China). The antagonistic bacterial SQR-7, SQR-29, and SQR-101 were grown separately in nutrient broth in a 5 L flask at 30 °C and 170 rpm for 24 h. The bacterial cells were harvested by centrifugation at 5000 × g and suspended in dH_20; the bacterial density was adjusted to 10^8 cfu/ml. The suspension of SQR-7 (6.3×10^8 cfu/ml), SQR-29 (7.8×10^8 cfu/ml), or SQR-101 (5.8×10^8 cfu/ml) was inoculated in the organic fertilizer at 10% (v/w) to obtain the bioorganic fertilizers BOF7, BOF29, and BOF101, respectively. After solid fermentation, antagonist SQR-7 reproduced to 6.8×10^8 cfu/g fertilizer in BOF7, SQR-29 reached 8.2×10^8 cfu/g fertilizer in BOF29, and SQR-101 reproduced to 7.5×10^8 cfu/g fertilizer in BOF101. The counts for the antagonistic agents were measured using selective media, as described below.

2.4. Pot experimental design

Pot experiments were conducted to examine the efficacy of three *Bacillus*-fortified organic fertilizers for bacterial wilt control and growth promotion effects on tobacco plants. Tobacco seeds (Honghuadajinyuan) (*Nicotiana tabacum* L.) were provided by Guizhou Tobacco Science Institute, Guizhou Province, China. The seeds were germinated in a nursery medium composed of perlite, vermiculite, and turf at a ratio of 3:3:4, according to the method of Dai et al. (2009).

The soil for the pot experiments was obtained from a field suffering from very severe TBW. The following treatments were designed: CK, the application of chemical fertilizer; OF, the application of organic fertilizer; BOF7, the application of bioorganic fertilizer BOF7; BOF29, the application of bioorganic fertilizer BOF29; and BOF101, the application of bioorganic fertilizer BOF101. The tobacco seedlings were transplanted into plastic pots (10 kg soil per pot) using 100 g bioorganic fertilizer or organic fertilizer thoroughly mixed with 1000 g soil that was evenly scattered