Crop Protection 96 (2017) 173–179

Contents lists available at ScienceDirect

Crop Protection

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

Screening and characterization of endophytic *Bacillus* for biocontrol of grapevine downy mildew

Xun Zhang, Yingying Zhou, Yan Li, Xuechi Fu, Qi Wang*

Key Laboratory of Plant Pathology, Ministry of Agriculture, Department of Plant Pathology, China Agricultural University, No.2 Yuanmingyuan West Road, Haidian District, Beijing 100193, China

ARTICLE INFO

Article history:
Received 24 October 2016
Received in revised form
12 February 2017
Accepted 14 February 2017

Keywords:
Bacillus
Plasmopara viticola
Biocontrol
Bacterial endophyte

ABSTRACT

Grapevine downy mildew, caused by *Plasmopara viticola,* results in a serious and persistent disease problem for the grapevine industry worldwide and is difficult to control through chemical and agricultural means. The aim of this study was to screen bacterial endophytes with potential applications for biocontrol of this disease. A total of 239 bacterial endophytes were isolated from grapevine leaves and screened by leaf disk assays. Two strains, GLB191 and GLB197, which exhibited the most robust preventive effects against *P. viticola*, were tested in the field during two successive years (2013–14). The results demonstrated a dramatic decrease in disease severity after eight spray treatments, suggesting both strains as potential biocontrol candidates for *P. viticola*. The 16S rDNA and the *gyrA*/*gyrB* gene analysis identified GLB191 and GLB197 as *Bacillus subtilis* and *B. pumilus,* respectively. Confocal microscopy showed that both endophytes could recolonize grapevine leaves. This study is the first to demonstrate that endophytic *Bacillus* can inhibit grapevine downy mildew disease.

1. Introduction

Grapevine downy mildew, a widespread and destructive grapevine disease caused by *Plasmopara viticola* ([Burk. & Curt.] Berl. & de Toni), is reported to be one of the top 10 oomycete pathogens in plant pathology (Gessler et al., 2011; Kamoun et al., 2015). This obligately biotrophic pathogen infects all green parts of the plant and develops rapidly in locations with warm and humid climatic conditions throughout the growing season (Yin et al., 2014). Controlling this disease is generally difficult through conventional fungicide treatments because the pathogen penetrates extensively into the leaf tissues by formation of infection hyphae and haustoria (Toffolatti et al., 2011). Therefore, a new and more effective measure for control of *P. viticola* is needed.

In recent years, endophytes have emerged as promising biological control agents (BCAs) for their ability to enter and survive within plant tissues infected by the pathogen (Compant et al., 2010). These endophytes, which colonize host tissues before the pathogens, may produce pathogen-inhibiting metabolites and other defense-related compounds that hinder disease infection (Musetti et al., 2006; Rosenblueth and Martinez-Romero, 2006). The endophytic fungus, *Alternaria alternata,* isolated from grapevine leaves has been reported to be efficacious in controlling downy mildew disease through the production of toxic diketopiperazines metabolites (Musetti et al., 2006). Several other microorganisms, isolated either from rhizosphere or grape fruit surfaces have also been selected as BCAs over the last decades to control *P. viticola*, such as *B. subtilis* KS1, *Lysobacter capsici* AZ78, *Trichoderma harzianum* T39 and *Fusarium proliferatum* G6 (Falk et al., 1996; Perazzolli et al., 2008; Furuya et al., 2011; Puopolo et al., 2014). However, no endophytic bacteria have been reported as BCAs against *P. viticola*, although a large diversity of endophytic bacteria have been isolated from grapevine tissues, including leaves, xylems, stems and reproductive organs (Piccolo et al., 2010; West et al., 2010; Compant et al., 2011). Our objective was to identify promising bacterial endophytes with potential applications in biocontrol of grapevine downy mildew disease.

2. Materials and methods

2.1. Leaf sample collection and isolation of bacterial endophytes

Healthy grapevine leaves were sampled for the isolation of endophytic bacteria. Leaves from ten vineyards in major grapevine-

* Corresponding author.
E-mail address: wangqi@cau.edu.cn (Q. Wang).

http://dx.doi.org/10.1016/j.cropro.2017.02.018
0261-2194/

growing regions of China, including Beijing, Hebei, Sichuan, Shandong and Liaoning, were collected over different growing seasons in 2012. All of the samples were stored at 4 °C and processed within 24 h.

The leaf samples were first washed with sterile distilled water and subsequently surface-sterilized by soaking in 70% ethanol for 30 s, immersed in 2% sodium hypochlorite for 3 min, and rinsed four times with sterile distilled water (1 min each time). The water used for the last rinse was checked by spreading out 100 μL on tryptone soybean agar (TSA) plate (1.5% tryptone, 0.5% soya peptone, 0.5% sodium chloride, 1.5% agar, pH 7.0) and subsequently examining the presence/absence of colonies formed on the plates. Only leaves with last rinse solutions that yielded no colonies were used for isolation. Approximately 2 g of surface-sterilized leaves were collected and ground in 8 mL sterilized water using a sterile mortar and pestle. The resultant suspension was plated on TSA after serial dilution. The plates were then incubated at 30 °C for 3 days. Colonies of different morphology were transferred onto Luria-Bertani (LB) plates (1.0% tryptone, 0.5% yeast extract, 1.0% sodium chloride, 1.5% agar, pH 7.0) for further characterization and identification. All isolates were purified and stored at −80 °C in a nutrient broth containing 15% glycerol.

