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Biological control of pathogenic bacteria by plant growth promoting rhizobacteria Bacillus amyloliquefaciens MG214652

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Abstract: Employing plant growth promoting rhizobacteria (PGPR) as biocontrol agents is of considerable interest. Some PGPR has the ability to produce one or more antibiotics that act as antimicrobial agent against pathogens. In this study, Bacillus MAP3 which was isolated from root nodules of Phaseolus vulgaris is evaluated as a biocontrol agent. The isolate MAP3 appeared milky white, opaque, irregular in shape, smooth and 2-4 mm in diameter after incubation at 37°C for 24 h and was found to be a Gram-positive spore-forming bacilli. The isolate MAP3 was tested for its antimicrobial activity against 9 pathogens. The antimicrobial activity was great against Erwinia carotovora and Bacillus subtilis (inhibition zone more than 25 mm), moderate against Candida albicans (inhibition zone 16 mm), weak against E. coli, Klebsiella spp, Staphylococcus epidermidis and Pseudomonas aeruginosa (inhibition zones were 11, 12, 10, 10 mm respectively) and there was no effect against Shigella spp. and Proteus vulgaris. The metabolites of this isolate showed antimicrobial activity when tested against Erwinia carotovora with an inhibition zone 20 mm. The results obtained put this isolate as a promising biocontrol agent that would be used against further pathogens.

keywords: PGPR, Bacillus amyloliquefaciens, Phaseolus vulgaris, antimicrobial activity, metabolites.

1.Introduction

Large number of plant growth promoting rhizobacteria (PGPR) were isolated from different legumes particularly root and nodule tissues including: Agrobacterium, Bacillus, Curtobacterium, Enterobacter, Erwinia, Herbaspirillum, Mycobacterium, Paenibacillus, Pseudomonas, Phyllobacterium, Ochrobactrum, Sphingomonas, Rhizobium, Ensifer, Mesorhizobium, Burkholderia, Phyllobacterium, and Devosia [1].

PGPR have direct and indirect mechanisms by which they interact positively with plants. The direct mechanisms are related to plant nutrition and development such as the production of plant growth regulators including abscisic acid [2], gibberellins (GAs), and indole acetic acid (IAA)[3-6]; nitrogen fixation that contributes to the accumulation of available nitrogen in soil; phosphate solubilization that makes P available for plant uptake[7, 8] ; and siderophore production that improves Fe acquisition[9, 10] . Indirect mechanisms principally include microbial antagonism/competitiveness[11] and enhancement of induced systemic resistance (ISR) and suppress the incidence of plant diseases[12]. This can be achieved by producing certain antimicrobials such as antibiotics, hydrogen cyanide, lipopeptide bio-surfactant, and production of siderophores.

The production of one or more antibiotics is the mechanism most commonly associated with the ability of plant growth-promoting bacteria to act as antimicrobial agents against phytopathogens[13]. The antibiotics, either volatile or non-volatile compounds produced by biocontrol agents during antagonism, inhibit the growth of pathogens

Antibiotics encompass a heterogeneous group of organic, low-molecular-weight compounds that are deleterious to the growth or metabolic activities of other microorganisms[14]. Numerous types of antibiotics have been isolated from PGPR and this diversity includes mechanisms of action

that inhibit synthesis of pathogen cell walls, influence membrane structures of cells and inhibit the formation of initiation complexes on the small subunit of the ribosome[15].

Production of antimicrobial metabolites is commonly observed during microbial interaction of antagonistic microorganisms and pathogens. Since these secondary metabolites are biologically synthesized, they are highly selective for target organism and hence, have little effect on beneficial organisms. Besides, as organic compounds, these metabolites are inherently biodegradable and often do not accumulate in nature and are safe to the environment[16].

Bacillus and *Pseudomonas* are the two most important genera among PGPR that are studied quite extensively for antimicrobial metabolites in the plant disease control programs. Bacillomycin, fengycin, iturin A, mycosubtilin and zwittermicin A are some of the important antibiotic secondary metabolites produced by *Bacillus* spp. and are widely used in plant disease control[17, 18].

Antibiotics, such as polymyxin, circulin and colistin, produced by the majority of *Bacillus* ssp. are active against Gram-positive and Gram-negative bacteria, as well as many pathogenic fungi[15].

