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Biological control tobacco bacterial wilt and black shank and root colonization by bio-organic fertilizer containing bacterium *Pseudomonas aeruginosa* NXHG29

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ABSTRACT

Tobacco bacterial wilt (TBW) and tobacco black shank (TBS) are two of the most devastating tobacco soil-borne diseases worldwide. In this study, *Pseudomonas aeruginosa* NXHG29 exhibited dually antagonistic activities against TBW and TBS *in vitro* assays. Pot experiments were performed to evaluate the capability of a novel bio-organic fertilizer (BOF) consisting of organic fertilizer with NXHG29 to control TBW and TBS. The results showed that application of BOF could more effectively decrease the disease incidence of TBW and TBS than the direct application of NXHG29. Higher amounts of BOF application (0.5% and 1% amendment) resulted in the more suppressive effects on tested pathogens when compared with a low amount of BOF application (0.1% amendment). To determine the antagonistic mechanism of NXHG29, we investigated the colonization pattern of NXHG29 on tobacco roots in a sand system and a natural soil system by tagging NXHG29 with a GFP-marked plasmid. Similar observations were obtained in the two systems. The results indicated that GFP-tagged NXHG29 colonized first the differentiation zones followed by the elongation and maturation zones of the primary roots and subsequently around the junctions of primary and lateral roots. The population dynamics of GFP-tagged NXHG29 on tobacco roots and in the rhizosphere were also monitored. The development of the BOF using dually antagonistic bacteria might provide new options for control strategies, especially with respect to managing both diseases simultaneously in the host plant, which should be more effective in the long term.

1. Introduction

Tobacco (*Nicotiana tabacum* L), is an economically important crop worldwide. Tobacco bacterial wilt (TBW) and tobacco black shank (TBS), respectively caused by *Ralstonia solanacearum* (Yabuuchi et al., 1995), and *Phytophthora nicotianae* (Lucas, 1975), are two of the most devastating soil-borne diseases resulting in severe losses in yield and quality of tobacco. However, traditional control methods such as agricultural and chemical control do not always show positive effects. Therefore, more recently, the use of microbial antagonists has been considered a promising strategy for the management of these soilborne diseases (Ling et al., 2010; Wei et al., 2011; Yang et al., 2011; Zhang et al., 2011; Zhao et al., 2011). In many tobacco-producing regions of China, bacterial wilt often co-occurred with black shank in the same plant and results in a disease complex that causes greater economic losses and renders the disease control more difficult than either pathogen acting alone (Liu et al., 2007; Wang et al., 2010). In general, a single biocontrol agent is usually used for biocontrol of plant disease against a single pathogen (Wilson and Backman, 1999). However, controlling just one of the pathogens might not fully solve the problem since different pathogens often co-occur on the same plant and result in a disease complex causing synergistic yield losses. An environmentally friendly alternative could be the use of an individual strain of microorganism with a broad spectrum of antagonism against multiple-pathogens, which might provide new options for control strategies for soilborne diseases. Consequently, recent research has focused on the

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discovery of biocontrol agents (BCA) with broad-spectrum activity to effectively control multiple plant pathogens in the same host plant. Dually antagonistic bacteria (DAB) refers to those bacteria in which one strain (or one species) of bacterium acts antagonistically against two diverse, co-occurring pathogens (Huang et al., 2015). The potential of DAB agents against disease complexes has been well demonstrated (Adam et al., 2014; Siddiqui and Ehteshamul-Haque, 2000; Son et al., 2009). However, no information is available concerning DAB agents against *R. solanacearum* and *P. nicotianae* in tobacco.

Directly deploying BCAs in the soil is the usual mode of application, but this mode may lead to poor activity of the functional agents. Many recent reports have shown that a combination of BCAs and organic amendments (e.g., compost, manure, plant solid waste) to create bio-organic fertilizers (BOFs) is a novel alternative method for biocontrol of soil-borne diseases (Luo et al., 2010; Saravanan et al., 2003; Yang et al., 2011) and is more efficient in inhibiting disease than using than using single BCAs (Huang et al., 2011; Liu et al., 2013). It is a promising strategy to control soilborne diseases which may be applied immediately for certain known functional microbial strains and organic amendments and may be further developed in the future. BOFs are not only a source of organic matter in soil which promote plant growth by improving soil structure, fertility, quality and nutrient use efficiency (Rivera-Cruz et al., 2008; Shen et al., 2013) but also supply nutrients for antagonists, allowing effective colonization and thus improving the suppressive capacity towards pathogens (Ling et al., 2010; Zhang et al., 2011). Studies have documented that the application of BOFs improved crop yields and successfully decreased bacterial disease incidences in many crops such as banana (Zhang et al., 2011), cucumber (Huang et al., 2011; Zhang et al., 2008), watermelon (Ling et al., 2010; Wu et al., 2009), tomato (Wei et al., 2011), tobacco (Ren et al., 2012), pepper (Wu et al., 2015), sweet melon (Zhao et al., 2011), and cotton (Lang et al., 2012). Nonetheless, reports on the effect of BOFs containing a combination of DAB strains with organic amendments as biocontrols for TBW and TBS are rare.

