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Effects of *Bacillus amyloliquefaciens* ZM9 on bacterial wilt and rhizosphere microbial communities of tobacco

Bo Wu[a,b], Xiao Wang[a], Liang Yang[a], Huan Yang[a], Heng Zeng[a], Yimin Qiu[a,c], Changjun Wang[d], Jun Yu[d], Jinping Li[d], Dihong Xu[a], Zhili He[a,b,**], Shouwen Chen[a,b,c,*]

[a] State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China
[b] Institute for Environmental Genomics and Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK 73019, USA
[c] Hubei Collaborative Innovation Center for Green Transformation of Bio-Resources, College of Life Sciences, Hubei University, Wuhan 430062, China
[d] Tobacco Research Institute of Hubei Province, Wuhan 430062, China

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ABSTRACT

Ralstonia solanacearum, is a known soil-borne pathogen and causative agent of tobacco (*Nicotiana tabacum*) bacterial wilt (TBW) worldwide. In this study, we evaluated the effect of different biocontrol agent inoculants on TBW and rhizosphere microbial communities. *Bacillus amyloliquefaciens* strain ZM9 was selected for its antagonistic characteristics against this pathogen *in vitro* and its ability to colonize crops in pot-based experiments. ZM9 was effective in the control of TBW, and its effects on the tobacco rhizosphere microbial community were accessed using liquid-state fermentation cultures (LSFC) and solid-state fermentation cultures (SSFC) methods. The biocontrol efficacy of LSFC and SSFC groups was increased by 23.51% and 38.20%, respectively compared to the control group. Sequencing of 16S rRNA gene amplicons from the tobacco rhizosphere revealed the tobacco rhizosphere communities were dominated by *Proteobacteria*,*Acidobacteria*, *Bacteroidetes*, *Gemmatimonadetes* and *Actinobacteria*, and the relative abundance of *R. solanacearum* decreased in the biocontrol groups relative to the control group, and was negatively correlated with the abundance of ZM9. The dominant OTUs affiliated with *Sphingosinicella*, *Gemmatimonas* and *Gp1* with a negative correlation to the abundance of *Ralstonia*, were the beneficial bacteria for TBW control. In addition, the relative abundance of plant growth promoting rhizobacteria increased in both biocontrol groups. These results suggest an antagonistic effect of ZM9 on bacterial wilt pathogen, thereby highlighting the application of this strain and other potential biocontrol agents for controlling TBW in the field.

1. Introduction

Bacterial wilt, caused by the soil-borne pathogen *Ralstonia solanacearum*, is a plant systemic vascular disease notorious for its devastating lethality (Yabuuchi et al., 1995), wide host range (Salanoubat et al., 2002), and worldwide distribution across tropical, subtropical, and temperate regions (Liu et al., 2009; Xie et al., 2012). Several virulence factors contribute to the increased pathogenicity of *R. solanacearum*, including the production of high molecular mass extracellular polysaccharides, motility of the bacterium, and longevity in soil or water microcosms (Genin and Denny, 2012). Moreover, infected lands could become bacterial wilt prone areas and may not be used again for susceptible crops for several years (Genin and Denny, 2012). Chemical disinfection, short rotation and resistant cultivars are some of the strategies employed to control bacterial wilt (Lemaga et al., 2001; Lwin and Ranamukhaarachchi, 2006; Pradhanang et al., 2005; Xu et al., 2012). However, these measures do not effectively reduce the negative impact of this pathogen (Gamliel et al., 2000). As a result, environmentally sound and sustainable crop production has garnered significant interests in combatting the problem (Alabouvette et al., 2006; Berg, 2009). Consequently, researchers have focused their efforts on biocontrol practices, and the use of beneficial microorganisms is considered as one of the most promising approaches for rational and safe crop managements (Ongena and Jacques, 2008; Pal and Gardener, 2006).

Biocontrol agents (BCAs) identified in previous studies targeting *R. solanacearum* include *Bacillus* species (Huang et al., 2013;

* Corresponding author at: State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China.
** Corresponding author at: Institute for Environmental Genomics and Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK 73019, USA.
E-mail addresses: zhili.he@ou.edu (Z. He), chenshouwen@hubu.edu.cn (S. Chen).

