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Antimicrobial Activity of *Bacillus* Spp. in the Biocontrol of Different Phytopathogenic *Agrobacterium* Isolates

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Abstract

The genus *Agrobacterium* consists of Gram-negative, soil-borne bacteria, both pathogenic and non-pathogenic for plants. Pathogenic strains include bacteria causing crown gall and hairy-roots diseases. Crown gall disease could cause fatal infection of young plants, and it is related to reduction in crop yield. Disease management in fruit crops worldwide is heavily dependent upon the application of synthetic fungicides for pathogen control. The use of microorganisms as biological control agents may represent an alternative method to control phytopathogenic bacteria. In this study we tested the

antagonistic effect of *Bacillus* spp. on *Agrobacterium* isolates originated from sour cherry, plum, blackberry, and grapevine.

The bacteria were isolated from collected samples of diseased sour cherry, plum, blackberry, and grapevine plants using young galls. Pathogenicity of the strains isolated from tumors was tested on sterilized and aseptically cut carrot disks by inoculation with bacterial suspension. The presence of galls was checked after four weeks. Different *Bacillus* spp. strains were tested for the production of compounds inhibitory to *Agrobacterium* strains using *In vitro* bioassay. A strain was scored positive if a clear inhibiton zone of at least 2 mm in diameter was observed.

Agrobacterium spp. were diagnosed as a pathogens of sour cherry, plum, blackberry, and grapevine using conventional methods based on the isolation on selective media, followed by pathogenicity tests on carrot disks. In vitro tests all of *Bacillus* spp. strains showed their antibacterial activity against different *Agrobacterium* isolates.

This study showed that *Bacillus* spp. Strains have potential as a agent in control of *Agrobacterium* spp.

Keywords:

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Introduction

The genus *Agrobacterium* consists of soil-borne bacteria, aerobic, motile, non spore forming, Gram-negative rods with perytrichous flagella. *Agrobacterium* spp. is a common disease of dicot plants including mainly stone and pome fruit trees, grapevines, roses and some ornamental plants. Members of the genus *Agrobacterium* are ubiquitous components of the soil microflora, the vast majority of which are saprophytic, surviving primarily on decaying organic matter. Pathogenic strains include bacteria causing crown gall and hairy-roots diseases. The crown gall bacteria can infect over 90 different plant families (De Cleene & De Ley, 1976) and are responsible for economic losses in nurseries of fruit trees and ornamental plants (Pulawska, 2010). This Bacteria induce tumors or hairy-roots, mainly on the underground part of the plant. One of the most important diseases caused by these bacteria is crown gall, responsible for extensive economic losses to nursery productions of fruit trees, roses and grapevines in many countries (Garrett, 1973; Kennedy & Alcorn, 1980; Sobiczewski *et al*., 1991). Bacteria causing tumors create a very heterogeneous group of strains classified to different species and biovars. Tumors inhibit plant physiological functions such as transport of water and nutrients. The disease seldom kills plants, but it can elicit lack of vigour and reduced growth. Crown gall and hairy root have been described as a the transfer and expression of a suite of *Agrobacterium* genes in a plant cell causes uncontrolled cell proliferation and the synthesis of nutritive compounds that can be metabolized specifically by the infecting bacteria (Schell et al., 1979). Thus, infection effectively creates a new niche specifically suited to *Agrobacterium* survival. The natural host range of *Agrobacterium* among species of the plant kingdom is rather extensive and includes members of most of the plant families. Crown gall disease is fatal infection for young plants, and is related to reduction in crop yield.

The mechanisms of biological control of plant pathogens by antagonistic bacteria and fungi have been the subjects of many studies (Janisiewicz et al. 2000). Antagonists are biocontrol agents such as bacteria, fungi, actinomycetes, viruses, and nematodes that reduce the number of disease producing activities of the pathogens (Whipps & Lumsden, 2001). Mechanisms of biocontrol of root and soil-borne pathogens are as a result of the direct action of antagonists on plant pathogens, through antibiosis, predation or parasitism, induced resistance of the host plant and direct competition for space and limited resources (Janisiewicz et al. 2000). Members of the genus *Bacillus* are known to produce a wide types of antimicrobial substances, including peptide and lipopeptide antibiotics, and bacteriocins (Stein, 2005). Members of the genus *Bacillus* are Gram-positive, aerobic and endospore-forming bacteria that are characterized by their rod-shaped cell morphology, catalase production and their ubiquitous distribution. The production of antimicrobial substances and sporulation capacity confer *Bacillus* strains with a double advantage in terms of their survival in different habitats. The aim of this study was to screen

Bacillus spp. isolates *in vitro* for antagonism against different *Agrobacterium* spp. isolates originated from sour cherry, plum, blackberry and grapevine.