Significant progress in the understanding of the mechanisms for biocontrol of soil-borne pathogens by BCAs has been made in the past two decades (Compant et al., 2005). Root colonization by BCAs is considered a prerequisite for successful biological control and is directly related to their efficacy in controlling soil-borne diseases (Maurer et al., 2013). Therefore, research on the colonization pattern of antagonistic agents and their survival in the rhizosphere when introduced into soil is needed to get positive results in the biological control of soil-borne pathogens. Currently, biocontrol of soil-borne diseases using plant-growth-promoting rhizobacteria (PGPR) is a promising research area (Bhattacharyya and Jha, 2012). PGPR strains of *Pseudomonas aeruginosa* have been widely used in the control of soil-borne diseases because of their excellent root colonizing ability, catabolic versatility, and ability to produce a wide range of metabolites that favor the plant to stand under varied biotic and abiotic stresses (Kumar et al., 2009; Illakkiam et al., 2013; Sathyapriya et al., 2012). These abilities make strains of *P. aeruginosa* excellent candidates for sustainable biocontrol. An antagonistic strain, *P. aeruginosa* NXHG29, was isolated from the rhizosphere of cucumber by State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, Yunnan University, Kunming, China and showed strong antifungal activity against banana wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* (Li et al., 2012). The present study was, therefore, carried out to evaluate the antagonistic activity of *P. aeruginosa* NXHG29 towards *R. solanacearum* causing TBW and *P. nicotianae* causing TBS *in vitro*. In addition, we developed a novel bioorganic fertilizer (BOF) by fermenting mature compost with *P. aeruginosa* NXHG29, and the capability of the BOF to control TBW and TBS of tobacco was evaluated in greenhouse experiments. Finally, the *P. aeruginosa* NXHG29 strain was tagged with GFP, and the colonization pattern of this bacterium on tobacco roots in a sand system and a natural soil system was studied by using confocal laser scanning microscopy (CLSM). This is the first report on the investigation of the colonization by GFP-tagged *P. aeruginosa* of tobacco roots.

2. Materials and methods

2.1. Strains, plasmids and culture conditions

P. aeruginosa NXHG29 (GenBank accession number HQ844486) was previously isolated and identified as well as stored in our laboratory (Li et al., 2012). This bacterium was grown in NB medium (L^{-1}: beef extract 3 g, peptone 10 g, NaCl 5 g, distilled water 1000 mL, pH 7.0–7.2) on a shaker at 180 rpm and 37 °C for 48 h. The cultures of this bacterium were maintained on nutrient agar (NA) plates and stored at −80 °C in nutrient agar broth (NB) +25% glycerol. Pathogens *P. nicotianae* HD1 and *R. solanacearum* K1 were kindly provided by Yunnan Tobacco Agriculture Science Research Institute of China, and cultured routinely using potato dextrose agar medium (PDA) and Kelman's tetrazolium chloride (TTC) agar medium (Kelman, 1954), respectively. A green fluorescent protein (GFP)-expressing plasmid, pSMC2, which constitutively expressed a bright mutant of GFP and stably maintained in *Pseudomonas* strains, was kindly provided by Dr. Guido V. Bloemberg (Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts) (Bloemberg et al., 1997).

2.2. Bio-organic fertilizer (BOF) preparation

The preparation of the organic fertilizer (OF) used for the BOF preparation was carried out following the procedure described previously (Ling et al., 2010; Wu et al., 2009; Zhang et al., 2008), with minor modification. The OF used for the preparation of BOF consisted of a mixture of amino acid fertilizer and cattle manure compost (1:1, *w/w*). The amino acid fertilizer was made from Sacha Inchi (*Plukenetia volubilis* L) seed oil residues that were microbe-enzymatically hydrolyzed for 8 days to obtain a mixed amino acid fertilizer. This amino acid fertilizer contained 43.50% organic matter, 10.14% total amino acids, small molecular peptides and oligopeptides, 4.87% nitrogen (N), 3.02% P_2O_5 and 0.94% K_2O. *P. aeruginosa* NXHG29 was served as a biocontrol strain and its cell concentration of the suspension was 1.0×10^9 CFU mL^{-1} in the BOF preparation. The cattle manure compost was made by composting cattle manure at a temperature range of 30–66 °C for 25 days. This compost contained 45.5% organic matter, 3.35% N, 2.44% P_2O_5 and 1.2% K_2O. The BOF product used in this experiment was prepared according to Ren et al. (2012) and Zhang et al. (2011). The preparation was as follows: 1000 mL suspension of 1×10^9 colony-forming units (CFU) of *P. aeruginosa* NXHG29 per milliliter and 5 kg OF were thoroughly mixed in a 600 × 400 × 200-mm plastic container for secondary fermentation. The mixture was maintained at 40–45% moisture by adding sterile water at room temperature (25–32 °C) for 6 days and manually turned every day. On the 7th day, the mixture was air-ventilated in an aerated room at 19–24 °C for 2 days to decrease the moisture to less than 30%. During the secondary fermentation stage, the temperature and bacterial density of the substrate were monitored every day. The density of *P. aeruginosa* NXHG29 in the finished BOF product was more than 1×10^9 CFU g^{-1} dry weight (DW) of BOF. The BOF product was stored at room temperature prior to use in pot experiments.

2.3. Plant material and growth conditions

Tobacco variety Hongda was used in the experiments, which is susceptible to bacterial wilt and black shank disease. Tobacco seeds were provided by Yunnan Tobacco Agriculture Science Research Institute, Yunnan Province, China and were surface-sterilized with 2% household sodium hypochlorite for 3 min, rinsed four times using sterile distilled water, and then germinated on a 90-mm sterile plate covered with sterile moistened filter papers at 25 °C for 48 h. The germinated seeds were sown in floating polystyrene tray. The trays were