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ISOLATION, CHARACTERIZATION AND FORMULATION OF ANTAGONISTIC BACTERIA AGAINST FUNGAL PLANT PATHOGENS

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ABSTRACT

Concerns regarding food safety and the environment have led to reduced use of agrochemicals and the development of sustainable agriculture. In this context, biological control of fungal plant pathogens can improve global food availability, one of the three pillars of food security, by reducing crop losses, particularly for low-income farmers. Antagonistic bacteria are common soil inhabitants with potential to be developed into biofungicides for the management of fungal plant pathogens. In this study, antagonistic bacterium was isolated from the commercial compost from a Resen factory for compost and screened for its growth inhibition of fungal pathogens in laboratory tests. The zone of inhibition (mm) was recorded by measuring the distance between the edges of the growing mycelium and the antagonistic bacterium. Five replications were maintained for each isolate. Based on phenotypic characteristics, biochemical tests, and sequence analysis of 16S rRNA, the antagonistic bacterium was identified as *Paenibacillus alvei* (strain DZ-3). The bacterium suppressed the growth of all five tested fungal plant pathogens (*Fusarium oxysporum*, *Rhizoctonia solani*, *Alternaria alternata*, *Botrytis cinerea* and *Plasmopara viticola*) in *in vitro* conditions over. The survival of antagonistic bacterium in peat and talc formulations decreased time at room temperature, but the populations remained above 10^8 CFU/g during the 180-day storage period. This study suggests that this bacterium can be developed and formulated as biofungicides for minimizing the crop losses caused by fungal plant pathogens and diseases caused by them.

Keywords: *biocontrol, fungal plant pathogens, biofungicides, antagonistic bacteria.*

INTRODUCTION

As agriculture struggles to support the rapidly growing global population, plant disease reduces the production and quality of food, fibre and biofuel crops. Farmers spend billions of dollars on disease management, often without adequate technical

support, resulting in poor disease control, pollution and harmful results. In addition, plant disease can devastate natural ecosystems, compounding environmental problems caused by habitat loss and poor land management. Disease losses can mean that communities become dependent on imported foods, often replacing a balanced diet with processed foods that create further health problems.

A variety of fungi are known to cause important plant diseases, resulting in a significant lost in agricultural crops. Fungal plant diseases are considered the most important microbial agents causing serious losses in the agriculture annually (Agrios, 1988). Plant diseases caused by a variety of fungi may cause significant losses on agricultural crops. All plants are attacked by several pathogenic fungi. Each pathogenic fungi can attack one or more plants. More than 10,000 species of fungi can cause disease in plants (Agrios, 2005).

The plant diseases need to be controlled to maintain the level of yield both quantitatively and qualitatively. Farmers often rely heavily on the use of synthetic fungicides to control the plant diseases. However, the environmental problems caused by excessive use and misuse of synthetic fungicide have led to considerable changes in people's attitudes towards the use of synthetic pesticides in agriculture. Today, there is an increased awareness about the healthy food and healthy environment. In response to this, some researchers have focused their efforts on the development of plant disease control methods alternative to the use of synthetic chemicals, such as biological control using microbial antagonists. Many microbial antagonists have been reported to possess antagonistic activities against plant fungal pathogens, such as *Pseudomonas fluorescens*, *Agrobacterium radiobacter*, *Bacillus subtilis*, *B. cereus*, *B. amyloliquefaciens*, *Trichoderma virens*, *Burkholderia cepacia*, *Saccharomyces* sp., *Gliocladium* sp. (Suprapta, 2012; Pal and Garderner, 2006). Biological control of plant diseases has been considered a viable alternative method to manage plant diseases (Cook, 1993). Biocontrol is environmentally safe and in some cases the only available option to protect plants against pathogens (Cook, 1993). Biological control employs natural antagonists of pathogens to eradicate or control their population. In broad terms, biological control is the suppression of damaging activities of one organism by one or more other organisms, often referred to as natural antagonists.

In recent years, research has lead to the development of a small commercial sector which produces a number of biocontrol products. The market share of biopesticides of the total pesticide market is less than three percent. However, significant expansion is expected the upcoming decades due to the increased demand for organic food, and safer pesticides in agriculture and forestry.

