



Bacillus spp As an Biocontrol Agent for Plant Disease Management

Vaishnavi Nagthane¹, Raut L. S.², Sanjay Dalvi³ and R. R. Rakh⁴

¹Department of Microbiology, NSB College Nanded.

²Department of Microbiology, Sant Tukaram Arts and Science College, Parbhani.

³Department of Botany, Shri Guru Buddhiswami Mahavidyalaya, Purna (Jn.)

⁴Department of Microbiology, Shri Guru Buddhiswami Mahavidyalaya, Purna (Jn.)

Abstract

The *Bacillus* spp survive in large number of different habitats. They are well known as producers of a wide array of antagonistic compounds of different structures, having between 5 to 8% of the total genome devoted to biosynthesis of secondary metabolites. Most important bioactive molecules from the genus *Bacillus*, are non-ribosomal synthesized peptides and lipopeptides, polyketide compounds, bacteriocins and siderophores. Lipopeptides from *Bacillus* have very complex mechanisms of biosynthesis catalysed by non-ribosomal peptide synthetases (NRPSs), large enzyme complexes with modular structure, with each module being in charge for the incorporation of particular amino acid. In general, these compounds have a broad spectrum of antagonistic activity against plant pathogenic bacteria, fungi, and viruses. Most important molecules from this group, circular lipopeptides from surfactin, iturin and fengycin families affect the target cells on the membrane level. *Bacillus* strains exhibit their biocontrol capacity predominantly through inhibitory activity on the growth of plant pathogens, as well as inducing systemic resistance in plants and competing for ecological niches with plant pathogens. Naturally present in the immediate vicinity of plant roots, *B. subtilis* is able to maintain stable contact with higher plants and promote their growth. In addition, due to its broad host range, its ability to form endospores and produce different biologically active compounds with a broad spectrum of activity, *B. subtilis* as well as other *Bacillus* spp. are potentially useful as biocontrol agents.

Keywords: *Bacillus*, Biocontrol, Lipopeptides, and Plant pathogens


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Introduction – Plant diseases, caused by various microorganisms, including fungi, bacteria, viruses, nematodes and protozoa, affect agricultural production and result in major yield losses (De Silva and Hyde, K. D. (2019). Approximately 20–40% of losses in crop yield are caused by pathogenic infections (Savary, *et al.*, (2012). Different strategies have been used to reduce the occurrence of plant diseases including pesticides, less susceptible cultivars, crop rotation, and other control measures, but their efficacy is usually insufficient due to the survival and resistance of soil-borne pathogens (Owen, M. D., & Zelaya, I. A. *et al.*, (2005). Moreover, the excessive use of synthetic pesticides has adverse effects on the environment and living organisms and disturbs ecosystem functioning and decreases agricultural sustainability (Kardol, P. *et al.*, (2018). Nowadays, research is directed to environmentally friendly alternatives for controlling plant pathogens and improving crop production, which are recommended within an integrated crop management system (ICMS) (Balešević-Tubić, S. *et al.*, (2020). As an important component of an ICMS, biological control is defined as the use of beneficial organisms to reduce the negative effects of plant pathogens and promote plant growth. The most common approach to biological control is the selection of antagonistic microorganisms, studies on their mechanisms of action and development of a biocontrol preparation (Balešević-Tubić, S. *et al.*, (2020). *Bacillus* species are among the most investigated biocontrol agents i.e., biopesticides which contribute to suppression of plant pathogens by antagonism and/or competition, the genus *Bacillus* represents a large group of Gram-positive bacteria that belongs to the Firmicutes phylum. They are rod-shaped, endospore-forming, catalase positive bacteria with aerobic or facultatively anaerobic metabolism. According to the latest edition of Bergey's Manual of Systematic Bacteriology (Logan and De Vos, 2009) 142 species within the genus *Bacillus* were identified, and the number is still growing. The bacteria from this group are exceptionally ubiquitous, since they can inhabit a large variety of ecological niches; they are present in soil, water, and air, as well as on surfaces and rhizosphere of plants, and in gastrointestinal tract of animals, as well as in many extreme environments (Connor *et al.*, 2010; Felske, 2004; Pignatelli *et al.*, 2009). From the biotechnological point of view, the most important feature of *Bacillus* species is their diverse secondary metabolism and the ability to produce a wide variety of structurally different antagonistic substances. Strains of *Bacillus subtilis* have approximately 4 to 5% of their whole genomes dedicated to synthesis of secondary metabolites, with the capability to produce more than two dozen structurally diverse antimicrobial compounds (Stein, 2005). Among other isolates, another typical example is the plant associated strain *B. amyloliquefaciens* FZB42, which has almost 8% of the genome involved in production of secondary metabolites, including bacteriocins, antimicrobial peptides and lipopeptides, polyketides and siderophores (Chen *et al.*, 2009; Ruckert *et al.*, 2011), which at the same time showed plant growth promoting properties (Chowdhury *et al.*, 2013; Idriess *et al.*, 2004).


