LIPOPEPTIDES: POWERFUL ANTIFUNGAL COMPOUNDS PRODUCED BY *BACILLUS* SPECIES, A REVIEW

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ABSTRACT

Phytopathogens affect the crops each year and hurt the economy of the country. Researchers' main concern is investigating biocontrol agents' potential as alternative sources to combat diseases. The *Bacillus* genus produced various plant growth-promoting compounds useful for managing plant diseases. These agents are harmless to the environment and proven effective antagonists against pathogens. The lipopeptides extracted by *Bacillus* spp. are effective germ killers, and the most notable among them are iturins, surfactin, fengycin, bacillomycin, and plipastatin, which exhibit potent antibacterial action against a wide variety of phytopathogens. Lipopeptides are non-toxic, biodegradable, and friendly to the natural world. Different lipopeptides have been reviewed for their function and mechanism of action in biocontrol in literature. The purpose of this review is to analyze the previous and recent research work and the mechanisms that involved for the management of disease by lipopeptides produced by *Bacillus* genus.

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INTRODUCTION

There is a great need to find out less harmful alternatives instead of chemical pesticides to battle dangerous plant diseases (Mukhtar et al., 2023; Shahbaz et al., 2023). The use of biocontrol agents has been expanded tremendously in recent years, due to concerns of ecosystem. The employment of hostile microorganisms as biopesticides proves competent and more secure, trustworthy, and sensible method to control the diseases (Fravel, 2005). There is a different approach to combating plant diseases, which is biological control. It promotes the sustainability of plants, is cost-effective, and should be environmentally friendly. There are various agents, such as different bacteria and yeast, which have been proven effective against various post-harvest diseases (Janisiewicz and Korsten, 2002). The genus *Bacillus* is considered

promising due to its ability to produce a wide variety of antimicrobial peptides with potent inhibitory effects against plant diseases (Emmert and Handelsman, 1999). This review discusses the use of the viable microorganism *Bacillus* bacteria for the management of plant infections (Tenover, 2006). *Bacillus* has the ability to produce spores that are resistant to drying out, making them suitable for use in long-lasting biopesticides. Lipopeptides extracted from certain species of the genus *Bacillus* are examples of antibacterial compounds that have gained significant importance nowadays. Surfactin, fengycin, bacillomycin, and iturin are members of the lipopeptide family that have received considerable attention due to their potential applications in biotechnology and pharmacology (Ongena and Jacques, 2008; Maalik et al., 2023). They can disrupt biological membranes directly (Patel et al., 2011) or indirectly by inducing host resistance and reducing diseases (Jourdan et al., 2009).

There are primarily three characteristics that make them promising biological control agents. The first trait is high ecological competence, which helps them colonize the rhizosphere and provides bacteria with an advantage in the competition for food and water. The other traits include their ability to produce antibacterial secondary metabolites and enhance the innate defense response to develop systemic acquired resistance against stress conditions and pathogens (Berendsen et al., 2012), making them remarkably resilient. Four to five percent of the genome of *Bacillus subtilis* is dedicated to antibiotic production, and this organism may generate over two dozen antimicrobial peptides (Stein, 2005).

Bacillus species produce a wide variety of amphiphilic peptides, including those known as lipopeptides. In a previous study, the species produced substantial amounts of the lipopeptides surfactin, fengycin, and iturin *in vitro* and in bioreactor experiments (Dietel et al., 2013). Lipopeptides are created through the esterase chain release mechanism or by non-ribosomal peptide synthetases (NRPS) (Calcott and Ackerley, 2014), and hybrid polyketide synthetases additionally contribute to their synthesis. Lipopeptides can be synthesized in a wide variety of structural orientations, in terms of type, amino acid sequence, and peptide cyclization, as a result of the aforementioned mechanisms. There are many different isoforms of these lipopeptides due to differences in their length, structure, amino acid substitution within peptide rings, and fatty acid branching sites (Kowall et al., 1998; Raaijmakers et al., 2010).