2.2. Preparation of P. viticola and other pathogens

The downy mildew pathogen *P. viticola* (Diez-Navajas et al., 2007) was isolated from naturally infected leaves of two-year-old *Vitis vinifera* cv. Muscat Hamburg grown in an untreated vineyard in Tianjin, China. The pathogen was maintained on Muscat Hamburg in a growth cabinet at 20–21 °C with a 12 h photoperiod and 90–99% relative humidity (RH) by subsequent weekly inoculations. The *P. viticola* inoculum was prepared by washing freshly sporulating lesions on the abaxial leaf surface with distilled water. The sporangial suspensions were then adjusted to a concentration of 1×10^5 sporangia/mL using ahemacytometer under a light microscope and were used in all the experiments.

Other phytopathogens listed in Table 2 were generously provided by Dr. Xinghong Li (Beijing Academy of Agriculture and Forestry, China), and the Department of Plant Pathology, China Agricultural University, China. All isolates were cultured on potato dextrose agar (PDA) medium (potato 200 g, glucose 20 g, sterile water to 1000 mL, pH neutral) at 25 °C.

2.3. Screening of endophyte isolates against P. viticola on leaf disks

The susceptible cultivar *V. vinifera* L. cv. Muscat Hamburg was used in the present study. Healthy leaves of susceptible cultivar Muscat Hamburg were used in the leaf disk assays. The fermentation broth of bacterial endophytes were prepared in LB medium in a shaker at 180 rpm for 2 days at 30 °C and diluted to 10^8 CFU/mL with sterile water. A total of 30 leaf disks (10 for each replicate) of 1.5 cm diameter were soaked in 100 mL of the prepared bacterial suspensions for 30 min. The same number of leaf disks soaked in sterile water were used as controls. All the leaf disks were then transferred to dishes with four-layer wet cotton gauze and covered by plastic wrap to retain moisture. *P. viticola* inoculum was sprayed onto the abaxial surface of the leaf disks using a hand sprayer 24 h later. Inoculated leaf disks were subsequently kept overnight in the dark at 20–21 °C and 90–99% RH for 4 h, and then subjected to a 12-h photoperiod.

Investigation of disease index (DI) was conducted 5 days post inoculation. The severity was assessed using the following 0–9 scale: 0 = no visible downy mildew development, 1 = 0–5%, 3 = 6–25%, 5 = 26–50%, 7 = 51–75%, 9= >76% leaf area affected (Reuveni, 1998). The DI was calculated according to the following formula: $DI = [\sum (f \times n)]/(N \times R)$, where f is the respective disease categories, n is the number of leaf disks of each class, N is the total number of leaf disks observed and R is the highest value of the evaluation scale (La Torre et al., 2014). The control efficacy was calculated using the following formula: Efficacy % = $[(DI_c - DI_t)/DI_c] \times 100$, where DI_c is the DI of control group, and DI_t is of the treated group.

Table 1
Biocontrol effect of GLB191 and GLB197 on grapevine downy mildew in leaf disk assays.

Treatment[a]	Disease index±SD[b]	Efficacy (%)
GLB191	13.7 ± 3.6b	69.0
GLB197	18.7 ± 3.1b	57.5
Water control	44.1 ± 11.1a	–

[a] The inocula concentration of GLB191 and GLB197 is 1×10^8 CFU/mL.
[b] The disease index was determined seven days after *P. viticola* inoculation. Values are representative of three experiments, and three replicates were used for each experiment. Different letters indicate significant differences according to Student's *t*-test ($P < 0.05$).

Table 2
Antagonist activity against various fungal plant pathogens by GLB191 and GLB197 *in vitro*.

Target fungal pathogens	Inhibitory activity[a]	
	GLB191	GLB197
Pestalotia menezesiana LXH02	+++	+
Colletotrichum gloeosporioides CBS 11299	++++	++++
Cryptosporella viticola LXH01	+++	+
Botryosphaeriaceae dothidea GS-08s1	++	+
Botrytis cinerea BCE4	+++	+++
Trichothecium roseum S24	++++	++++
Alternaria alternata BJ-MYD-T2	+++	+
Monilinia fructigena CXY11	++++	+
Botryosphaeria dothidea YL1	++	+
Valsa mali ZYY08	+	+
Fusarium roseum GS-TG-05	+	–
Alternaria solani BJ-YQ-KX-P2	+++	+
*Fusarinum moniliforme*HLJ-XC-X	++	–
Verticillium dahliae XJ14	+++	–

[a] +, ++, +++ and ++++ represent relative inhibition rates against mycelia growth of each fungal colony on the PDA medium to the extent of 50%–60%, 61%–70%, 71%–80% and >80%, respectively. - represents no inhibition rates.

2.4. In vitro antagonism tests

The spectrum of antagonistic activity of GLB191 and GLB197 against 14 phytopathogens was assessed on PDA plates by dual-culture method (Zhang et al., 2016). Plates measuring 9 cm in diameter, each containing 15–20 mL of PDA medium, were used. A pathogen disc (5 mm diameter) was placed in the center of each plate, and the plates were incubated for 2 days at 25 °C. The antagonistic strain GLB191 or GLB197 was then spotted at 2.5 cm from the center of the PDA plate. Plates inoculated only with the pathogen were used as the control. Each treatment was replicated three times. Following the inoculation, plates were incubated at 25 °C in the dark. The antagonistic activity was expressed as the inhibition rates against mycelia growth compared to the control. It was calculated by the formula: (R1 - R2)/R1 × 100, where R1 and R2 were the mycelial radial growth of the pathogen in the control and in the presence of the antagonist, respectively.

2.5. Field trial

The field trials were designed according to *GB/T 17980.*