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In recent years, the interest in biological control of plant pathogens has significantly increased, due to the need for introduction of more environmentally friendly alternatives to the massive use of chemical pesticides (Ongena and Jacques, 2008). Besides the effect of protection, many strains of microorganisms have growth promoting properties on plants as well having the most important role in biological control as soil and plant associated microorganisms. If defined as the products that consist of living microorganisms that are used in combat against plant pathogens, until the last decade biocontrol products made up only 1% of sales on the global market of all agricultural potential biological agent against various plant diseases worldwide (Cavaglieri *et al.*, 2005; De Jensen *et al.*, 2002; Sharma and Sharma, 2008; Scherm *et al.*, 2004).

Bacillus subtilis is common in nature, nontoxic and harmless to humans and other animals, and nonpathogenic to plants (Acea *et al.*, 1988). The bacterium produces antimicrobial compounds in vitro, including the antibiotics zwittermicin-A and kanosamine (Leifert *et al.*, 1995), lipopeptides (Ahimoua *et al.*, 2000; PalBais *et al.*, 2004; Stelle and VlamiM de Souza, 2002) and antifungal protein bacisubin (Liu *et al.*, 2007). As a group, *Bacillus* species also offer several advantages over other bacteria for protection against root pathogens. These advantages include the broad-spectrum activity of their antibiotics and their ability to form endospores. The ability to form endospores facilitates long-term storage and commercialization. One disease that may potentially be controlled by *B. subtilis* is sheath blight of rice (*Oryza sativa*), caused by *Rhizoctonia solani*. Rice is an important crop worldwide, and over half of the world population relies on it for food (Qin and Zhang, 2005).

Mechanism of biocontrol

Antibiotics –The antagonistic activities of *Bacillus* spp. are frequently related to the production of secondary metabolites with antibiotic properties. These compounds mainly involve peptides with low molecular weight that are generated ribosomally (bacteriocins) or non-ribosomally (lipopeptides, peptides, polyketides). Bacteriocins are ribosomally synthesized peptides produced by bacteria.

Bacteriocin act against target cells. by interfering with the synthesis of the cell wall or by forming pores in the cell membrane. The antimicrobial mechanisms of bacteriocins are usually directed against the species which are the same or closely related to the producers, with a narrow spectrum of action. due to the production of bacteriocins, *Bacillus* spp. exhibit a broad-spectrum of antibacterial activity [Some reports identify bacteriocins and bacteriocin-like substances (BLs) (amylolysin, amysin, subtilin, subtilisin A, subtilisin B, thuricin) isolated from various *Bacillus* spp., including *B. amyloliquefaciens*, *B. subtilis*, *B. thuringiensis*, *B. cereus*, and *B. coagulans*.



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However, *Bacillus* spp., which produce non-ribosomally synthesized lipopeptides and peptides, exhibit much stronger antimicrobial activity. Several *Bacillus* spp. are able to produce other non-ribosomally synthesized antibiotics, such as peptides (bacilysin, rhizoctin, amicoumacin, mycobacillin, diketopiperazines) and polyketides (bacillaene, dihydrobacillaene, di_cidin, macrolactin), with diverse antifungal and antibacterial activities. The most commonly used biocontrol agents, *B. subtilis* and *B. amyloliquefaciens*, dedicate 4–5% and 8.5% of total genetic capacity to synthesis of secondary metabolites, with the potential to produce more than two dozen structurally diverse antimicrobial compounds. Nowadays, gene clusters encoding for bacteriocins, as well as peptides and polyketides, can be readily identified in genomic sequences by genome mining. In total, 583 putative bacteriocin gene clusters were identified from 328 strains of 57 *Bacillales* species, while 1231 putative non-ribosomal antimicrobial gene clusters were detected and sub-grouped into 23 types of peptide and five types of polyketide compounds distributed over 49 species of *Bacillales*.

