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Soil *Bacillus* spp. – a potent cell factory for antimicrobials against phytopathogens

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Abstract

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Many plant diseases are caused by phytopathogenic bacteria which greatly determine the quality of plant production. The biological control of plant pathogenic bacteria is an alternative method to the application of chemicals, which may be accomplished through the destruction of existing inoculums, exclusion from the host, or the suppression or displacement of the pathogen after infection. It offers an environmentally friendly approach to the management of plant disease and can be incorporated with cultural and physical control and limited chemical usage for an effective and integrated disease-management system. Biological control includes the use of beneficial microorganisms, such as specialized fungi and bacteria, to attack and control plant pathogens and the diseases they cause. Biological control is an innovative, cost effective and eco-friendly approach for control of many plant diseases.

Bacteria of the genus *Bacillus* has showed antimicrobial activity against plant pathogenic microorganisms. *Bacillus* spp. are natural inhabitants of the phyllosphere and rhizosphere. These bacteria are involved in the control of plant diseases through a variety of mechanisms of action, such as competition, systemic resistance induction and antibiotic production. The mechanism of antibiosis has been shown to be one of the most important. *Bacillus* spp. have the advantage of being already adapted to the environment where they can be applied as biological control. They have the characteristics of having high thermal tolerance, showing rapid growth in liquid culture, and readily form resistant spores. It is considered safe biological agents and their potential as biocontrol agents is considered to be high.

This research is based on the isolation and screening of biocontrol activities of soil *Bacillus* sp. against several phytopathogens in *in vitro* study. Since tested isolate has showed the production of antimicrobials against the growth of selected phytopathogens, in further work, all of this trial need to be supported by evaluation of antimicrobial activity in *in vivo*.

Keywords: biocontrol; *Bacillus* spp.; antimicrobial effects; phytopathogens; *in vitro* study

Introduction

Annually the Earth's population increases by about 1.6%, and so does the demand for plant products of every kind (91/414/EEC; 128/2009/CE). People need food, and a crop is not food until it is eaten. On the other hand, maintenance of food security is a key EU policy driver. The future expectation of crop production is high as domestic production of protein, vegetable oil and energy increases within EU under the confounding pressures of sustainable intensification, reduced and sustainable pesticide application (91/414/EEC; 128/2009/CE), and a changing climate.

But, phytopathogens frequently depress yield and reduce crop quality. The protection of crops against plant diseases has a vital role to play in meeting the growing demand for food quality and quantity (Strange & Scott, 2005).

Different plant pathogens can cause enormous losses in yield and quality of field crops, fruits, and other edible plant material, and this becomes increasingly a more important issue to human health and the global economy in this century, with increasing human populations and climate change threats to arable land. In terms of economic value, plant diseases alone cost the global economy around US$220 billion annually (Agrios, 2005).

The immense diversity of plant pathogens, which include viruses, bacteria, fungi, nematodes, and insects, approximates 7100 species. Most of these are bacterial and fungal species that cause diseases to plants. The major ways that these pathogens cause plant diseases are by obtaining nutrients one or more host plants for their own growth; using specific mechanisms to secrete proteins and other molecules to locations on, in, and near their hosts; and by exploiting these proteins and other molecules modulate or avoid plant defense circuitry to enable parasitic colonization (Chisholm et al., 2006; Davis et al., 2008).

Also, after harvest, losses of fruits, vegetables and crops can be very high. In developing countries these losses are over 50%, while in industrialized countries they reach over 25%. Most of these losses are caused by these phytopathogens that develop due to high amount of nutrients and water in fruits, low pH and loss of intrinsic resistance of the plant (Agrios, 2005). Postharvest loss have been managed by postharvest bactericide and fungicide applications, postharvest management practices and by storage at low temperature.

Today, still world-wide, the most prevalent disease management practice is the application of chemical treatments, in conjunction with other practices for managing plant health, including, for example, the reduction of over-crowding and over-watering of plants, the rapid removal of diseased plants and plant debris, the constant monitoring of plants to ensure healthy production stocks, and more recently the progressive use of biocontrol agents in the field (Maas, 2004).