It has also been observed that several viruses, such as herpes viruses and retroviruses, are significantly inhibited by lipopeptides. Lipopeptides are hydrophobic, and they exert a non-specific detergent action through which they target and destroy the virus envelope and virus particles (Kracht et al., 1999). Biodegradability, low toxicity, environmental compatibility, and greatly increased activity at severe temperatures, salinity, and pH are just a few of the benefits of lipopeptides that have piqued the interest of scientists. Finding and identifying the gene sequences that code for the generation of lipopeptides with potent antibacterial action is of paramount importance. Recent developments have been made to investigate the use of lipopeptides for the control of plant diseases, as well as the function of key lipopeptides in the biocontrol of plant diseases and their mode of action.

TYPES OF LIPOPEPTIDES

The lipopeptides produced by *Bacillus* species have significant biocontrol potential against various plant diseases. There are different types of lipopeptides which are given below.

Surfactin

It was discovered in 1968 that *Bacillus cereus* produced surfactin compounds in broth culture. Previous research revealed that the *B. subtilis* sp. *biosynthesis* was initiated by the non-ribosomal, multi-enzyme-catalyzed system that comprises surfactin synthetase. Surfactin is made via a nucleic acid-independent multi-enzyme-catalyzed mechanism called the surfactin synthetase complex (Steller et al., 2004). Surfactin is a 1.36 kDa cyclic lipopeptide (Maget-Dana and Ptak, 1990), which is produced by *Bacillus* spp. and consists of a loop of 7 amino acids attached to a hydrophobic-hydroxy fatty acid (C13-C16). The seven amino acids are glutamine, asparagine, leucine, valine, isoleucine, and leucine, with valine and leucine found at positions 2, 3, 4, and 7, and the hydrophilic glutamyl and aspartyl occurring at positions 1 and 5. Surfactin homologs are often present in the cell as a mixture of several peptide variations with varying aliphatic chain lengths (Tang et al., 2007). The hydroxy fatty acid content and amino acid orientation of surfactin are mostly determined by the bacterial strain used in production and the conditions under which it was grown. The hydrogen bonds produced intramolecularly create the turn, and intermolecularly

create the sheet (Zou et al., 2010).

Surfactins are the most effective biosurfactants at reducing the surface tension of water due to their unique structural arrangement (Peypoux et al., 1999). They act as bioemulsifiers and boost the solubility of water-soluble substances by increasing their surface area. They also increase the availability of hydrophobic substrates, which bacteria use to cling to and then separate from the surfaces of substrates (Rosenberg and Ron, 1999). They may be useful in the creation of biopesticides.

The formation of C14-C15 surfactins by the *Bacillus* genus was reported by Nazari et al. (2016). Tobacco cell cultures treated with *Bacillus* species, especially the strain ACCT21332, resulted in increased production of reactive oxygen species (ROS) and the ability to produce surfactin. Due to its amphipathic character and substantial inhibition of the F strain FJAT-14262, it was further validated by oil-spreading experiments (Hussein et al., 2016). It was found that *Bacillus* inhibits Tomato mosaic virus (ToMV). Tomatoes developed systemic resistance to Tomato mosaic virus (ToMV) after being exposed to *B. subtilis* strain BMG02. Surfactant molecules produced by *B. subtilis* (Osouli and Afsharmanesh, 2016), M419, have been shown to be hemolytically active against *Papilio demoleus* in both the first and second instar stages.

Iturins

Iturins are a class of cyclic lipopeptides produced by different strains of *Bacillus* that have a molecular mass of 1.1 kDa, such as *B. amyloliquefaciens* (Souto et al., 2004). The amino fatty acid lipid moiety is a diagnostic feature of iturins, and they exhibit potent antifungal and hemolytic activity. The heptapeptide molecule consists of seven amino acid residues and one amino acid residue. The antifungal action of *Bacillus* spp. against *Pythium ultimum*, *Eudrilus eugeniae*, and others has been documented in the literature (Constantinsecue, 2001; Tsuge et al., 2001), which determined that the iturin A operon is 38-40 kb in size and contains the four open reading frames ituA, ituB, ituC, and ituD. The ituA gene encodes a protein with a mass of 449 kDa, structurally related to peptide synthetase, amino acid transferase, and fatty acid synthase. Peptide synthetases ItuB and ItuC each have a molecular weight of 609 kDa. The remarkable antifungal role iturins play is attributed to their broad spectrum of action. According to Chen et al. (2016), the isolated biosurfactant from *B. subtilis* resembles iturin-A. *B. amyloliquefaciens* IUMC-7, an antifungal strain used to treat *Ralstonia solanacearum* in *Lycopersicum* (Sotoyama et al., 2016), was cited in the literature as possessing strong antifungal effects against *Penicillium expansum*, *Alternaria alternata*, and *Botrytis cinerea*. Additionally, it has potent destabilizing action and significant surface activity (Cozzolino et al., 2020).

