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PLGENE-00066; No. of pages: 8; 4C:

Plant Gene xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Plant Gene

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

Rapid marker-assisted selection of antifungal *Bacillus* species from the canola rhizosphere

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ARTICLE INFO

Article history:
Received 20 July 2016
Received in revised form 20 October 2016
Accepted 30 October 2016
Available online xxxx

Keywords:
Biological control
Rhizosphere bacteria
Bacillus
Marker assisted selection

ABSTRACT

A marker-assisted approach was adopted to search for *Bacillus* spp. with potential as biocontrol agents against stem rot disease of canola caused by *Sclerotinia sclerotiorum*. Bacterial strains were isolated from the rhizosphere of canola and screened using multiplex PCR for the presence of surfactin, iturin A and bacillomycin D peptide synthetase biosynthetic genes. Among the 96 isolates screened, only CS-42 harbored all three genes and was subsequently identified as *Bacillus cereus* using 16S rRNA gene sequencing. This strain was found to be effective in significantly inhibiting the growth of *S. sclerotiorum in vitro* and *in planta*. Scanning electron microscopy studies at the dual culture interaction region revealed that mycelial growth was curtailed in the vicinity of bacterial metabolites. Complete destruction of the outmost melanised rind layer of sclerotia was observed when treated with the bacterium. Transmission electron microscopy of ultrathin sections challenged with CS-42 showed partially vacuolated hyphae as well as degradation of organelles in the sclerotial cells. These findings suggested that genetic marker-assisted selection may provide opportunities for rapid and efficient selection of pathogen-suppressing *Bacillus* strains for the development of microbial biopesticides.

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1. Introduction

The rhizosphere has been considered as a “Black Box” of microorganisms that provides a front line defence and protection of plant roots against pathogen invasion (Bais et al., 2006; Weller, 1988). A number of plant and soil associated beneficial bacteria are able to suppress plant pathogens through multiple modes of action, including antagonism (Pal and Gardener, 2006). The genus *Bacillus* occurs ubiquitously in soil and many strains are able to produce versatile metabolites involved in the control of several plant pathogens (Ongena and Jacques, 2008). The persistence and sporulation under harsh environmental conditions as well as the production of a broad spectrum of antifungal metabolites make the genus a suitable candidate for biological control (Jacobsen et al., 2004). Members of the *Bacillus* genus possess the ability to produce heat and desiccation resistant spores which make them potential candidates for developing efficient biopesticide products. These spores are often considered as factories of biologically active metabolites which in turn have been shown to suppress a wide range of plant pathogens. *Bacillus* spp. are known to produce cyclic lipopeptide antibiotics such as surfactin (Kluge et al., 1988), iturin A (Yu et al., 2002) and bacillomycin D and exhibit strong antifungal activity against a number of plant pathogens. The selection and evaluation of these bacteria from natural environments using direct dual culture techniques is a time consuming task. The discovery of lipopeptide synthetases genes associated with novel biocontrol and the development of gene specific markers has provided the opportunity to detect antagonistic *Bacillus* species from large numbers of environmental samples (Joshi and McSpadden Gardener, 2006).

A number of PCR-based methods have been developed for screening antagonistic bacteria (Raaijmakers et al., 1997; Park et al., 2013; Giacomodonato et al., 2001; Gardener et al., 2001). Recently, a high-throughput assay was developed to screen for antagonistic bacterial isolates for the management of *Fusarium verticillioides* in maize (Figueroa-López et al., 2014). There is no doubt of the contribution of these studies to accelerate the selection of potential biocontrol organisms. However, a more efficient and affordable molecular based approach is crucial for the detection of bacterial antagonists that can inhibit fungal growth, disintegrate cellular contents and protect plants from pathogen invasion. Therefore, the objective of this investigation was to develop a rapid and reliable screening method to identify *Bacillus* isolates from the canola rhizosphere with antagonistic properties towards *S. sclerotiorum*.

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http://dx.doi.org/10.1016/j.plgene.2016.10.002
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Please cite this article as: Kamal, M.M., et al., Rapid marker-assisted selection of antifungal *Bacillus* species from the canola rhizosphere, (2016), http://dx.doi.org/10.1016/j.plgene.2016.10.002

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The antagonism of marker selected bacteria was further evaluated against *S. sclerotiorum in vitro* and in glasshouse grown canola plants. In addition, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) studies were conducted to explore the inhibitory effect of bacteria on cell organelles in mycelium and sclerotia.

2. Materials and methods

2.1. Rhizosphere sampling

The root system of whole canola plants from Wagga Wagga (35°07008″S, 147°22008″E) were collected to a depth of 15 cm using a shovel and immediately placed in plastic bags for transport to the laboratory. The samples were immediately processed and the remaining placed in a cold room (8 °C) within 3 h of sampling and stored for a maximum of 3 days prior to processing. Soil samples from 10 individual canola rhizopheres were processed by removing the intact root system and adhering soil from each shoot using a clean scalpel. Each rhizosphere sample of 3 g was placed in a falcon tube containing 30 ml of sterile distilled water. To dislodge the bacteria and adhering soil from the roots each sample was vortexed four times for 15 s, followed by a 1 min sonication. Aliquots of 1 ml of the suspensions were stored in individually labelled microfuge tubes for further investigation.