However, the problems of pathogen resistance to many bactericides and fungicides, and effects of these products on human health and the environment have promoted restricted use and the need to find alternative methods to control pre- and postaharvest diseases. As a result, biological control with biopesticides has emerged as an effective tool for management of plant diseases.

Biopesticides have several advantages over synthetic pesticides: their dagradation in the environment is much faster and they are less toxic to non-target organisms. Moreover, modes of action of biological pesticides usually differ from conventional pesticides and therefore, they can reduce resistant populations of pathogens. Bacteria with antimicrobial potential occur in many genera, such as *Bacillus, Streptomyces, Pseudomonas, Xanthomonas, Rhizobium, Enterobacter* etc.

The biocontrol of plant pathogens with microorganisms has been studied for more than 70 years (Massart & Jijakli, 2007). Today, biocontrol is becoming a feasible alternative but, although much knowledge has been accumulated from studies conducted in recent years, there is still a long way to go before a realistic and effective alternative to the use of chemical products is available.

Microorganisms introduced to control disease must interact with the crop plants, with potential pathogens, with environmental variables, and with indigenous organisms under prevailing microclimate conditions. The biocontrol agent is assumed to be a natural colonizer of plant, and to be either non-pathogenic or saprophytic, and to be capable of interacting successfully with the plant, microbiological and other environmental conditions, and cultivation systems (Maas, 2004).

Among the microorganisms that can act as biocontrol agents, *Bacillus* spp. are a favorable alternative for the control of various plant diseases. Species belonging to the genus *Bacillus* are gram-positive, spore-forming, rod-shaped, motile bacteria that are present in diverse environmental conditions but mostly in the soil. Their long-term survival in different harsh conditions is attributed to the production of endospores by the simple and rapid development of different reliable formulations (Nicholson et al., 2000). These species provide protection to plants and fish against microbial infections through diverse mode of actions, e.g., through secretion of antibiotics, enzymes, volatile compounds, etc. (Romero et al., 2004; Santoyo et al., 2012; Shrestha & Karki, 2016). The use of *Bacillus* as a BCA has also reported to cut down the cost of agriculture by suppressing the need for fertilizers and pesticides (Saha et al., 2012).

The present study focused on screening antibiotic-producing *Bacillus* sp. that could be used as an alternative to commercial herbicides and pesticides against a broad range of microbes, especially those responsible for the production losses in agriculture.

Material and Methods

Sampling procedure

Soil samples were collected from agricultural land from Skopje Region, North Macedonia, with 5–10 cm depth using some clean, dry and sterile polythene bag along with sterile spatula. All the samples were transferred to lab. Then, the samples were air dried by heating at 70°C for 1 h in a dryer.

Isolation of Bacillus spp.

For the isolation of *Bacillus* sp., serial dilution technique was used considering different aqueous dilutions (10^{-1} to

10^{-4}) using phosphate buffered saline (PBS, pH 7.2). A sample from each dilution was then streaked on a nutrient agar (NA) plate amended with cycloheximide (100 mg mL^{-1}) to prevent fungal growth and incubated at 37°C for 24 h (Cazorla et al., 2007).

Biochemical identification of Bacillus species

The suspected *Bacillus* colonies were identified on the basis of morphology and Gram staining. Subsequent identification tests included hemolysis, starch hydrolysis, gelatin hydrolysis, citrate test, Voges–Proskauer (VP) test, and growth at different pH and temperature (Wulff et al., 2002).

Phytopathogens

Nine representative isolates of phytopathogenic fungi and two isolates of phytopathogenic bacteria were tested. Stock cultures of each isolate were maintained on potato

Table 1. Phytopathogens used in this study

	Fungus	**Attack**	**Diseases**
1	*Phytophthora infestans*	potato, tomato	late blight
2	*Fusarium oxysporum*	tomato, tobacco, legumes, cucurbits, sweet potatoes, banana, eggplant and pepper plants	fusarium wilt
3	*Botrytis cinerea*	chickpeas, lettuce, broccoli, beans, grape, strawberry, and raspberry	gray mold
4	Alternaria alternate	tomato, tobacco, apple, cherry, bean, strawberry	leaf spot
5	*Plasmopara viticola*	grape	brown rot; downy mildew of grapevine; grey rot
6	*Aspergillus flavus*	preharvest and postharvest seed crops, oil-containingcrops such as maize, peanut, and cottonseed	aspergillosis, production of aflatoxin
7	*Aspergillus niger*	fruits and vegetables such as grapes, apricots, onions, and peanuts	black mold
8	*Penicillium aurantiogriseum*	grain, asparagus, strawberry	
9	Penicillium digitatum	citrus, other fruit	green rot or green mould
Bacteria			
1	*Erwinia amylovora*	apple, pear	fire blight
2	*Pseudomonas* spp.	lettuce; brassicas; cucurbits; tomato; capsicum; potato; sweetpotato; carrots;herbs	bacterial soft rot