Fengycin

Fengycin belongs to the third family of compounds, following surfactin and iturins. The fengycin molecule is produced through a lactonization process, resulting in a cyclic lipodecapeptide with a lactone ring in the hydroxy fatty acid chain. It is connected to the fatty acid chain via a variable-length side chain consisting of 16-19 carbon atoms (Akpa et al., 2001). Additionally, fengycin contains 4 D-amino acids and ornithine in its peptide chain (Koumoutsi et al., 2004). Based on the position of amino acids in the peptide ring, fengycins can be classified into two main classes: fengycin A, which has Ala at position 6, and fengycin B, which contains Val at position 6. Similar to the lipopeptide plipastatin, fengycin exhibits modest fluctuations in response to varying salt concentrations (Honma et al., 2012). It inhibits the growth of filamentous fungi primarily and suppresses phospholipase A2 activity (Nishikori et al., 1986). In their findings, the authors reported that fengycin produced by strain B5 of *B. atrophaeus* sp. reduced anthracnose disease in avocados (Guardado-Valdivia et al., 2018).

Bacillomycin

The effective antibiotics, such as bacillomycin F, D, and L, belong to the lipopeptide class known as bacillomycin. They consist of seven amino acid residues and one amino fatty acid residue. These lipopeptides are included in the iturin family. The function of this family is to exert pressure on biological membranes, attacking the sterols and phospholipids of fungi and rendering the pathogen incapable of causing infection (Volpon et al., 1999). In *B. subtilis*, the bacillomycin gene cluster is effectively produced using a quick and easy strategy for heterologous production of biosynthetic pathways through direct cloning (Liu et al., 2016). The pathogenic fungus *Botryosphaeria dothidea*, which causes gummosis disease in peach fruits, is particularly susceptible to the powerful antibacterial effect of bacillomycin, followed by fengycin and surfactin compounds (Li et al., 2016).

Plipastatin

Plipastatin is a non-ribosomal peptide that inhibits fungal growth and is composed of 10 amino acids and 1

hydroxy fatty acid chain. Two forms of plipastatin have been identified: plipastatin A and B, containing either valine or alanine in position 6. It is a co-linear chain made up of five different non-ribosomal peptide synthetase subunits: ppsA (289 kDa), ppsB (290 kDa), ppsC (287 kDa), ppsD (407 kDa), and ppsE (145 kDa) (Umezawa et al., 1986; Sun et al., 2006).

The role of lipopeptides in the colonization of plant tissues

In the rhizosphere, plants release a variety of low-molecular-weight compounds that provide a specialized food source for soil microbes (Bais et al., 2006). Bacteria, such as *Bacillus* species, utilize chemotaxis triggered by certain plant-released chemical compounds. The root exudates are utilized by *Bacillus* species for growth and survival (de Weert et al., 2002). Lipopeptides are an integral part of the intricate process by which *Bacillus* species colonize plant roots. To colonize plant roots, rhizobacteria create micro-colonies in the rhizosphere (Lugtenberg et al., 2001; Ali et al., 2014). Some researchers refer to these communities as biofilms (Ramey et al., 2004). Surfactin has been shown to play an important role in pellicle production at the air-water interface (Kinsinger et al., 2003). Different *Bacillus* strains, such as *Bacillus* strain ATCC21332, died off into the pellicles quickly compared to *Bacillus* strain ATCC9943, which colonized the rhizosphere extensively, formed micro-colonies, and produced abundant lipopeptides. The importance of *B. subtilis*-made surfactins was reported by Bais et al. (2004), showing the formation of biofilm on the roots of the plant *Arabidopsis* using *B. subtilis* strain 6051. The bacteria colonize different areas of the rhizosphere to look for food and shelter. This is made possible by the synthesis of biosurfactants, which, in turn, facilitates the bacterium's swarming activity-a flagellum-driven movement that permits the bacteria to spread over the root zones (Daniels et al., 2004). Several reports have established a role for lipopeptides in surface motility. Since then, *Bacillus* species lipopeptides have been shown to prevent harmful microbes from forming biofilms (Rivardo et al., 2009).