Biological control agents are generally formulated as wetable powders, dusts, granules and aqueous or oil-based liquid products using different mineral, organic or inert carriers (Ardakani et al., 2009). Despite of a lot of research on biological control of plant diseases, the number of available products is limited and their market share is marginal. The market for biological control products is not only determined by agricultural aspects such as the number of diseases controlled by one biocontrol product in different crops but also by economic aspects as cost-

effective mass production, easy registration and the availability of competitive means of control including fungicides. The future development of low-chemical input sustainable agriculture and organic farming will determine the eventual role of biological control in agriculture.

The paper describes the method of isolation, characterization, biocontrol potential and formulation of antagonistic bacteria against several fungal plant pathogens.

MATERIAL AND METHODS

Origin of the bacteria- Isolation of potentially antagonistic microorganisms

Fifty grams of compost from the composting plant in Resen, Macedonia, were taken and added to 250 ml sterile distilled water in a 500 ml Erlenmeyer flask. The flask was shaken on an orbital shaker for 30 min at 27°C and serial dilutions from 10^{-1} to 10^{-6} were performed. From each dilution, 0.5 ml of sample was taken and placed on Muller Hinton agar (MHA) medium along with antimycotic cycloheximide (5 g mL^{-1}) using pour plate technique and incubated at 27°C for 1 week. After the incubation period, the plates were observed for microbial colonies which had formed a clear zone of inhibition. The colony with the greatest zone of inhibition was selected and picked up by a sterilized wire loop and sub-cultured on MHA to obtain pure bacterial colonies. The pure culture was preserved on agar slants of Muller Hinton medium for further studies.

Molecular characterization of antagonistic agents

The phenotypic properties of the selected strain wase determined using the methods described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The selected antimicrobial strain wase identified by sequencing of the 16S rRNA gene. First, DNA from each strain was isolated. Pure colony was grown overnight in the appropriate medium, cells were harvested by centrifugation (14000 rpm, 10 min), washed twice with 1xPBS buffer (140 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 1.8 mM KH_2PO4, pH 7.3) and kept at -20^{o}C until further processing. DNA extraction was done using PrepManUltra reagent (Applied Biosystems), following the protocol for culture broth samples. The concentration of DNA was determined spectrophotometrically. DNA working solution of 2.7 – 3.1 ng/µl was prepared by diluting the stock DNA. The sequence of the 16S ribosomal RNA gene (rDNA) of bacterial strains was determined using MicroSeq Full Gene Kit (Applied Biosystems), composed of two parts: MicroSeq® Full Gene 16S rDNA Bacterial Identification PCR Kit and MicroSeq® Full Gene 16S rDNA Bacterial Identification Sequencing Kit. Amplification of the three fragments of the 16S ribosomal RNA gene was done using 7.5 µl DNA working solution in a reaction volume of 15 µl on 2720 Thermal Cycler (Applied Biosystems). Purification of the amplified products was done using ExoSAP-IT® reagent (USB) according to the manufacturer's instructions prior to sequencing. The cycle sequencing was performed with forward and reverse primers for each amplified product according to the instructions provided by the kit with one exception: the final volume of the sequencing reactions was 10 µl. After cycle sequencing, excess

dye terminators and primers were removed from the cycle sequencing reactions by precipitation in separate tubes with 2 µl 5M Na-acetate and 50 µl ethanol. After incubation at room temperature for 30 min, the tubes were centrifuged at 14000 rpm for 30 min, the supernatant was discarded, the precipitate was dried for 5 min at room temperature and re-suspended in 20 µl of Hi-Di™ Formamide. Sequence analyses were performed on a 3500 Genetic Analyzer (Applied Biosystems).

Plant pathogens

Phytopathogenic strains (*Botrytis cinerea* FNS- FCC 23, *Fusarium oxysporum* FNS- FCC 103, *Plasmopara viticola* FNS- FCC 65, *Alternaria alternata* FNS- FCC 624, *Rhizoctonia solani* FNS- FCC 218) were supplied by the Culture Collection of the Department of Microbiology and Microbial Biotechnology, Faculty of Natural Sciences and Mathematics, Skopje, Macedonia. Fungal cultures of phytopathogenic strains were kept on Sabouraud Dextrose Agar (SDA) at 4°C.