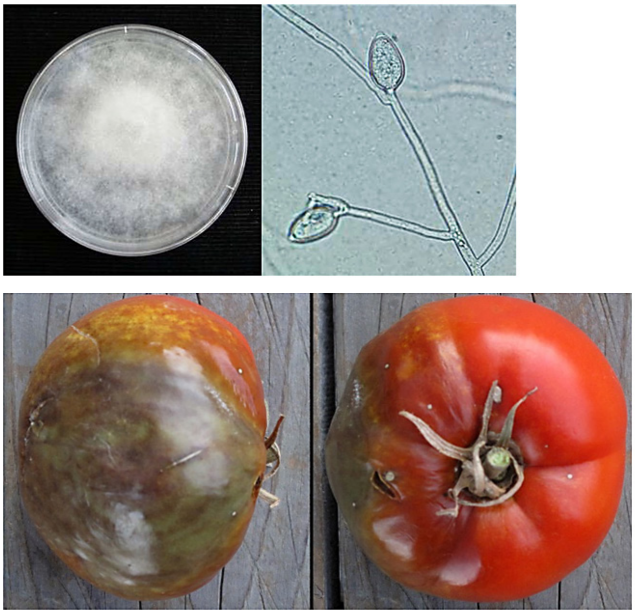


Fig. 1. ***Phytophthora infestans***
(on plate, under microscope, on tomato)

Fig. 2. ***Fusarium oxysporum***
(on plate, under microscope, on tomato)

Fig. 3. ***Botrytis cinerea***
(on plate, under microscope, on strawberry)

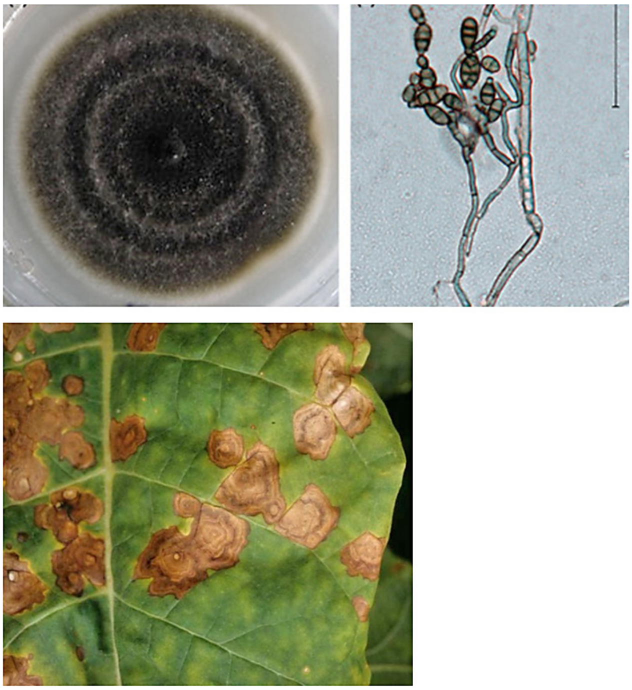


Fig. 4. ***Alternaria alternata***
(on plate, under microscope, on tobacco leaf)

dextrose agar (PDA) (for fungi) and on tryptic soy agar (TSA) (for bacteria) at 4°C. Working cultures were established by transferring each isolate onto PDA or TSA in Petri dishes and incubating for 7 days in darkness at 25°C (Table 1; Figures 1–8).