Direct antagonism of pathogens by lipopeptides

Bacillus species are effective in the biological control of plant diseases once they have colonized the rhizosphere. There are hardly any papers that solely suggest that lipopeptides have biocontrol activity *in vitro*. Since then, Bais et al. (2004) concluded from their experiments that surfactin molecules aided in rhizosphere colonization

and protected *Arabidopsis* plants from *Pseudomonas syringae* infection. They concluded that increased lipopeptide surfactin production in the root zone was responsible for the decline in disease. While it was evident that it prevented pathogens from attaching to the root zone and forming biofilm, it was not clear whether this was due to the compound's direct antibacterial activity.

Iturin and fengycin have been found to have antibiosis activity against a wide range of plant diseases. The negative impact of *Podosphaera fusca* on melon leaves has recently been reported to be counteracted by iturins and fengycins, generated by *Bacillus* species (Romero et al., 2007). The identification and isolation of iturins and fengycins were reported as the primary antagonistic agents that inhibit conidial germination and subsequent fungal growth on leaves treated with bacterial lipopeptides. The production of surfactin, iturin, and a wide variety of fengycin molecules by *B. subtilis* strain S499 has been reported (Jacques et al., 1999; Ongena et al., 2005a). Since then, experiments have verified the antagonistic action of fengycin when *B. subtilis* was used as a test organism. Using the *B. subtilis* S499 strain, it has been able to successfully treat infected apples and prevent the spread of grey mold disease caused by *Botrytis cinerea*. The high detection of fengycin following treatment of the infected fruit with lipopeptide-rich extracts supported the antagonistic effect.

Lipopeptides as signaling molecules

Activating systemic acquired resistance is one of the important ways through which fengycin and surfactin might interact with plants to elicit an immune response. The results from Ongena et al. (2007) revealed that fengycin and surfactin in bean and tomato plants have protective and defense-stimulating activities. *Bacillus* strains prone to producing excess lipopeptides upregulated key enzymes in the "oxylipin pathway", a metabolic route initiated by the lipoxygenase enzyme (Blee, 2002; Fatima et al., 2023).

The application of fengycin on potato tuber cells resulted in the accumulation of phenolic chemicals produced in the phenylpropanoid pathway (Ongena et al., 2005b). Interestingly, a previous study observed that fengycin alone, as compared to surfactin and iturin, boosts the formation of phenolic chemicals. *B. subtilis* S499 prevented cucumber culprits from damaging the plants. The author Tran et al. (2007) also validated the lipopeptides' potential to improve plant defense

response. They found that the *Pseudomonas fluorescens* cyclic massetolide A lipopeptide might stimulate a defensive response in tomato plants resistant to *Phytophthora infestans*. However, research on the impact of lipopeptides on plant cells found no evidence of any phytotoxicity. To explain the anti-proliferative action of surfactin lipopeptides on cancer cells that did not undergo leakage or lysis but rather underwent regulation of substantial changes in the cell necessary for cell survival, a similar theory was recently proposed (Kim et al., 2007; Mahmood et al., 2022; Bibi et al., 2017). Changes generated by lipopeptides have not yet been fully understood at the molecular level. However, it can be concluded that lipopeptides play a substantial role in triggering a systemic defense response in plant cells, based on prior studies of defense activation by lipopeptides. For instance, the molecule surfactin may treat the infection of tomatoes, beans, and tobacco, but its effect on potato disease has not been observed in the past. Fengycin, on the other hand, may have an effect on all three members of the solanaceae family. So far, not a single lipopeptide has been found to have any effect on cucumber plants. Tomato, potato, and tobacco cells were resistant to iturin locally but not systemically. It has also been discovered that iturin's action against fungi depends on the presence of ergosterol in the fungi. Without phytosterols, lipopeptides may not be able to break membranes as effectively.