Disc diffusion method

Disc diffusion method was used to check the antifungal properties of the isolated bacterial strain against selected fungal pathogens. Petri plates containing equal volumes of MHA and SDA (7.5+7.5 ml) medium were inoculated with a standardized bacterial isolate. A filter disc containing 20 µl of bacterial suspension was placed on a Petri plate pre-seeded with the fungal pathogen. The plates were initially kept at 4°C for 2h to allow the diffusion of the isolate, and later incubated at 28 ± 1°C. The zones of inhibition were measured after five days of incubation and the mean values were calculated. Five replications were maintained for each isolate. The zone of inhibition between pathogen and the bacterial isolate was rated as significant (+++) if the inhibition zone was >10mm wide, moderate (++) if the zone of inhibition was 2 to 10 mm wide, and poor (+) if it was <2 mm wide.

Antagonistic activity of isolated bacterium against phytopathogenic fungi

The suppressive effect and antagonistic activity of isolated bacterium against phytopathogenic fungi was demonstrated using the technique of Landa et al. (1997). Growth inhibition was expressed as the ratio of the radius of hyphal growth relative to the radius of growth on a control plate without antagonist. Values were conveniently corrected so they could be expressed in a scale from 0 (no inhibition) to 1 (maximum inhibition).

Development of talc and peat formulations of antagonistic bacteria

The formulations of selected bacterial isolate DZ-3 was prepared in talc powder and irradiated peat. The MHB broth was inoculated with a loopful of bacterium, and the flask was incubated on a rotary shaker at 150 r/min for 72 h at room temperature (24±2°C). The broth containing 8×10^8 colony-forming units (CFU)/mL, determined spectrophotometrically and by dilution plating on MHB plates, was used for the preparation of talc and peat formulation. The talc formulation was prepared with sterilized talc powder following the method

described by Vidhyasekaran and Muthamilan (1995). To 400 mL of MHB broth, the following were added under sterile conditions: 1 kg of talc powder sterilized at 105°C for 12 h, 15 g of calcium carbonate to adjust the pH to neutral, and 10 g carboxymethyl cellulose (CMC) as adhesive. The peat formulation was prepared with sterile irradiated peat. To 70 mL of MHB broth, 120 g of irradiated peat and 5 mL of bacterial culture were added under sterile conditions. The formulated products were air-dried in a laminar flow hood to a workable (15%–20%) moisture level and kept in polyethylene bags and used for the treatments immediately or as needed. The population of bacteria was around 2.5×10^8 CFU/g in both talc and peat formulations at the time of application.

Shelf life of formulated antagonistic bacteria

The shelf life of the products stored at room temperature (24±2°C) for 6 months was studied by monitoring the viability of antagonistic bacterium in peat and talc formulations by a serial dilution technique. One gram of the sample drawn from each formulation periodically at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 150, and 180 days of storage period was mixed with 9 mL of sterile distilled water (SDW). From this, serial dilutions were made. A 1 mL aliquot of each dilution was pipetted out into sterilized Petri plates, and 15 mL of MHB was added and incubated at room temperature. The bacterial colonies were counted 3 days after plating and expressed as the number of CFU per gram of peat or talc formulation (Vidhyasekaran and Muthamilan 1995).

RESULTS AND DISCUSSION

Biological control of soil borne pathogens by introduced microorganisms has been studied over 80 years, but most of the time it has not been considered commercially feasible. However interest and research in this area increased steadily. There is a shift toward the important role of biological control in agriculture in the future. Several companies now have programs to develop biocontrol agents as commercial products. Morphological studies showed that the isolate with the greatest zone of inhibition was Gram-positive, sporulating, rod shaped bacterium. Alignment of the 16S rRNA sequences of the bacterial species revealed identity of 99% to the genus *Bacillus*. Isolate DZ-3 was identified as *Paenibacillus alvei*. Inoculated on MHA, *P. alvei* produced large, circular, rough, white-yellowish colonies with irregular margins. The spores of *P. alvei* are smooth, spherical and green in color using the Schaeffer and Fulton staining method. *Paenibacillus alvei* are Gram-positive, rod-shaped, motile, spore-forming and catalase-positive bacteria (Najafi et al., 2011). The first report of antimicrobial peptide production by these bacteria was by Anandaraj et al., 2009, who isolated a strain from fermented tomato fruit and detected two antimicrobial peptides, Paenibacillin P and Paenibacillin N. The isolated bacterial strain from compost was screened for secondary metabolites with antimicrobial activity by diffusion agar method. *Paenibacillus alvei* DZ-3 showed potential antifungal activity against all tested fungi, with the highest zones of *Alternaria alternata* FNS- FCC 624 (Table 1).

