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Biocontrol of *Phytophthora infestans*-induced late blight using *Bacillus velezensis*-derived iturin A based bioformulation

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ABSTRACT

Phytophthora infestans, the causal agent of late blight disease, poses a significant threat to tomato and potato crops worldwide. The present study investigated the potential of *Bacillus velezensis*-derived iturin A as a biocontrol agent against *P. infestans*, both *in vitro* and in greenhouse pot experiments. First, *P. infestans* was isolated and identified from infected leaf samples collected from fields. Concurrently, *B. velezensis* was isolated from the same soil and characterized through molecular techniques. Subsequently, the inhibitory activity *of B. velezensis* living cells and cell suspension against *P. infestans* was evaluated, showing significant inhibition rates of 85.4% and 95.6%, respectively. Extraction and identification of iturin A from *B. velezensis* revealed a concentration of 210 mg per gram of dried cyclic lipopeptides. Mass spectrometry and NMR analysis confirmed the presence and chemical characteristics of iturin A, validating its abundance in the *B. velezensis* strain. Antagonism tests of bioformulations containing varying concentrations of iturin A exhibited varying degrees of inhibition, with higher concentrations achieving complete suppression of *P. infestans* mycelium growth. Notably, bioformulations RPB4 containing iturin A (20 mg per ml) showed promising efficacy in greenhouse pot experiments, demonstrating the highest efficacy in reducing disease incidence and severity index, comparable to the chemical fungicide metalaxyl. Hence, the study deals with developing a sustainable alternative to chemical fungicides.

1. INTRODUCTION

Global food security remains a paramount concern with the pressure of a growing population and the emphasis on sustainable agriculture practices. One of the major hurdles of contemporary agriculture is the management of plant diseases, which profoundly impact crop productivity [1]. Late blight caused by *Phytophthora infestans* epitomizes such challenges by affecting globally popular plants like tomato (*Solanum lycopersicum*), leading to substantial loss in the agriculture sector [2]. The pathogen infects various parts of plants, including roots, tubers, and shoots, leading to substantial yield loss [3]. The traditional means of controlling this disease is by using fungicides, which led to multiple issues in the long term, such as environmental pollution, negative effects on non-target organisms, and the emergence of fungicide-resistant microorganisms [4].

Recent research has increasingly focused on the potential of biocontrol agents, particularly iturin A, a cyclic lipopeptide (CLP) produced by various *Bacillus* species such as *Bacillus velezensis, Bacillus amyloliquefaciens* and *Bacillus atrophaeus* [5–7]. Iturin A has been identified as the main active antimicrobial and antifungal compound in *Bacillus* strains, showcasing its potential as a biocontrol agent for combating a broad spectrum of bacterial and fungal diseases [7,8]. Studies have shown that iturin A effectively inhibits the growth of several fungal species such as *Fusarium oxysporum,* and *Colletotrichum acutatum*, by affecting their mycelial morphology, conidia germination, and cell membrane integrity [9,10,8,11]. The compound's mechanism of action appears to involve disrupting fungal cell membranes, increasing reactive oxygen species levels, and affecting gene expression related to fungal growth and toxin production, ultimately leading to fungal cell death [9,12].

Moreover, iturin A has proven effective in suppressing plant diseases caused by various pathogens, including *Phytophthora* species, which are responsible for root rot and blight, as well as controlling diseases like Verticillium wilt in cotton and Fusarium wilt in lettuce, and rice blast in rice [8,11–13]. These findings underscore the compound's potential as a sustainable biocontrol strategy for managing plant diseases. While the precise mechanism of iturin A's antimicrobial activity is not fully understood, it is believed to involve the disruption of microbial cell membrane integrity, resulting in cell lysis and death [11]. Additionally, iturin A possesses surfactant properties, which enhance the spreadability of liquids, making it a valuable component in various industrial applications, including the formulation of agricultural products and cleaning agents [13,14]. Its properties also

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suggest a promising role as a biopesticide for controlling fungal diseases [8,13,15].