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Xue et al., 2013; Yuan et al., 2014), *Brevibacillus brevis* (Chen et al., 2012), bacteriophages (Bae et al., 2012; Chappell et al., 2013; Fujiwara et al., 2011), nonpathogenic mutants of *R. solanacearum* (Chen et al., 2015; Trigalet and Trigalet-Demery, 1990), and *Streptomycete* strains (Tan et al., 2011). Among the above BCAs, *Bacillus* strains produce a broad spectrum of bioactive peptides with potentially inhibitory effects on phytopathogens (Baysal et al., 2013), and as spore-forming bacteria, those organisms are more easily stored and transported as stable products. Therefore, *Bacillus* strains are the best candidates for developing efficient biopesticide products (Ongena and Jacques, 2008), and some other important commercially available biocontrol agents include *Bacillus subtilis* strain GB03 (Kodiak), *Bacillus pumilus* strain GB34 (YieldShield; Gustafson), *Bacillus licheniformis* strain SB3086 (EcoGuard; Novozymes), a mixture of *B. subtilis* strain GB122 and *B. amyloliquefaciens* strain GB99 (BioYield; Gustafson), and several *Bacillus* spp. (*e.g.*, *B. subtilis* B916, *B. subtilis* B908) (Haas and Défago, 2005).

Sufficient production of antimicrobial metabolites and the ability to colonize the crop rhizosphere, are two key factors for successful protection against soil borne pathogens (Chowdhury et al., 2013). A wide arsenal of antimicrobial compounds produced by *Bacillus* strains are well studied, which include lipopeptides (*e.g.*, surfactin, iturin, fengycin) (Ongena and Jacques, 2008), bacteriocin (Abriouel et al., 2011), and macrolides (*e.g.*, macrolactin) (Mondol et al., 2012). Recently, subsequent real-time qPCR analysis was used to investigate the antibacterial related mechanisms of the *Bacillus* stains, and revealed that the expression of *ituC* (iturin A synthetase C) and *srfAA* (surfactin synthetase subunit 1) genes in *Bacillus* strains Am1 and D16 was remarkably up-regulated during *in vitro* interactions with *R. solanacearum*. These results suggest these lipopeptides have a potential to inhibit growth of *R. solanacearum* in the surrounding niche (Almoneafy et al., 2014). A recent study on the biocontrol efficacy of *B. amyloliquefaciens* BZ6-1 against the peanut bacterial wilt pathogen *R. solanacearum* indicated that the antimicrobial substances of surfactin and fengycin A both played an important role in inhibition of *R. solanacearum* growth (Wang and Liang, 2014). Root colonization by biocontrol bacteria has been considered as a prerequisite for successful biological control (Chowdhury et al., 2013), which may be largely dependent on the formation of biofilms (Bais et al., 2004; Weng et al., 2013). When the global transcriptional regulator (*abrB*), that negatively controls the biofilm formation was disrupted, the *abrB* mutant exhibited a stronger colonization activity than the wild-type strain (Weng et al., 2013). Thus, as the *Bacillus* strains possess these properties for producing antimicrobial metabolites and colonizing crops, they are considered as promising BCA candidates.