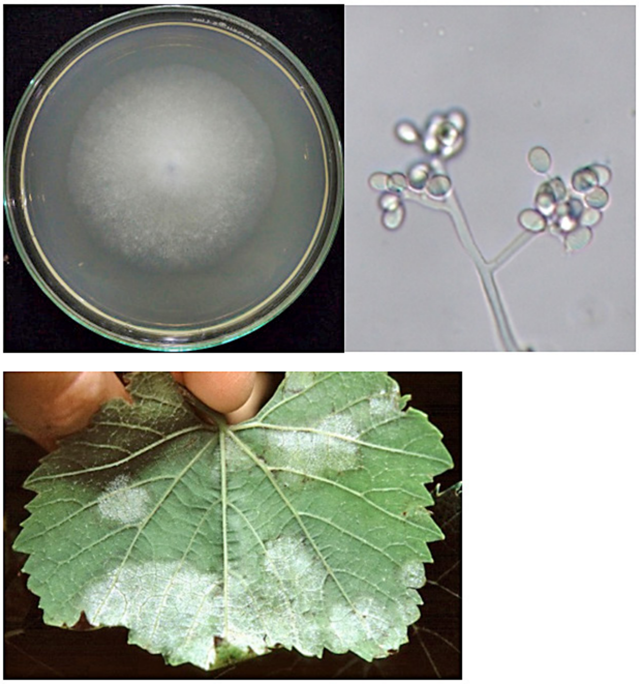


Fig. 5. ***Plasmopara viticola***
(on plate, under microscope, on grape)

Fig. 6. ***Aspergillus niger***
(on plate, under microscope, on onion)

Fig. 7. ***Penicillium digitatum***
(on plate, under microscope, on lemon)

In vitro antagonistic activity assay

The assay for antagonism was performed on PDA and TSA on Petri dishes by the well diffusion method. One layer of PDA or TSA medium, with added plant pathogen, was spread on 90 mm petri dishes. After solidification, five wells per plate with a diameter of 8 mm were made. In each well, 100 μl of test bacteria was added. In the first well it was added original, non-dilluted *Bacillus* sp. with 0.5 McFarland, in the second – 1:10 (diluted with sterille PBS), in the third – 1:20 (diluted with sterille PBS), in the forth – 1:30 (diluted with sterille PBS), and in the last one – sterille PBS. Activity against each phytopathogens was tested in three replicates.

The assesment of antagonistic activity was done after 48–96 h incubation at 25°C by measuring radius of inhibition zones (mm) – zones around wells with no visible growth of tested microorganism.

Fig. 8. ***Pseudomonas*** **spp.**
(on plate, under microscope, on tomato)

Results and Discussion

Isolation and identification of bacteria

From NA culture, the colonies of prospective *Bacillus* sp. were identified according to Bergey's manual for the identification of *Bacillus* species modified by Wulff et al. (2002). Based on the test results (Table 2), one *Bacillus* species was selected for the further *in vitro* study.

Antagonistic activity of Bacillus sp. IS1

Results from the well diffusion assay showed that tested *Bacillus* sp. IS1 showed potential inhibitory effect against all the tested microorganisms in the *in vitro* study.

Among bacteria, *Erwinia amylovora* seems to be more

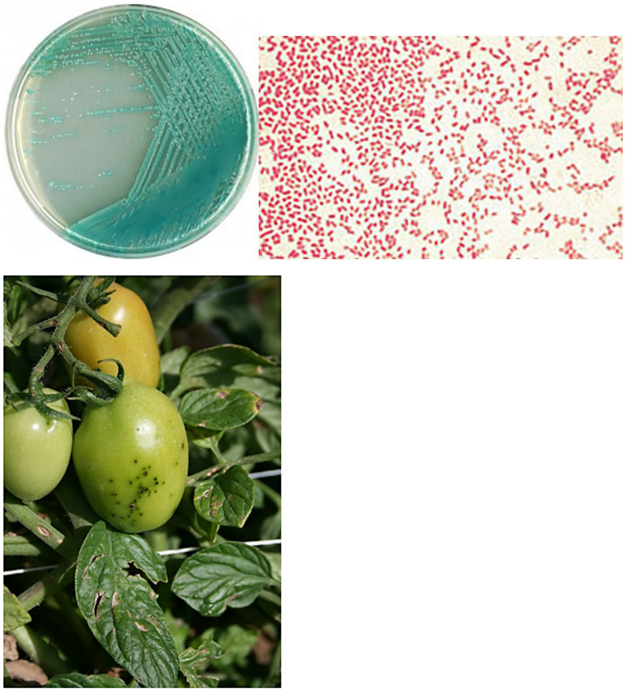


Table 2. Biochemical characterization of selected *Bacillus* sp. isolate for the study