Plant defensive responses triggered by lipopeptides

In addition to their direct antagonistic activity, lipopeptides also tend to stimulate a defense response in plants, protecting them from pathogen damage (Ongena and Jacques, 2008). Another type of systemic acquired resistance is induced systemic resistance (ISR), which is typically triggered by non-pathogenic microorganisms such as plant growth-promoting fungi and rhizobacteria. After the identification of pathogen-specific MAMPs, ISR is triggered (Abramovitch et al., 2006; van Loon et al., 2006). The entire plant receives a signal that causes a change in gene expression and resistance to pathogen invasion (Van Wees et al., 2008).

Bacillus spp., such as *B. amyloliquefaciens*, *B. previouseurri*, *B. Bacteroides*, and *B. cereus*, are involved in this process (Kloepper et al., 2004). Recently, Garcia-Gutiérrez et al. (2013) suggested that compounds like surfactin and fengycin can induce a strong defensive response in plants. However, the failure of an iturin produced by the B6633 strain of *B. subtilis* to

overproduce protein led to the conclusion that iturins have no ISR effect (Leclére et al., 2004).

Lipopeptide-mediated plant defense requires jasmonic acid, salicylic acid, and ethylene signaling (Falardeau et al., 2013; Garcia-Gutiérrez et al., 2013). Many studies have found evidence for a correlation between the expression of lipopeptides and triggering a defensive reaction. Surfactin and fengycin overexpression in plants increased their tolerance (Ongena et al., 2007).

Defense elicitation processes at the molecular level, however, are not well understood. Recent research has revealed several potential mechanisms by which lipopeptide interact with plants to activate defenses. Surfactin-induced ISR in plants was triggered by the insertion of single molecules of surfactin into lipid membranes, resulting in minor plasma membrane disturbances. Surfactin-induced ISR resulted in a rise in reactive oxygen species; however, contrary to expectations from typical elicitors, ROS did not play a role in cell death. It seems that surfactin's structure and integrity were also required for membrane binding (Jourdan et al., 2009).

The significance of fengycin in ISR in plants is less clear, while surfactin is thought to be the primary factor (Coway et al., 2013). A recent finding confirms the central role of surfactin in defense elicitation. *B. Subtilis* lipopeptide-producing mutant strains were evaluated for their ability to elicit a defensive reaction in plants (Garcia-Gutiérrez et al., 2013). Ryegrass (*Lolium perenne* L.) showed a robust ISR response to *Magnaporthe oryzae* when treated with the *B. amyloliquefaciens* FZB42-AK3 strain, providing evidence of increased peroxidase activity, callose and phenolic compound deposition, and hydrogen peroxide buildup (Rahman et al., 2015). Surfactin-induced ISR was also associated with increased expression of various defense-related genes, including LpPrx, LpOXO4, LpPAL, LpLOXa, LpTHb, and LpDEFa (Rahman et al., 2015).

Co-production of different classes of lipopeptides by single *Bacillus* specie

Previous studies have revealed that some *Bacillus* species produce more than one type of cyclic lipopeptide molecule. In a study by Zhao et al. (2014), inhibitory activity against *Fusarium oxysporum* was demonstrated by *B. amyloliquefaciens* strain Q-426, attributed to Fengycin A, B, and bacillomycin D. For example, *B. velezensis* GH1-13 possesses biosynthetic genes for antifungal compounds such as bacillycin, iturin,

bacillomycin, surfactin, and fengycin (Ben Ayed et al., 2017). Previous research findings have shown that isolated strains of *B. subtilis* and *B. amyloliquefacien*s have the ability to produce surfactin, bacillomycin, and fengycin, exhibiting potent antibacterial and antioxidant activity against a wide range of fungi, as well as several strains of both gram-positive and gram-negative bacteria. In a study with 96 different *Bacillus* species isolates (Kamal et al., 2017), marker-assisted selection was employed to identify bioactive genes for iturin, surfactin, and bacillomycin D peptide synthetase. Among the 96 isolates, only the CS-42 strain contained all three bioactive genes and significantly reduced the development of *S. sclerotiorum*, the causative agent of canola stem rot disease, both *in vitro* and *in vivo*.