There is an extensive body of literature that describes the effect of BCAs on pathogen abundance and the occurrence of active compounds in the rhizosphere soil (Jacobsen et al., 2004; Yi et al., 2007; Zhao et al., 2014), but crop health is not solely affected by pathogens or BCAs; the interactions between members of rhizosphere-associated microbes (*e.g.* plant growth promoting rhizobacteria (PGPR)) often directly or indirectly impact crop health (Berg, 2009; Bonfante and Anca, 2009; Pal and Gardener, 2006). PGRP includes a wide range of soil bacterial genera such as *Pseudomonas* sp., *Rhizobium* sp., *Mesorhizobium* sp., *Acinetobacter* sp., *Psychrobacter* sp., *Bradyrhizobium* sp., *Bacillus* sp., *Stenotrophomonas* sp., *Azospirillum* sp., *Burkholderia* sp., *Azotobacter* sp., *Proteus* sp., and *Serratia* sp. which are involved in promoting plant growth and development via production and secretion of various regulatory chemicals in the vicinity of the rhizosphere (Ahemad and Kibret, 2014; Bhattacharyya and Jha, 2012). Additionally, a previous study on PGPR against tobacco blue mold disease caused by *Peronospora tabacina* indicated that five PGRP strains *Serratia marcescens* 90–166, *Bacillus pumilus* SE34, *Pseudomonas fluorescens* 89B-61, *B. pumilus* T4, and *B. pasteurii* C-9 elicited significant disease reduction (Zhang et al., 2002). Concurrently, the disturbance of indigenous rhizosphere microbial communities caused by BCAs has become a focal point in the use of biocontrol agents (Grosch et al., 2012; Kröber et al., 2014). A previous study showed that the treatment with a BCA, *Bacillus amyloliquefaciens* FZB42 did not have a major impact on the indigenous rhizosphere bacterial community based on 16S rRNA gene analysis with terminal restriction fragment length polymorphism (T-RFLP) (Chowdhury et al., 2013), and a later study about effects of the *B. amyloliquefaciens* FZB42 on the rhizosphere microbial community of lettuce by Illumina sequencing confirmed the previous results (Kröber et al., 2014). In addition, other studies used high throughput sequencing including ABI technology (Romero et al., 2015) and 454 pyrosequencing (Köberl et al., 2013; Qiu et al., 2012; Rosenzweig et al., 2012; Schreiter et al., 2014), to investigate the rhizosphere microbial communities. Those studies were focused on the influence of organic agriculture on microbial communities (Köberl et al., 2013), the soil type-dependent effects of biocontrol inoculant (Schreiter et al., 2014), microbial communities in the disease suppressive soil (Rosenzweig et al., 2012), and/or responses of microbial communities to the application of bio-organic fertilizer (Qiu et al., 2012). However, little is known about how BCA inoculants affect the abundance of soil pathogens, composition, and structure of the rhizosphere microbial communities of tobacco. Furthermore, it is unknown exactly how BCAs contribute to the dynamic changes in the tobacco rhizosphere community composition and structure as well as ecosystem functioning and stability (*e.g.*, crop yield, quality).

To expand our understanding about the dynamics of the indigenous rhizosphere community associated with the tobacco growth in a TBW prone area and the responses of rhizosphere microbial communities to applied BCA inoculants, a field trial was carried out at a TBW prone area in Enshi of Hubei Province, China. The main objectives of this study were to (1) screen the *Bacillus* biocontrol agent that could inhibit the tobacco bacterial wilt (TBW); (2) compare the biocontrol efficacy of two different formulas of ZM9; and (3) assess the impact of biocontrol inoculant on the crop and rhizosphere microbiota. Through MiSeq sequencing of 16S rRNA gene amplicons, we not only detected specific OTUs that significantly increased with the decreased pathogen abundance, but also identified significantly increased OTUs derived from known beneficial microorganisms such as plant growth promoting rhizobacteria (PGPRs). This study provides new insights into our understanding of the dynamics of tobacco rhizosphere microbial communities in response to biocontrol agent inoculation, highlighting the application of this strain and other potential biocontrol agents for controlling TBW in the field.

2. Materials and methods

2.1. Pathogen strain, soil samples and plant samples

R. solanacearum 9-2 (collection No.: ACCC 19857; 16S rRNA gene Genbank accession number: KP729638) was used as the pathogen in this study, which was provided by Hubei Tobacco Research Institute. Rhizosphere soil samples and plant samples were taken from a healthy crop zone and a sub-healthy crop zone in a TBW prone area in Xuanen County (109°26′20″ E, 29°59′55″ N), Enshi City, China. Three tobacco plants in the maturity stage were uprooted in the healthly and sub-healthly crop zone, respectively. Then, the rhizosphere soil was shaken off from the plant, and plants and soil were collected into sealed plastic pockets, and placed into an icebox.