Material and Methods

Pathogen Isolation

The bacteria were isolated from collected samples of diseased sour cherry, plum, blackberry, and grapevine plants using young galls. Gall surface was washed under running water, surface sterilized for 10 minutes in 1% sodium hypochlorite solution and rinsed with sterile water prior to isolation. The small pieces of tumor tissue were macerated, soaked in sterile distilled water to allow difussion of bacteria into the liquid and bacterial suspension was streaked on nutrient agar plates (NA) (Milijašević et al., 2007). Plates were incubated at 25°C and examined after 2-3 days. Presumptive colonies were purified by streaking onto PDA medium with 0.5% $CaCO_3$ and PYGA medium (0.3% peptone, 0.5% yeast extract, 1% glicerol and 2% agar). Single cell colonies were transferred on to NA slants and stored at 4°C. The strains CFBP 2621 (*A. vitis*), CFBP 4442 (*A. tumefaciens*) from the French collection of phytopathogenic bacteria, were used in this work as referent strains.

Pathogenicity Tests

Pathogenicity of the strains isolated from tumors was tested on sterilised and aseptically cut carrot disks by inoculation with bacterial suspension (containing 10^7 cfu/ml). Carrot disks treated in the same way with distilled water served as negative control and disks inoculated with the reference strains were used as positive control. Inoculated carrot slices were placed on moistened sterile filter paper in Petri dishes and kept at room temperature for three weeks. Plants inoculated with water served as negative control, while plants inoculated with the reference strain served as positive control. The presence of galls was checked after four weeks.

Polymerase Chain Reaction (PCR)

Total genomic DNA was prepared by using a modification of the procedure of Ausubel et al. (1992). Cultures were grown on YDC medium for 48 h at 25°C. Bacterial cells were washed with sterile distilled water and centrifuged at 4,000 × g for 10 min at 4°C. The pellet was washed twice in 0.85% NaCl and once in 0.1M $NaPO_4$ buffer, pH 6.8. Cells were treated with 10% sodium dodecyl sulfate (SDS) and mixed with proteinase K at 37°C for 1h. DNA was purified using a 5M NaCl and solution of 10% hexadecyltrimethyl ammonium bromide (CTAB) in 1M NaCl at 65°C for 10 min, followed by phenol-chloroform and chloroform extractions. The DNA was recovered by isopropanol precipitation, redissolved in Tris-EDTA (TE, 10mM Tris, 1 mM EDTA, pH 8.0), and quantified spectrophotometrically at 260 nm. PCR was conducted using primers specific for detection of tumorigenic agrobacteria (complementary to the *tms*2 gene) with the following

sequence: tms2F1 (5` TTT CAG CTG CTA GGG CCA CAT CAG 3`) and tms2R2 (5` TCG CCA TGG AAA CGC CGG AGT AGG 3`) (Pulawska and Sobiczewski, 2005) where the expected PCR products are 617 base pairs. DNA amplification was performed in a total volume of 25 µl. All reactions contained: 1 x PCR Master mix (Fermentas, Lithuania) (0.625 U Taq polymerase, 2 mM $MgCl_2$, 0.2 mM each dNTPs), 1 µl of each primer (20 µM) and 1 µl of template DNA. Sterile deionized water was used as negative control, and the reference strains were used as positive control. Amplification conditions were: initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, extension at 72°C for 1.5 min, and a final extension step at 72°C for 10 min. (Milijašević, et al., 2007). Amplified PCR products were separated by gel electrophoresis on 1% agarose gels in 0.5 X TAE buffer for 1 h at 5 V/cm, stained with ethidium bromide, and visualized under UV illumination.

In Vitro Bioassay

Different Bacillus sp. strains were tested for the production of compounds inhibitory to Agrobacterium spp. strains as described elsewhere (Harris et al., 1989). *Bacillus* isolates used in this study originates from The Collection of The Laboratory of Microbiology, Faculty of Biology, University of Belgrade. A collection of 203 different *Bacillus spp*. isolates were tested *in vitro* for inhibitory activity against *Agrobacterium* isolates using agar well diffusion assay. Cultures of *Bacillus* were grown in Luria Bertani (LB) medium for 24 h at 30°C. Luria Agar (LA7) soft agar (0.7% m/v) containing *Agrobacterium* isolates were overlaid onto LA plates. Wells were made in the lawn of solidified Luria Agar (LA) medium to which aliquots (50 µl) of supernatant of overnight culture were added. The plates were than incubated overnight at 30°C. The zone of inhibition was measured after 24 h of incubation. The appearance of a clear zone representing growth inhibition of a sensitive strain around the well was taken as a positive signal for inhibitory activity. Microbial interactions were analyzed by determination of the size and shape of inhibition zone.