2.2. Bacillus culture and maintenance

Microfuge tubes containing 1 ml of each soil suspension were incubated in a water bath at 80 °C for 15 min and then allowed to cool to 40 °C. Using a sterile glass rod, 50 μl of the soil suspension was then spread onto tryptic soy agar (TSA) and incubated for 24–48 h at room temperature. Following incubation, individual colonies were inoculated into 96 well microtiter plates (Costar) containing 200 μl of tryptic soy broth (TSB) and incubated at 40 °C for 24 h. A 10 μl aliquot of each liquid culture was transferred to fresh TSB in a new microtiter plate and incubated at room temperature overnight. A Genesys 20 spectrophotometer (Thermo Scientific) was used to assess the optical density of bacterial growth at 600 nm with a reading of ≥0.05 being scored as positive. Replicate plates were prepared by mixing individual cultures with 35% sterile glycerol (*v*/v) which were stored at −80 °C.

2.3. Preparation of combined isolates and lysis of bacterial cell

The bacterial isolates from 12 wells of the TSB cultures were combined by transferring 10 μl of each into a single well of a 96 well PCR plate (BioRad). This resulted in 8 groups from a total of 96 isolates. DNA was isolated from each combined sample using a freeze thaw extraction protocol as described by Gardener et al. (2001). Briefly, 90 μl of 1/5× PCR buffer (Promega) was placed into each well of a PCR plate and 10 μl of combined liquid culture was added and mixed thoroughly. The PCR plates were then placed into a −80 °C freezer for 8 min before being transferred to a FTS 960 thermocyler (Corbert Research) at 94 °C for 2 min. The incubation procedure was repeated thrice and the lysed cells stored at -20 °C.

2.4. Development of PCR assays

The target specific primers used in this study were as described by Ramarathnam et al. (2007). The combined template DNA (representing 12 isolates) was amplified using a combination of three gene specific primers corresponding to iturin A (~647 bp), bacillomycin D (~875 bp) and the surfactin lipopeptide antibiotic biosynthetase operon (~441 bp) (Table 1). DNA of individual isolates from combinations resulting in positive amplification was then re-amplified with the same primers to detect target isolate/s. The PCR reaction volume of 25 μl comprised of 12.5 μl of 2× PCR master mix (Promega), 0.5 μl mixture of primers (1.8 mmole/L), 2 μl (20 ng) of mixed template DNA and 10 μl of nuclease free water. The targets were amplified using the cycling conditions as initial denaturation 94 °C for 3 min, 35 cycles of at 94 °C for 1 min, annealing at 60 °C for 30s, extension at 72 °C for 1 min 45 s and final extension 72 °C for 6 min. The re-amplification of the positive strain group was conducted with individual template DNA using the same conditions. Amplified PCR reactions were separated on 1.5% agarose gels stained with gel red (Bio-Rad) in Trisacetate-EDTA buffer and gel images were visualized with a Gel Doc XR+ system (Bio-Rad). A 100-bp DNA size standard (Promega) was used to measure the size of the amplified products.

2.5. Identification of genes and bacteria

Each amplicon gel was excised and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. The purified DNA fragments were sequenced by the Australian Genome Research Facility (AGRF, Brisbane, Queensland). All sequences were aligned using MEGA v. 6.2 (Tamura et al., 2013) then corresponding genes were identified through a comparative similarity search of all publicly available GenBank entries using BLAST (Altschul et al., 1997). Bacterial DNA extracted from isolates that positively amplified with all three target primers were subjected to 16S rRNA gene sequencing following partial amplification using universal primers 8F and 1492R (Turner et al., 1999). The amplified DNA was purified and sequenced as described earlier. The sequence data was analyzed by BLASTn software and compared with available gene sequences within the NCBI nucleotide database. Strains were identified to species level when the similarity to a type reference strain in GenBank was above 99%.

2.6. Bio-assay of bacterial strains in vitro

The inhibition spectrum of marker selected *Bacillus* strains against mycelial growth and sclerotial germination were conducted according to the method described by Kamal et al. (2015b). *S. sclerotiorum* used in this study was collected from a severely diseased canola plant as described in Kamal et al. (2015a). The isolate was subcultured onto potato dextrose agar (PDA) and incubated at 25 °C for 3 days. Agar plugs (5 mm diameter) colonized with fungal mycelium were transferred to fresh PDA and incubated at room temperature in the dark. The marker-selected bacterial strain CS-42 was cultured in TSB at 28 °C. After 24 h fresh juvenile cultures of bacterial isolates were measured using a Genesys 20 spectrophotometer (Thermo Scientific) and diluted to 10^8 cells ml^{-1} which was subsequently confirmed by dilution plating. A 10 μl aliquot of two different bacterial strains were dropped at two equidistant positions along the perimeter of the assay plate and incubated at room temperature in the dark for 7 days. The inhibition of sclerotial germination by selected bacterial isolates was also investigated. Sclerotia grown on baked bean agar (Hind-Lanoiselet, 2006) were dipped into the bacterial suspensions and placed onto PDA and incubated at 28 °C. Sclerotia dipped into sterile broth were considered as controls. After 7 days incubation the germination of sclerotia was observed using a Nikon SMZ745T zoom stereomicroscope (Nikon Instruments). All challenged sclerotia were transferred onto fresh PDA without the presence of bacteria to observe germination capability. Both experiments were conducted in a randomized block design with five replications and assayed three times. The inhibition index for each replicate was calculated as follows:

Inhibition (%) $= \frac{R1-R2}{R1} \times 100$, R1 = Radial mycelial colony growth of *S. sclerotiorum* in control plate, R2 = Radial mycelial colony growth of *S. sclerotiorum* in bacteria treated plate.

2.7. Disease inhibition bioassay in planta

Antagonism of the potential biocontrol strain was evaluated against *S. sclerotiorum* on 50 day old canola plants (cv. AV Garnet) in the

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