Table 1. Growth inhibition of *Paenibacillus alvei* DZ-3 on tested phytopathogenic fungi with disc diffusion method.
(Five replications were maintained for each fungus; the mean values were calculated).

phytopathogenic fungus	inhibition zone (mm)			
	Paenibacillus alvei DZ-3	+ positive control (0.5 gL^{-1} nystatine)	+ positive control (0.5 gL^{-1} cycloheximide)	- negative control (sd H_2O)
Botrytis cinerea FNS-FCC 23	11.3	5.3	7.3	0
Fusarium oxysporum FNS- FCC 103	12.1	5.4	8.7	0
Plasmopara viticola FNS- FCC 65	14.6	6.5	6.1	0
Alternaria alternata FNS- FCC 624	22.7	6.9	7.6	0
Rhizoctonia solani FNS- FCC 218	19.2	5.4	5.6	0

significant activity (+++) (inhibition zone > 10 mm)
moderate activity (++) (inhibition zone 2–10 mm)
poor activity (+) (inhibition zone <2 mm)

There are many different types of Gram positive and Gram negative bacteria (such as *Bacillus* spp. and *Pseudomonas* spp.) exhibiting antifungal activities especially toward different phytopathogenic fungi (Kobayashi et al., 2000; Gupta et al., 2001). In this group we can add our antifungal isolate *Paenibacillus alvei* DZ-3, who showed a wide range of antifungal activities toward phytopathogenic fungi.
Isolates of *Pseudomonas* were evaluated for antifungal activity against five fungal plant pathogens, i.e. *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus flavus*, *Alternaria alternata* and *Erysiphe cruciferarum* (Singh et al., 2011). All tested fungal strains showed significant reduction in terms of radial diameter after the treatment with *Pseudomonas* cultures, in comparison with the controls. Out of the five fungal pathogens studied, *Fusarium oxysporum* showed maximum extent of inhibition (% control inhibition = 51.76%) followed by *Aspergillus niger* (50.14%), and least by *Erysiphe cruciferarum* (22.27%). The antagonistic effect of *Pseudomonas* might be explained on the basis of its antifungal secondary metabolites that are capable of lysing chitin which is the most important component of fungal cell wall (Singh et al., 2011).
Biological control of plant diseases is a result of many different types of interaction among microorganisms and can occur through different mechanisms, which are generally classified as: parasitism/predation, antibiosis, competition, lytic enzymes, and induced resistance (Pal and Gardener, 2006). The most effective biocontrol active microorganisms studied appear to antagonize plant pathogen employing several modes of actions. For example, *Pseudomonas* known to produce the antibiotic 2,4-diacetylphloroglucinol (DAPG) may also induce host defenses.

Since inhibition indexes directly obtained from antagonist-phytopathogen confrontations came in different scales, they were appropriately corrected so they ranged from 0 (no pathogen inhibition) to 1 (maximum pathogen inhibition) in order to facilitate comparisons. Inhibition indexes varied widely and showed indexes from 0.12458, for *Botrytis cinerea* FNS- FCC 23 to 0.94513 for *Alternaria alternata* FNS- FCC 624 (Table 2). According to these we can conclude that the results corresponds with that from disc diffusion method.

Table 2. Inhibition indexes obtained from antagonist-phytopathogen confrontations.
(Five replications were maintained for each fungus; the mean values were calculated).

phytopathogenic fungus	*Paenibacillus alvei* DZ-3	
	inhibition index*	corrected inhibition index
Botrytis cinerea FNS- FCC 23	0.12458	0.2
Fusarium oxysporum FNS- FCC 103	0.26936	0.4
Plasmopara viticola FNS- FCC 65	0.48378	0.8
Alternaria alternata FNS- FCC 624	0.94513	1.0
Rhizoctonia solani FNS- FCC 218	0.86923	0.6

* ratio of the radius of hyphal growth relative to the radius of growth on a control plate without antagonist
0- no pathogen inhibition
1- maximum pathogen inhibition

The initial bacterial isolate *Paenibacillus alvei* DZ-3 were higher in irradiated peat than in talc powder formulation, although both products were inoculated with the same bacterial concentrations. These population densities declined over time in both formulations during the 180 days of storage at room temperature but remained above 10^8 CFU/g (Figure 1). In peat formulation, the viability of the bacterial isolate during the first 60 days of storage did not decline significantly compared with their respective initial populations of 9.4 log CFU/g at day 0 (Figure 1). The populations of antagonistic bacterium dropped to 8.72 log CFU/g after 60 days (in talk powder formulation) and to 9.1 log CFU/g (in peat formulation).