Results

Pathogenicity Test

All tested strains caused small, green tumors on carrot disks three weeks after inoculation. No changes were observed on carrot slices inoculated with water, while the positive control strains CFBP 2621 (*A. vitis*), CFBP 4442 (*A. tumefaciens*) induced tiny, green tumors three weeks after inoculation.

Polymerase Chain Reaction (PCR)

The appropriate amplification bands was obtained after amplification with primers for the tms2 gene. Using tms2F1 and tms2R2 primers, 617 bp PCR products specific for tumorigenic *Agrobacterium* strains were detected.

Therefore, we confirmed that the strains isolated from tumors in sour cherry, plum, blackberry, and grapevine plants were tumorigenic *Agrobacterium* strains.

In Vitro Bioassay

Different Bacillus spp. strains were tested for in vitro inhibitory activity against Agrobacterium spp. isolates. Results from the *in vitro* bioassay showed that out of 203 *Bacillus* strains, nine strains showed antimicrobial activity against *Agrobacterium* isolates originated from sour cherry, plum, blackberry, and grapevine, with varying efficiencies. Distinct inhibition zones were observed when *Bacillus* strains were used against *Agrobacterium* isolates. Three *Bacillus* strains showed strong and very strong inhibition zones to the *Agrobacterium* isolates, strain 12.6 (10-16 mm), strain 13.1 (5-10 mm), and strain 51.1 (5-8 mm). Very strong inhibition zones were revealed after 24h of incubation.

Discussion

Agrobacterium spp. is a common disease of dicot plants including many woody shrubs and various herbaceous plants including mainly stone and pome fruit trees, grapevines, roses and some ornamental plants (Rhouma et al., 2006). The bacteria that induce crown gall are responsible for great losses, first of all in nursery production of fruit trees, roses and grapevines worldwide (Kennedy & Alcorn, 1980). *Agrobacterium* were diagnosed using conventional methods based on the isolation on selective media, followed by pathogenicity tests on carrot disks. PCR amplifications was conducted with specific sets of primers and were confirmed the presence of *Agrobacterium* spp. on sour cherry, plum, blackberry, and grapevine in Serbia. Trends in research include the increased use of biorational screening processes to identify microorganisms with the potential for biocontrol. In previous studies have been suggested that employment of microorganisms with antimicrobial activity may be an alternative or supplementary method to chemical plant protection. Mechanisms of biological control agents are diverse and include the induction of plant resistance by elicitors, the interference of pathogen infection pathways with antagonistic microorganisms and direct suppression of pathogens with antimicrobial natural compounds. These different, yet complementary, approaches for disease control have shown great promise in laboratory conditions, but many have failed to realise their potential in the field due to various unforeseen biological, technical, economic or regulatory hurdles (Fravel, 1999). As one of ideal candidates for use as a biocontrol agent appeared *Bacillus*. The strains of bacteria *Bacillus* have been widely used against a number of economically important plant pathogenic bacteria. *Bacillus* spp. has special characteristics that make them good candidates as biological control agents (Beric et al., 2012). *Bacillus* inhibited growth by establishing a clear inhibition zone in a *in vitro* bioassay test. The inhibition of growth by the

forming of an inhibition zone against *Agrobacterium* is considered as metabolite production. Screening is a critical step in the development of biological control agents. The success of all subsequent stages depends on the ability of a screening procedure to identify an appropriate candidate. Several recent studies have shown that antagonistic microorganisms from the genus *Bacillus* can help limit pathogen damage in various fruits (Živković et al., 2010). Our results support these findings by showing that *Bacillus* restrict the *in vitro* growth of *Agrobacterium*, economically important pathogens of fruits trees in nurseries. The *in vitro* results do not necessarily translate to what occurs *in planta*. Nonetheless, this study and the results are particularly useful for identifying likely candidates for biocontrol and for making educated guesses concerning the mechanisms by which they reduce pathogen damage. Our results demonstrated the prospect of *Bacillus* spp. as a potentially promising biocontrol agent for *Agrobacterium* spp. caused disease.

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