Biological Control 88 (2015) 1–7

Contents lists available at ScienceDirect

Biological Control

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

Bacillus amyloliquefaciens GB1 can effectively control apple valsa canker

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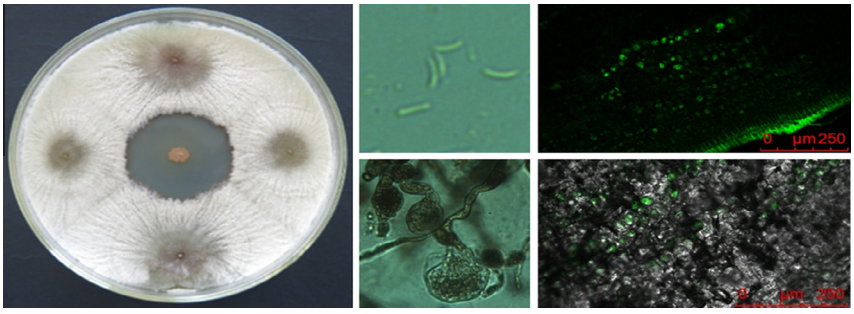
HIGHLIGHTS

- *B. amyloliquefaciens* GB1 inhibits significantly development of *Valsa mali*.
- *B. amyloliquefaciens* GB1 can form the biofilms over the wound of apple twigs by colonization.
- Our results demonstrate the strain GB1 as a promising agent for biocontrol of Apple valsa canker.
- Our results have provided new insights into the ability of *Bacillus* spp. to colonize the apple trees.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 January 2015
Accepted 22 April 2015
Available online 2 May 2015

Keywords:
Valsa
Green fluorescent protein (GFP)
Biological control
Bacillus
Cytospora

ABSTRACT

Apple valsa canker (AVC), caused by *Valsa mali*, is one of the most serious diseases of apple trees in eastern Asia, and the most important factor limiting apple production in China. This disease is difficult to control by chemical and agricultural measures, thus biocontrol may constitute a desirable alternative strategy. A *Bacillus amyloliquefaciens* strain denoted GB1 isolated from ageing cucumber stems, exhibited a strong antagonistic activity against *V. mali*, inhibiting significantly the germination of conidia and the growth of hyphae. GB1 conidial suspensions (above 10^6 CFU/ml) applied prior to wound inoculation of apple twigs with *V. mali* resulted in total inhibition of infection. Strain GB1 colonized xylem and phloem tissues surrounding the wounds made on apple twigs and formed biofilms over them. Results indicate that *B. amyloliquefaciens* GB1 may be a promising agent for the biocontrol of AVC, and provide new insights into the ability of *B. amyloliquefaciens* to colonize apple trees.

1. Introduction

Valsa mali Miyabe & Yamada (anamorph, *Cytospora mandshurica*), an ascomycetous fungus, is the agent of apple valsa canker (AVC), an ubiquitous destructive disease of apple trees in eastern Asia (Abe et al., 2007; Adams et al., 2006; Uhm and Sohn, 1995; Wang et al., 2005). Cankers caused by *V. mali* result in the death of twigs, limbs and stems and, eventually, of the entire tree (Ke et al., 2012). Because of repeated, serious outbreaks, AVC is one of the important factors limiting apple production in China (Wang et al., 2005), Japan (Abe et al., 2011) and Korea (Lee et al., 2006). Although the effect of several fungicides against *V. mali* has been studied in apple (Wang et al., 2009), AVC is difficult to control by chemical treatments since the pathogen invades the host phloem and xylem, where it is not accessible to conventional fungicide treatments (Yin et al., 2013). Furthermore, all current apple cultivars are susceptible to infection (Bessho et al., 1994; Liu et al., 2011), so AVC cannot be controlled by breeding for resistance.

In view of the need for reducing environmental pollution due to fungicide treatment against AVC, biological control is regarded as a most desirable practice, in line with the principles of sustainable agriculture. Biocontrol is based on the use of populations of antagonistic microorganisms, among which the *Bacillus*-based preparations have become important tools for disease suppression

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http://dx.doi.org/10.1016/j.biocontrol.2015.04.022
1049-9644/

(Jacobsen et al., 2004). *Bacillus* spp. synthetize different types of antimicrobial compounds against fungi, and favor the growth and defense responses of host plants. In addition, *Bacillus* spp. produce spores that allow them to resist adverse environmental conditions and permit an easy formulation and storage of the commercial products (Perez-Garcia et al., 2011).

Wounds elicited by frost damage, sunscalds, pruning ends and other mechanical injuries are regarded as the main infection routes for AVC (Adams et al., 2006; Kepley and Jacobi, 2000), thus their protection is a most effective manner to prevent invasion by *V. mali*. Wound-invading pathogens are vulnerable to biological control, because the antagonists can be applied directly to the targeted area (Janisiewicz and Korsten, 2002). There are a number of reports on the effective ability of *Bacillus* spp. in controlling the important plant fungal diseases, including root diseases (reviewed by Noble and Coventry, 2005), foliar diseases (reviewed by Jacobsen et al., 2004) and postharvest diseases (reviewed by Sharma et al., 2009). Compared to the long-standing interest in biological control of these diseases, research into biological control in trunk disease of fruit trees is in its infancy (Ren et al., 2013). In principle *Bacillus*-based biocontrol has an enormous potential to prevent *V. mali* infections. However, since relatively little is known about *Bacillus* spp. as an effective AVC biocontrol agent, this study was carried out with the aim of isolating and characterizing *Bacillus* spp. that antagonize *V. mali*, and assessing their potential as biocontrol agents.