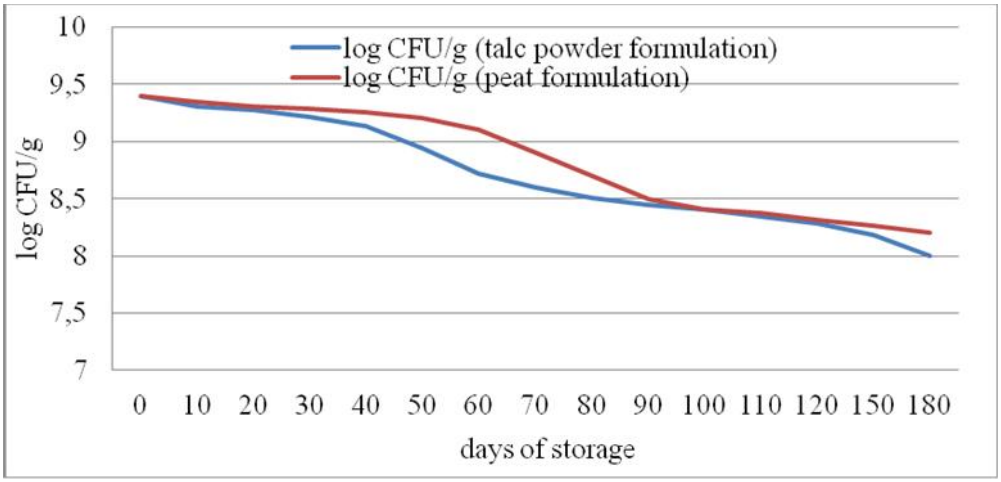

Figure 1. Population densities of antagonistic bacteria, *Paenibacillus alvei* DZ-3 in irradiated peat formulation and talc powder formulation during 180 days of storage at room temperature (24±2°C).

The main focus of the study was the performance of antifungal activity and storage stability of *Paenibacillus alvei* DZ-3, which were superior in *in vitro* conditions. In terms of the formulation for showing the storage stability it was clear that both, irradiated peat formulation and talc powder, formulation were suitable. The survival of antagonistic bacterium in peat and talc formulations decreased over time at room temperature, but the populations remained still above 10^8 CFU/g during the 180-day storage period on room temperature.

Today, the market share of biocontrol formulations is increasing and it occupies 1% of the overall pesticide sales. Montesinos (2003) and Fravel (2005) have drawn up lists of biocontrol products and strains registered by the United States Environmental Protection Agency (USEPA) and the European Protection Agency (EPA). These strains mainly belong to Bacillus and Pseudomonas bacterial genera and Aspergillus and Trichoderma fungal genera. Microbial pesticides are seen as a tool for developing a more rational pesticide use strategy and future products should have improved balance between efficiency and cost (El-Said, 2005; Rao et al., 2007; Glare et al., 2012; Khater, 2012).

Additional studies on the mechanism(s) of action of newly discovered antagonist against the tested phytopathogenic fungi are necessary to fully understand the potential beneficial role of *Paenibacillus alvei* DZ-3. In addition, field experiments are needed, particularly in regard to season long control of phytopathogenic fungi. Generally, the cost and complexity of studies for the registration of microbial pesticides is a barrier to the transfer of laboratory knowledge to the commercialization of these substances.

CONCLUSION

In order to have more effective biological control strategies in the future, it is crucial to carry out further research on certain less developed aspects of biocontrol, including development of novel formulations, understanding the impact of environmental factors on biocontrol agents, mass production of biocontrol microorganisms and the use of biotechnology and nano-technology in improvement of biocontrol mechanisms and strategies. Biocontrol of plant diseases has a bright and promising future, due to the growing demand for biocontrol products by the farmers. In addition, it is possible to use biological control as an effective strategy to manage plant diseases, increase yield, protect the environment and biological resources, and establish a sustainable agricultural system.

The study suggests that *Paenibacillus alvei* can be developed and formulated as biofungicide for minimizing crop losses and diseases caused by fungal plant pathogens .

The challenge is to develop a formulation and application method which can be implemented on a commercial scale. It must be effective, reliable, consistent, economically feasible, and with a wider spectrum. Continuous laboratory research followed by field experiments are needed to develop excellent biocontrol agents, particularly against plant fungal pathogens.

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