Bacillus amyloliquefaciens is closely related to *B. subtilis* and is able to produce a variety of structurally diverse antimicrobial compounds which are used in biotechnology and biomedical applications. Many strains of *Bacillus amyloliquefaciens* are known to suppress fungal and bacterial growth *in vitro* by the production of several antimicrobial compounds[19].

This study aims to assess B. MAP3 which was recently isolated as a biocontrol agent against several pathogenic bacteria.

2. Materials and methods

Bacterial strains

The bacterial isolate *Bacillus* MAP3 was generously donated by Mona Agha (Assistant lecturer at Botany Department, Faculty of Science, Mansoura University) as it was isolated from the root nodules of *Phaseolus vulgaris*. 16s rRNA analysis indicated a high level of identity (99%) to members of genus *Bacillus amyloliquefaciens* in GenBank database in the National Center for Biotechnology Information (NCBI) and it has been given the accession number MG214652 on this database. The strain was kept in 30% glycerol stocks at -20°C for further experiments. LB medium was used to refresh the growth at 37°C for 24 h.

Morphological characterization of *Bacillus* MAP3

Characteristics of the bacterial isolate as colony form, color, margin, texture, polysaccharides and pigments formation was taken into account for preliminary characterization of the isolate by examining the growth of the isolates on solid media. Cells were stained with Gram's stain and examined under a microscope at 100X [20].

Antibacterial activity of *Bacillus* MAP3

Whole cell antimicrobial assay

The isolate MAP3 was cultured in LB medium at 37°c for 3 days for antibacterial assay. The following test organisms obtained from Genetic Engineering and the Biotechnology unit, Faculty of Science, Mansoura University were used for antibacterial assay: E.coli, Klebsiella spp, Erwinia carotovora, Bacillus subtilis, Staphylococcus epidermidis, Pseudomonas aeruginosa, Shigella spp., Proteus vulgaris, and Candida albicans. The test organisms were cultured on LB media for 24 h before antibacterial assay and the OD were around to be 1. The antibacterial activity was evaluated using the disc diffusion method [21]. 15-cm Petri dishes LB media were inoculated with the test organisms and streaked evenly in 3 planes onto the surface of the medium with a sterilized cotton swab to ensure growth spreading. Then 5 mm sterilized filter paper discs immersed in *Bacillus* MAP3 culture were placed on the previously inoculated plates with the tested organisms. Sterilized discs immersed in sterilized LB culture were used as a negative control. The plates were incubated at 37 °C for 24 hours, then the diameters of the inhibition zones were observed.

Metabolites antimicrobial assay

A 30 ml LB broth inoculated with *Bacillus* MAP3 was incubated for 5 days at 37°C. After centrifugation at 10,000 rpm for 20 min the supernatant was further filtered through a 0.45 µm pore size syringe filter to ensure the absence of cells under aseptic conditions. The cell-free culture supernatant containing the active metabolites of *Bacillus* MAP3 was used for the antibacterial assay.

The antibacterial activity of cell-free supernatant was evaluated using agar well diffusion method [22]. *Erwinia carotovora* was used as a tested organism in this experiment. The LB agar plate surface was inoculated by *Erwinia carotovora* over the entire agar surface. Then, 3 holes with a diameter of 6 mm have been punched aseptically with a sterile tip, and a volume of 100 µL of the bacterial culture and cell-free supernatant were introduced into the wells. Uninoculated LB medium was used as a negative control. The plates were incubated at 37°C for 2-3 days for inhibition zone development

3. Results and discussion

The isolate used in this study was obtained from the root nodule of *Phaseolus vulgaris*, a work that has been done by Mona Agha and the isolate was identified as Bacillus with very close homology to *Bacillus amyloliquefaciens* (accession number MG214652) and so it has been given the name *Bacillus* MAP3. **Figure 1** shows the phylogenetic tree of this strain with the closest type strains.