Isolate	Source	Shape	Motility	Hemolysis	Starch hydrolysis	Gelatin hydrolysis	Vp test	Citrate test	Growth at 6% NaCl	Growth at different Ph				Growth at different temperatures, °C			
										2	5	8	10	10	25	45	55
Bacillus sp. IS1	soil	rod	+	a	+	+	+	–	–	–	+	+	+	-	+	+	+

sensitive (16 mm at non-diluted product against 14 mm in *Pseudomonas* spp.) to tested isolate of *Bacillus* sp. (Table 3) (Figure 9).

Among fungi it was observed also inhibition against majority of tested microorganisms Distinct inhibition zones were observed when *Bacillus* sp. IS1 was used against *Phytophtora infestans* (18–11 mm), *Penicillium digitatum* (16–10 mm), *Penicillium aurantiogriseum* (15–12 mm), *Aspergillus flavus* (15–11 mm) and *Alternaria alternata* (15–9 mm) (Table 2) (Figure 10).

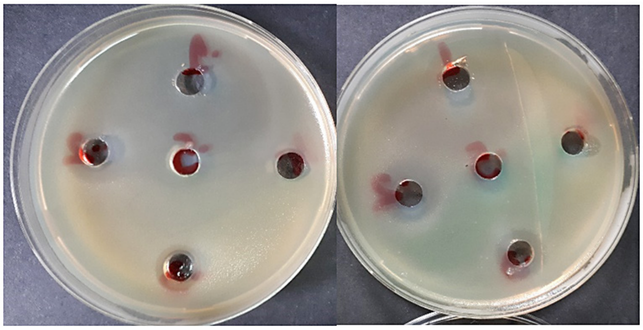
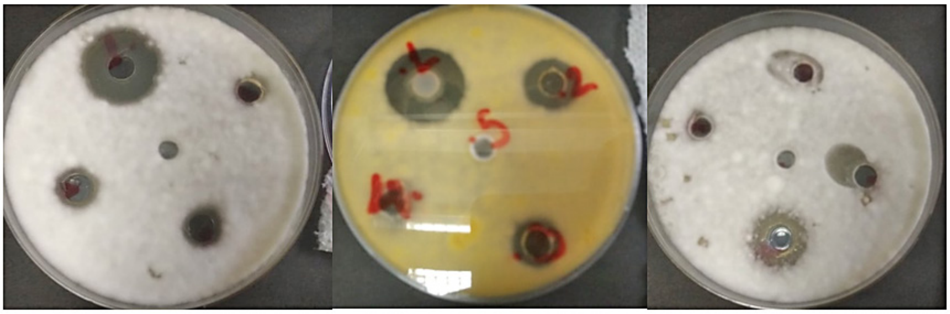


Fig. 9. Comparative well diffusion assay with *E. amylovora* and *Pseudomonas* spp

Slightly weak inhibition zones were revealed between our *Bacillus* sp. IS1 and *Fusarium oxysporum*, *Botrytis cinerea*, *Plasmopara viticola*, *Aspergillus niger*, but also there were inhibition zones (10–13 mm) after 96 hours of incubation. No distinct inhibition zones were observed between *Bacillus* sp. IS1 and tested microorganisms when bacterial suspension was diluted 1:30 (Table 3) (Figure 11).

For many years, as chemical pesticides are expensive, threaten both human health and environment, and some extracts of pathogens are resistant to them, recently, biological control agents are used to fighting plant pathogens. Microorganisms, are a types of biological control agents, have attracted a great deal of attention due to the ability of some species to suppress different plant diseases through different mechanisms and the possibility of combining with other control methods.