Lytic Enzymes –

Antimicrobial activity of *Bacillus* spp. could also be due to the production of hydrolytic enzymes such as chitinases, chitosanases, glucanases, cellulases, lipases, and proteases, which efficiently hydrolyze the major components of the fungal and bacterial cell walls. Chitinases (EC3.2.1.14) are glycoside hydrolases (GHs) which degrade the β -1,4-glycosidic bonds in chitin, the second most abundant naturally available polysaccharide after cellulose, and the main component of the fungal cell wall (Pechsrichuang, P. (2017).

Recently, several reports have documented the production of lytic enzymes from *Bacillus* spp. biocontrol agents (Table 1). Chitinase-producing *B. subtilis* was effective against *Rhizoctonia solani* (Balešević-Tubić, S. *et al.*, (2020). Crude and purified protease of *B. amyloliquefaciens* showed efficacy in biocontrol of *Fusarium oxysporum* Shirkot, C. K. *et al.*, (2016). The potential of *B. amyloliquefaciens* for biocontrol of *Clavibacter michiganensis* ssp. *michiganensis* was attributed to the production of lytic enzymes (cellulase, lipase, protease, chitinase) (Shirkot, C. K. *et al.* (2019). Hydrolytic enzymes (protease, glucanase, chitinase) produced by *Bacillus* sp. were responsible for a strong inhibitory activity against *Fusarium verticillioides* causing stalk and ear rot of maize (de Oliveira-Paiva, C. A. *et al.*, (2022). The strength of hydrolase activity (protease, chitinase, cellulase, glucanase) was the key factor of *B. velezensis* in control of pepper gray mold caused by *Botrytis cinerea*. Generally, it has been found that strains of *Bacillus* spp. which have the ability to produce cell wall hydrolases are more effective in the suppression of plant pathogens (Balešević-Tubić, S. *et al.*, (2020). In search of efficient biocontrol agents, isolation and characterization of enzyme-producing *Bacillus* spp. should be done in order to achieve maximum survival of bacteria under detrimental environmental conditions and intrusion of pathogens.


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Siderophores –

Siderophores are metal-chelating, non-ribosomal peptides with low molecular weight produced by some microorganisms and plants, especially under iron starvation conditions (alešević-Tubić, S.*et al.*, (2020) Iron (Fe) is an essential element for different biological processes such as oxygen metabolism, DNA and RNA syntheses, electrontransfer, and enzymatic processes. The primary role of siderophore sequesters Fe, allowing its solubilization and extraction from minerals and organic compounds. The significance of siderophores in biological control is based on competition for Fe in order to reduce its availability for pathogens (Balešević-Tubić, S.*et al.*, (2020). the more, microbial siderophores can be reduced to donate Fe to the transport system of a plant or chelate Fe from soils, and then, do a ligand exchange with phytosiderophores, microorganisms 2020, 8, 1037 6 of 19 thus, providing plants with this essential element so as to enhance their growth. In addition to Fe, siderophores also have the ability to bind a variety of metals in the environment, thereby acting as bioremediation agents. Siderophores are grouped into three main families, depending on the functional group, including hydroxamates, catecholates, and carboxylates. Most of the bacterial siderophores are catecholates, such as bacillibactin produced by several *Bacillus* spp. Including *B. subtilis*, *B. amyloliquefaciens*, *B. cereus*, *B. thuringiensis*, etc., Besides bacillibactin, *Bacillus* spp. produce a wide variety of siderophores such as pyoverdine, pyochelin, schizokinen, petrobactin, etc., *Bacillus* spp. were better producers of siderophores than other bacterial isolates from the maize rhizosphere. Siderophores produced by *Bacillus* spp. have been involved in suppression of several plant diseases (Table 1). For instance, siderophore-producing *B. subtilis* reduced the incidence of *Fusarium* wilt, and enhanced the growth and yield of pepper (Yu, X., Ai, C., Xin, L., & Zhou, G.*et al.*, (2011). Several studies indicated synergistic antimicrobial effects of siderophores along with lipopeptides and/or lytic enzymes [42, 49, 50]. Similarly, *B. subtilis* is a promising biological control agent against *Bipolaris sorokiniana* due to production of siderophores, chitinase, and cellulase Balešević-Tubić, S.*et al.*, (2020).