Morphological and physiological characterization of *Bacillus* MAP3

Morphological characters of isolate MAP3 grown on LB media were observed after 48 hours of incubation at 37ºC. The colonies were milky white, opaque, irregular in shape, smooth and 2-4 mm in diameter. Polysaccharides secretion and pigmentation were not observed. Staining showed that the isolate is Gram-positive spore-forming bacilli as shown in **Figure 2**

Antimicrobial activity of *Bacillus* MAP3

Whole cell antimicrobial assay

During its isolation for the first time, the strain *Bacillus* MAP3 shoed the dominance in growth and inhibition of other neighboring stains. For that it was tested for its antimicrobial activity using the disc diffusion method against nine selected pathogens. The strain showed antimicrobial activity against a broad range of pathogens. As shown in **Table 1** it was effective against *E.coli, Klebsiella spp, Erwinia carotovora, Bacillus subtilis, Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Candida albicans*. The antimicrobial activity was most effective against *Erwinia carotovora* and *Bacillus subtilis* (inhibition zone more than 25mm for both as shown in **Figure 3**) and were moderately effective against *Candida albicans.* However, a weak effect was observed against *E.coli, Klebsiella spp, Staphylococcus epidermidis and Pseudomonas aeruginosa* and there was no effect against *Shigella spp*. and *Proteus vulgaris.*

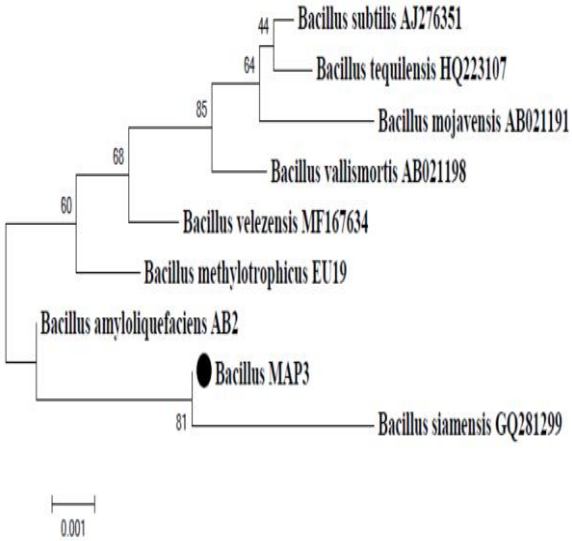

Figure 1: The Phylogenetic tree of Bacillus MAP3 based on 16 s rRNA analysis with the closest type strains

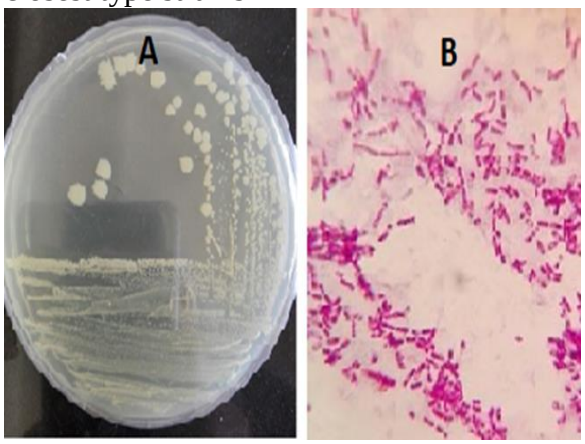

Figure 2: Characteristic of the isolate MAP3 **A)** morphology of colonies on LB media. **B)** Gram staining shows that the isolate is Gram positive "violet color" with spore forming

ability. Black arrow indicates the spore and this photo was taken under 100X oil immersion lens

B. MAP3 metabolites antimicrobial assay

The metabolites of *B.* MAP3 were extracted and tested for their antimicrobial activity using agar well diffusion method. The crude metabolites of the isolate P3 showed more consistent activity against the target bacteria *Erwinia carotovora*. It showed a zone of inhibition (20 mm) against the target bacteria. The zone of inhibition obtained for crude metabolites are shown in **Figure 4**

Table 1: The antimicrobial activity of *Bacillus* MAP3 against 9 pathogens

Pathogenic strain	Antibacterial activity	Average inhibition diameter (mm)
E.coli	+	11
Klebsiella spp	+	12
Erwinia carotovora	+++	27
Bacillus subtilis	+++	>35
Staphylococcus epidermidis	+	10
Pseudomonas aeruginosa	+	10
Shigella spp.	-	0
Proteus vulgaris	-	0
Candida albicans	++	16