According to the findings of Jasim et al. (2016) and Mahmood et al. (2022), powerful antimicrobial lipopeptides, such as fengycin and surfactin, may be produced by *B. mojavensis*. LC-MS/MS has been employed to identify the formation of both molecules, showcasing the extensive antibacterial properties of the biocontrol potential of *B. mojavensis*. Li et al. (2016) reported, through agar diffusion tests, that *B. amyloliquefaciens* SYBC H47 exhibited antifungal activity against *F. oxygeneum*. The principal antifungal compounds were bacillomycin L, fengycin, and surfactin, as evaluated by HPLC LC/ESI-MS/MS. Similarly, according to Mosquera et al. (2014), lipopeptides fengycin C and iturin A account for the majority of the growth inhibition caused by Bs EA-CB0015 in *Mycosphaerella fijiensis*.

Advances in research

It is necessary to conduct consistent research that has led to the development of rapid, efficient, and reproducible chromatography and mass spectrometry techniques for the simultaneous detection of several lipopeptides in a range of *Bacillus* strains. Geissler et al. (2017) created a high-efficiency thin-layer chromatography method during their work with *Bacillus* strains such as *B. subtilis* Strain DSM 10T and *Bacillus* sp. *amyloliquefaciens* DSM 7T to identify and quantify cyclic lipopeptides iturin A, surfactin, and fengycin in a single experiment. Deng et al. (2016) developed a sensitive method for simultaneously identifying and quantifying iturin and surfactin cultured by *B. natto* NT-6 using liquid chromatography (LC) tandem mass spectrometry. The synthesis of lipopeptides in *Bacillus* species varies among different species, depending on external factors.

Monteiro et al. (2016) studied how various factors, such as pH, temperature, and growth conditions, affected the ability of *B. subtilis* to produce the lipopeptides surfactin, iturin, and fengycin A. They discovered that environmental factors, including temperature, pH, and growth medium, had a significant impact on the yield of these lipopeptides.

Finally, managing the cost becomes challenging when attempting to manufacture these compounds into useful biopesticides as an alternative to synthetic fungicides. Additionally, the processes associated with separation and purification should be taken into consideration.

CONCLUSION

To benefit from the *Bacillus* genus, it is crucial to explore synergistic interactions and identify potentially harmful interactions. Further research is needed to implement the aforementioned technique. In previous studies, numerous *Bacillus* spp. have significantly contributed to the development of Plant Growth Promoting Bacteria, focusing on biocontrol management of diseases. However, new potential strains are still awaiting emergence to enhance our understanding of the various processes supporting plant growth and pathogen suppression.

The biocontrol action of *Bacillus* species is primarily regulated through lipopeptides. The production of biopesticides can leverage the extraordinary potential of *Bacillus* species producing lipopeptides, given their intense antifungal and antibacterial impact. The biocontrol activity of *Bacillus* species in managing a wide range of plant diseases has been strengthened by their coproduction of lipopeptides.

The purpose of this current review is to contribute knowledge to ongoing research projects supporting the use of *Bacillus* spp. lipopeptides as potential bio-pesticides for enhancing biological control of numerous diseases with significant agricultural and horticultural impacts. To make biocontrol agents more widely applicable as bio-pesticides in the future, a greater understanding of the factors at play, including the signaling relationships among antagonists, pathogens, soil, and plants, is still to be discovered.

AUTHORS' CONTRIBUTION

All the authors contributed equally to the collection, arrangement, compilation, and proofreading of the article.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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