Systemically Induced Disease Resistance -

Plants adapt to constant pathogen exposure through defense mechanisms. Resistance to pathogens, developed after proper stimulation, represents an improvement in the defense capacity of the plant. Infected plants increased their levels of signaling molecules which coordinate the activation genes for appropriate syntheses

Plant defense mechanisms, such as induced systemic resistance (ISR), can be initiated by external agents before infection or triggered by a localized infection, resulting in systemic acquired resistance (SAR) (Shoresh, M., Yedidia, I., & Chet, I.*et al.*, (2005). Both biotic and abiotic factors have been used for inducing ISR in plants against different plant pathogens.

Mechanisms of Plant Growth Promotion –

Nutrient Availability –

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Bacillus spp. produce numerous metabolites which can increase nutrient availability to plants, and thus, directly promote plant growth and yield. Most of the plant essential nutrients are supplied through mineral fertilization, a practice which causes major economic losses, as well as posing significant problems to the environment. The use of biofertilizers which contain N₂-fixing and/or P-solubilizing *Bacillus* spp. is a reasonable approach to reducing the negative impacts of synthetic fertilizers without compromising food safety. N₂-fixing and P-solubilizing *Bacillus* spp. are directly related to nutrient uptake and the subsequent growth promotion in different plants

• **Phytohormone Production –**

Bacillus spp. may directly increase plant yield through mechanisms that impart the production of phytohormones or plant growth regulators (PGRs), such as auxins, cytokinins, gibberellins, ethylene, and abscisic acid. Plant hormones are organic substances that influence the physiology and development of plants at very low concentrations. Plant hormone biosynthesis by *Bacillus* spp. has been directly related to subsequent growth promotion in different plants biocontrol.

In vitro Antifungal Activity of Volatile Compounds Produced by *Bacillus* spp.

The ten most effective strains of *Bacillus* spp., as determined by the results of the dual plate assay, were used in this study. The efficacy of volatile compounds produced by *Bacillus* spp. were assessed using the partition plate technique (Fernando et al., 2005). *Bacillus* spp., were challenged with *S. sclerotiorum* on partition plates which enables the movement of volatiles alone without any direct contact between the microbes. The pathogen inoculated alone into the partition plate was maintained as control and incubated at $20 \pm 2^\circ\text{C}$ for 7 days. The experiment was replicated thrice. After 7 days of incubation, the percent inhibition of sclerotial production was calculated as proposed by Dennis and Webster (1971).

In vitro inhibition of the fungi by strain NJ-18

Rhizoctonia solani and *S. sclerotiorum* were grown on PDA plates at 25 C. After 1 day for *R. solani* and 3 days for *S. sclerotiorum*, 0.5cm-diameter mycelial discs were transferred from these plates to new PDA plates (9-cm diameter, four disks per plate). The mycelial discs were added in a circular pattern, equidistant between the plate edge and center, so that the bacteria added to the center were 2.25 cm distant from each disk. Each new PDA plate was inoculated

immediately in the center with a 150- μl suspension of strain NJ18. The suspension contained 0.0, 2.5107, 5.0107, 1.0108, 2.0108, 4.0108, or 1.0109 cfu/ml. Each of the six concentrations of strain NJ-18 plus the control was replicated using four plates for each fungus, and the experiment was performed three times. The plates were incubated at 25 C. The diameters of the fungal colonies were measured when the mycelia had grown over half of the plate in the control. The inhibition zone was defined as the mean diameter of the fungal colony in the control plates minus the diameter of the fungal colony in each plate inoculated with strain NJ-18 (Karthikeyan and Gnanamanicham,

2008). After the diameters of fungal colonies were measured, a small section of mycelium was removed from the edge of control colonies or colonies treated with 0.0, 2.5107, 1.0109 cfumll of strain NJ-18. The sections (three sections per treatment) were placed on a glass microscope slide, covered with a coverslip, and examined with bright-field optics on a Leica microscope (Leica DMR, Germany). Photographs were taken with a Samsung Color Camera (SAC-410 PA, Korea) and computer.