Non-pathogenic bacteria with the antagonistic ability of plant pathogenic microorganisms' disease prevention are an



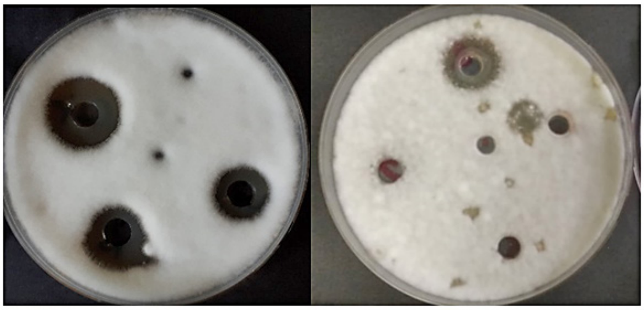


Fig. 10. Comparative well diffusion assay with *Phytophtora infestans* (18–11 mm), *Penicillium digitatum* (16–10 mm), *Penicillium aurantiogriseum* (15–12 mm), *Aspergillus flavus* (15–11 mm) and *Alternaria alternata* (15–9 mm)

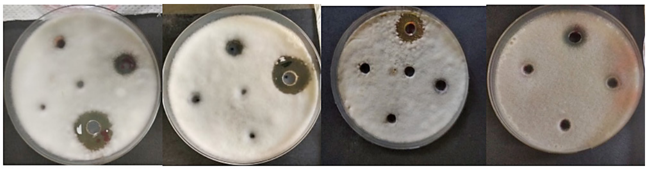


Fig. 11. Comparative well diffusion assay with *Fusarium oxysporum*, *Botrytis cinerea*, *Plasmopara viticola*, *Aspergillus niger*, with inhibition zones (10-13 mm) after 96 h of incubation

appropriate alternative to chemical fungicides and are one of the most important factors in establishing and sustaining agricultural systems. Biological control reduces the effects of pesticide in the long run, leads to a balance between harmful plant pathogens and their natural enemies. In this regard, antagonistic bacteria and fungi are widely used to control plant diseases. In comparison with chemical controls, biological control is a healthier and safer control since unlike some toxic substances, microorganisms are not stored and accumulated in food chains. Biological control is carried out through mechanisms such as competition, antibiosis, predation, and parasitism.

Our isolate of *Bacillus* sp. inhibited radial growth by establishing a clear inhibition zone in a well diffusion test. Several mechanisms are responsible for the suppression of pathogens by microorganism, including competition, antibiotic and metabolite production (Compant et al., 2005). The microorganisms for biological control produced several kinds of antimicrobial peptide substances such as subtilin, bacilysin, mycobacillisyn, and iturin (Yoshida et al., 2001).

These microorganisms can control bacterial and fungal pathogens by competition, direct antibiosis and induced resistance. In the rhizosphere, competition takes place for space at the root surface and for nutrients, noticeably those released as seed or root exudates. Competitive colonisation of the rhizosphere and successful establishment in the root zone is a prerequisite for effective biocontrol, regardless of the mechanism(s) involved.

The diseases of crop plants and fruits present one of the biggest challenges and threats to global food security. These have significant financial repercussions in turn. The use of natural enemies of microbial pathogens is one of the alternative management strategies being used to stop the loss. The isolate, IS01, found in the present study belongs to the genus *Bacillus* and was confirmed by the identification method of Wang et al. (2009) (method for identification of eight *Bacillus* species and subspecies). Most recent studies also reported successful use of soil *Bacillus* species as a BCA to control plant pathogen mycotoxigenic *Fusarium* species causing head blight (Lee et al., 2017), *Botryosphaeria berengeriana* associated with pear ring rot (Snn et al., 2017), and *Rhizoctonia solani* involved in wilt and root rot (Agarwal et al., 2017). Chemical pesticides and antibiotics have long been used to control the spread of diseases; however, their effects on environment and ecosystem have always remained questionable. Moreover, the frequent use of antibiotics and chemical pesticides in farmland and culture ponds increases the chances of production of antibioticresistant strains (Li et al., 2009). Several studies exist on the use of *Bacillus* and *Streptomyces* genera for controlling plant and human pathogens (Saha et al., 2012; Hassain et al., 2014).