+ = (10-12 mm), ++ = (15-17mm), +++ = more than 25mm, - no inhibition zone

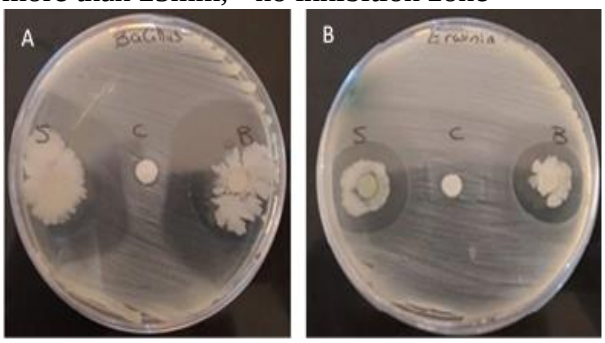

Figure 3: The antimicrobial activity of *Bacillus MAP3* against **A**) *Bacillus subtilis* and **B**) *Erwinia carotovora* using the supernatant (S) and the bacterial cells (B). C refers to the control (liquid LB media).

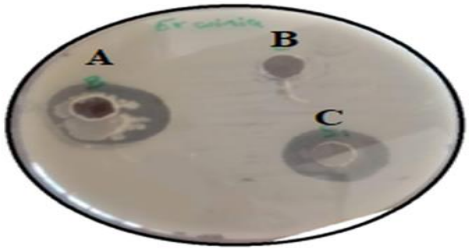

Figure 4: The antimicrobial activity of *B.* MAP3 metabolites against *Erwinia carotovora* **A**) Whole B. MAP3 cells "positive control", **B**) LB media "negative control" and **C**) crude metabolites

Discussion

Bacillus amyloliquefaciens is a Gram-positive model bacterium that is used to study plant–microbe interactions. It is also utilized commercially in agriculture as a biofertilizer and biocontrol agent because of their effectiveness in combating a variety of plant diseases using a complex strategy [23-25]. This bacterium is a root associated one as it has been isolated from roots of different types of plants [26-28]. In this study, *B.* MAP3 has been isolated from the nodules of *Phaseolus vulgaris.* This strain was previously isolated from the nodules of soybean [29] and chickpea [30]. The 16s rRNA gene analysis of this strain showed that it shared a very high sequence identity with Bacillus *amyloliquefaciens* AB2.

B. amyloliquefaciens is known for its antibacterial, antifungal, antilarval and antioxidant activity [31-33]. The tested strain in this study showed the inhibition effect against *E. coli, Klebsiella spp, Erwinia carotovora, Bacillus subtilis, Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Candida albicans*. The antimicrobial activity was most effective against *E. carotovora* and *B. subtilis.* Previously, it has been reported that the post-harvest soft rot caused by *E. carotovora* is controlled by *B. amyloliquefaciens.* The anti-Ecc metabolites was not affected by high temperature, UV-light and protease K[34]. However, the activity against *B. subtilis* has not been reported before and it was found that subtilosin, the antimicrobial factor of *B. amyloliquefaciens*, has been transferred via inter-species horizontal gene transfer from *B. subtilis* [35]. The moderate activity reported in this study against *E. coli, Klebsiella spp, Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Candida albicans* was reported elsewhere [36-38]. However, *E.coli* presence in the growth media enhanced the anti-microbial activity of *B. amyloliquefaciens* [39].

The genome *B. amyloliquefaciens* FZB42 was found to have a number of large gene clusters that are involved in the production of antifungal, antibacterial, and nematocidal lipopeptides and polyketides. Five gene

clusters, srf, bmy, fen, nrs, and dhb, totaling 137 kb, were discovered to drive the production of the cyclic lipopeptides surfactin, bacillomycin, fengycin, an unknown peptide, and the iron-siderophore bacillibactin respectively. In addition, this strain has one gene cluster that encodes enzymes involved in the production and export of the antibacterial dipeptide bacilysin. The antibacterial active polyketides macrolactin, bacillaene, and difficidin are directed by three gene clusters, mln, bae, and dfn, with a total size of 199 kb. In all, this strain devotes roughly 8.5 % of the entire genome directing the synthesis of secondary metabolites [40]

Conclusion

B. MAP3 is a good candidate against further pathogens. Several studies are ongoing for further characterization of this strain and for its evaluation in agricultural applications

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