In vitro antagonistic activity assay

Bacillus subtilis strain SB24 was selected from a total of 140 strains isolated from soybean roots in a long term crop rotation field at the Central Experimental Farm (CEF), Ottawa, Canada in 2006, based on the production and size of inhibition zones against *S. sclerotiorum* in dual culture on PDA (data not shown). The bacterial strain was identified based on morphology, Gram staining, potassium hydroxide and other biochemical tests and confirmed by 16S rRNA gene sequence followed by a

similarity search against all publicly available GenBank entries using BLAST (Basic Local Alignment Search Tool). In the present study, the effectiveness of SB24 against mycelial growth and sclerotial production of *S. sclerotiorum* on PDA was examined in two experiments. In the dual-culture experiment, a 6 mm filter paper disc was soaked in the bacterial suspension at 1×10^8 cells mL⁻¹ for 2 min and placed on PDA at the centre of a 9 cm Petri dish. Two 6 mm agar plugs containing 5-day-old *S. sclerotiorum* mycelia were placed 2 cm from the bacterized filter paper disc, one on each side. A Petridish with *S. sclerotiorum* alone on PDA served as a positive control. The cultures were incubated at 25°C, and sclerotial number was counted and inhibition of fungal growth was recorded by measuring the clear zone between the *S. sclerotiorum* plug and bacterized paper disc 10 days after inoculation. In a second experiment, the effects of bacterial cell-free filtrates on mycelial growth and sclerotial production of *S. sclerotiorum* were evaluated on PDA. The bacterial cell-free filtrates were obtained by centrifugation of a bacterial broth culture dilution with 1×10^8 cells mL⁻¹ at 10 242 g and passing the supernatant through a 0.22 µm filter (Millipore Corporation). The 100 µL cell-free filtrate of SB24 was spread on a PDA plate and a 6 mm mycelial agar plug of *S. sclerotiorum* was placed at the centre of the plate. A plate containing no cell-free filtrate, but 100 µL sterile distilled water and inoculated with a *S. sclerotiorum* mycelial plug alone served as a positive control. After incubation at 25°C for 10 days and sclerotial number and colony diameter were measured as described above. The experiments were conducted twice, each with four replicates.

Effect of *Bacillus* spp. in Management of Stem Rot –

Earlier work revealed that application of *B. amyloliquefaciens* (VB7) by root dip followed by soil drenching at 0.5% (5ml/l) resulted in a minimal stem rot incidence of 4.60%, which was reduced 87.9% relative to the control. *B. amyloliquefaciens* (VB2) and *B. cereus* (BSC 5) followed with the next highest efficacies significantly different from each other. For reference, stem rot incidence in the untreated control was 38.24% (Figure 11). Studies

on the yield parameters indicated that the mean shoot number, stalk length, and flower yield in the plants treated with *B. amyloliquefaciens* (VB7) were relatively higher than that of the untreated control.

Conclusion

The present review article state that the *Bacillus* spp. represent an environmentally friendly strategy for crop production improvement through different mechanisms of biological control, biofertilization and biostimulation. Although possibilities to use *Bacillus* spp. for disease incidence reduction and crop production improvement are well known, their application is not a widespread practice, mostly because of inconsistent efficiency under different conditions. The ability of *Bacillus* spp. to exhibit beneficial traits depends on the interaction of bacteria with plant and/or pathogen, and the environment. Given the great economic and ecological importance of *Bacillus* spp., it is necessary to increase the number of practically important species and find advanced methods for the irrapid and comprehensive research and efficient application.

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**Co-ordinator
IQAC**

Shri Guru Buddhiswami Mahavidyalaya
Purna (Jn) Dist. Parbhani - 431511 (M.S.)



PRINCIPAL

Shri Guru Buddhiswami Mahavidyalaya
Purna (Jn.) Dist. Parbhani