Table 3. Average value of zone of inhibition (mm) of *Bacillus* sp. IS1 against different phytopatogenic microorganisms

		1 – non-dilluted *Bacillus* sp. IS1	2 – 1:10 dilluted *Bacillus* sp. IS1	3 – 1:20 dilluted *Bacillus* sp. IS1	4 – 1:30 dilluted *Bacillus* sp. IS1	5 – sterille PBS
	Fungi					
1	*Phytophtora infestans*	18	13	11	9	0
2	*Fusarium oxysporum*	10	9	9	9	0
3	*Botrytis cinerea*	10	9	9	9	0
4	*Alternaria alternata*	15	11	9	9	0
5	*Plasmopara viticola*	11	10	9	9	0
6	*Aspergillus flavus*	15	13	11	9	0
7	*Aspergillus niger*	13	11	10	9	0
8	*Penicillium aurantiogriseum*	15	13	12	10	0
9	Penicillium digitatum	16	12	10	9	0
	Bacteria					
1	*Erwinia amylovora*	16	13	10	9	0
2	*Pseudomonas* spp.	14	11	10	9	0

Growth inhibition of molds could be due to the ability of our tested bacteria to produce antimicrobial compounds such as bacillomycin, mycosubtilin, fungimycin and zwittermicin that inhibit the growth of various microorganisms including fungi (Pal & Gardener, 2006; Madhaiyan et al., 2010; Zhang et al., 2012). In this regard, Edwards et al. (1994) mention that antibiosis is the most common mode of action in the *Bacillus* genus. Iturin group antibiotics are secreted by most strains of *Bacillus* spp. and inhibit mycelial growth and sporulation of the fungus by altering membrane permeability and fungal cell lipid composition (Romero et al., 2007; Li et al., 2009).

Several species of the *Bacillus* genus (*B. subtilis*, *B. pumilus*, *B. amyloliquefaciens* and *B. licheniformis*) are widely studied to mitigate the incidence of diseases of importance to agriculture (Raaijmakers & Mazzola, 2012). Some of the main ways in which these strains avoid the establishment and development of phytopathogenic organisms is through different mechanisms, which include A) the excretion of antibiotics, B) siderophores, C) lytic enzymes, D) toxins and E) inducing the systemic resistance of the plant (ISR) (Layton et al., 2011; Tejera-Hernández et al., 2011).

The tested *Bacillus* sp. IS01 is effective against a broad spectrum of plant pathogens and it can be used either as foliar application or root application before trasplanting. In case of soil or root application the ability of the specific strain to colonise and permanently establish on the roots of the specific crop is crucial. Sometime the colonization is not simply crop-specific, but cultivar-specific. Root application of our product should be preferably targeted to improve resistance of the plant or to protect the early stage of seed germination, rather to directly control soil-borne inoculum of pathogens.

The advantage of this bacteria for use as biological control is his long shelf-life at room temperature, given by the fact that it produces endospores. Biological control of plant pathogens is considered an attractive alternative to chemical-based treatments because it has a minimal impact on the environment.

Therefore, it seems that bacteria *Bacillus* sp. IS01, applied as biofertilizers formulated with single strains or with a consortia of isolates combining different beneficial effects, could serve as a possible solution to feed the world while protecting ecosystems and improving food quality. Consequently the establishment of a dialogue among scientists, politicians and farmers as well as the existence of research programs and policies should be occurring oftenly in order to join efforts for the development effective and safe products, which will bring benefits not just for producers, but for the whole human being as well as for the entire Planet.

Conclusions

Numerous agricultural crops are influenced by various pathogenic mo. Because of serious environmental and health problems a widespread synthetic pesticides have been created in the world, we have to search alternative approach to control the phytopathogenic microorganisms. Biocontrol is a significant strategy to control the pest in an eco-friendly way without affecting fauna and flora. The screening of soil bacteria for novel bioactive compounds, for the antibacterial and antifungal activity, tends to enrich compounds that are already known and abundantly present in environment. Bacterial antagonism has great potential to improve safety in disease management. *Bacillus* spp. IS01 represent an environmentally friendly strategy for crop production improvement. Therefore, the wide antagonistic spectrum of antibiotics produced by the isolate against several pathogenic microbes makes it a potential study strain. It can thus be utilized as a source of environment-friendly, prospective antibiotic-producing bacterial strain for controlling diseases in farmland. Further research on this trial need to be supported by evaluation of antimicrobial activity in *in vivo* to utilize it as an effective biological control agent.

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