2. Materials and methods

2.1. Isolation and identification of the pathogen

V. mali was recovered from an apple tree of cv. Fuji growing in the germplasm repository of the Research Institute of Pomology, Chinese Academy of Agricultural Sciences, Xingcheng (China). Single-spore isolates were obtained to ensure nuclear homogeneity (Choi et al., 1999) and the isolate SXP1 was identified by molecular tools. Total DNA was extracted and purified from SXP1 mycelium using the cetyltrimethylammonium bromide (CTAB) protocol (He, 2000). The extracted DNA was used as template for PCR amplification of the partial translation elongation factor-1a (TEF) gene with the primers EF1 and EF2 (Ali et al., 2008; O'Donnell et al., 1998), and the ribosomal DNA internal transcribed spacer (rDNA ITS) region with the universal primers pITS1/pITS4 (Lee et al., 2000), as described by the respective authors. Amplification products were purified using the MinElute Gel Extraction Kit (Sangon Biotech, Shanghai, China). The eluted fragments were ligated into pEASY-T3 simple vector (Trans, China) following the supplier's instructions, transformed into competent *Escherichia coli* TG1, then sequenced by the Beijing Genomics Institute (BGI, China). The sequences were aligned with the NCBI online database using the Basic Local Alignment Search Tool (NCBI BLAST) program.

2.2. Isolation of the antagonistic bacillus strains

The *Bacillus* strains were isolated from ageing cucumber stems which were cut into 1 cm-long pieces, soaked in 75% alcohol for 2 min and washed twice with sterilized saline (0.9% NaCl) prior to homogenization in 10 ml saline with mortar and pestle. The homogenate was transferred to 5.0 ml eppendorf tubes, incubated for at 80 °C for 10 min in a water bath, then serially diluted and plated onto LC (8 g of NaCl, 10 g of tryptone and 5 g of yeast extract in 1 l water) agar plates (Michielse et al., 2008). After culturing at 37 °C for 1 day, single bacterial colonies were picked, and cultured in LC broth.

Preliminary screening for *Bacillus* strains with antagonistic activity was conducted as described by Lin et al. (2014) with minor modifications. Conidial suspensions (1×10^6/ml, 100 μl) of *V. mali* were spread on potato dextrose agar (PDA) plates, and then obtained putative *Bacillus* strains were inoculated on the surface of the culture plate with toothpicks, allowing the microorganisms to encounter the pathogens directly. After incubated at 25 °C for about 4 days, the strains in the inhibition zone have tentatively been identified as the target microorganisms. The microorganisms were isolated from the inhibition zone on the PDA medium, and were streaked on a fresh LB plate for purification. After incubation for 1 day at 37 °C, the single colonies were picked and preserved, and then further examined for antagonism as the above methods.

2.3. Antagonistic activities on the dual culture

One of the strong antifungal strains, GB1, was further assessed using the dual culture method described by Raio et al. (2011) with minor modifications. Aliquots of 0.2 μl of the bacterial culture were placed at the center of PDA plates. At the same time, 1-mm diameter plugs were excised from the edge of actively growing colonies and placed at a distance of 3 cm from the bacterial colony. After incubation at 25 °C for eight days, the inhibition effect on fungal growth was assessed. Strain GB1 was also used for testing its inhibitory activity on the following phytopathogenic fungi, *Alternaria mali* (agent of apple spot leaf drop), *Glomerella cingulata* (Glomerella leaf spot of apple), *Botryosphaeria berengriana* (apple ring rot) and *Colletotrichum gloeosporioides* (apple anthracnose). Four plates were used for the experiment while four plates inoculated with the fungus alone served as control. The inhibitory activity was determined by comparing the mean diameters of the inhibition zone with the size of fungal colonies.

2.4. Identification of the bacillus strain GB1

The strain GB1 was identified by molecular tools. DNA was extracted from the LC-cultured bacterial cells as described (Sessitsch et al., 2003). 16S rDNA, DNA gyrase subunit A (*gyrA*) sequence and the histidine kinase (*cheA*) gene were amplified using primer pairs P0/P6 (Cello et al., 1997), GyrA-f/ GyrA-r and CheA_MF/CheA_MR (Reva et al., 2004), respectively, and the amplification conditions described by the respective authors. PCR products were purified and ligated into pEASY-T3 vector, transformed into competent *E. coli* TG1, and sequenced by BGI (China). The obtained DNA sequences were compared with those retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

2.5. Effect on conidial germination

The effect of strain GB1 on the germination of *V. mali* conidia was evaluated according to Moreira et al. (2014) with minor modifications. Conidia of isolate SXP1 were suspended in PD medium and 10 μl aliquots of conidial suspension (1×10^5/ml) adjusted using a hemocytometer were placed on microscope slides in the center of water agar (15%) plates. Fermentation broth, produced by strain GB1 growing in 10 ml of LC liquid medium on a rotary shaker at 180 rpm for 24 h at 37 °C, was diluted to 1×10^5, 1×10^6 and 1×10^7 CFU/ml, respectively. An equal volume of diluted fermentation broth was added to 10 μl aliquots of conidial suspensions. A 10 μl aliquot of strain GB1 suspension diluted to 1×10^7 CFU/ml was used as negative control, while 10 μl of conidial suspension adjusted 1×10^5/ml was the positive control. Petri plates containing the fungal-bacterial suspensions were incubated at 25 °C in the dark for 24 h. Conidial germination was determined by counting 50 conidia for each repetition, and germination rate was calculated using the formula: (mean germination number of control − mean germination number of the treatment)/mean germination number of the control × 100%. The experiment was performed four times.