Several chemical fungicides such as Polyversum and Biochikol 020 PC are known to significantly reduce *P. infestans* infestation in potato plants [16]. Similarly, Kurzawińska and Mazur reported decreased canopy infection and tuber infestation in potatoes treated with these biotechnical preparations [17]. Similarly, Tomar *et al.* [17] identified *Pseudomonas aeruginosa*-1 as a promising biocontrol agent against *P. infestans*, which inhibits disease in whole potato plant tests [18]. Ndala *et al.* [19] evaluated the efficacy of plant extracts of *Plectranthus barbatus, Lantana camara,* and *Sphaeranthus suaveolens* showing comparable results to a commercial fungicide in managing late blight disease in tomatoes [19]. Despite these promising findings, there remains a gap in developing a comprehensive bioformulation utilizing these agents. Therefore, the present study deals with the intent to utilize the bioactive potential of iturin A derived from *B. velezensis* for the development of a bioformulation specifically targeting the management of *P. infestans*-induced late blight in tomato (*S. lycopersicum*) cultivation, offering a sustainable alternative to traditional chemical fungicides.

2. MATERIAL AND METHODS

2.1. Inhibition of pathogen *P. infestans* by *B. velezensis*

2.1.1. Collection of samples

Kolar tomato field, at latitude 13.1362° N, longitude 78.1291°, situated in Karnataka, India was explored for late blight symptoms (green to light brown or tan patches) on tomato leaves in the month of June 2022. infected leaf samples were collected and stored in sterile plastic bags for transportation to the laboratory. Bacteria were isolated and purified from the soil of the same field where healthy plants were spread for at least 20m×20m block. The soil samples were collected and placed in sterile plastic bags, ensuring their cleanliness, and promptly storing them in a cold environment using ice packs.

2.1.2. Isolation of P. infestans

Infected leaves were dried and cut into small pieces, which were cultivated on potato dextrose agar (PDA) plates fortified with tetracycline (10 mg/l) at 20ºC under dark conditions as described by Rakesh and Shivkumar [20]. The oomycete *P. infestans* was isolated for further study.

2.1.3. Isolation of B. velezensis

Three different culture media compositions were standardized and used for growing *B. velezensis*. All chemicals were purchased from Himedia Laboratories Pvt Ltd, India. In the M3A medium, the main components include malt extract at 6 g/l, glucose at a high concentration of 40 g/l, and yeast extract at 5 g/l, supplemented with 5 g/l each of K_2HPO_4 and $MgSO_4$, along with a trace of $ZnSO_4$ (1 g/l) in 1,000 ml of water. The F medium relied on 25 g/l of ragi flour as a complex carbon source, alongside 10 g/l of $CaCO_3$ and KH_2PO_4, and only 2 g/l of glucose, in 1,000 ml of water. Another GMK medium was composed of 10 g/l glucose and 5 g/l L-monosodium glutamate as primary nutrients, supported by several salts: 0.5 g/l $MgSO_4$, 0.78 g/l KCl, 1 g/l KH_2PO_4, 0.05 g/l $FeSO_4$, 5 g/l $MnSO_4$, and 0.16 g/l $CuSO_4$ in distilled water, with the pH adjusted to 7.2. Each of these media provided a unique composition to support the growth and metabolic activity of *B. velezensis*.

Initially, 2 g of soil was mixed with 2 ml of sterile phosphate buffered saline and allowed to incubate for 2 h. Following this, the tubes underwent centrifugation at 5,000 rpm for 5 min, and subsequently, 100 µl of the supernatant were inoculated into each of the culture media in triplicate. Incubation took place at 30°C for 2 days [21]. Morphologically different colonies were isolated and maintained in the same media. The species were identified by molecular characterization, and sequences were submitted to NCBI.

2.1.4. Molecular characterization of B. velezensis

Genomic DNA was isolated from bacterial colonies using a bacterial DNA isolation kit (Qiagen, New Delhi). Genomic DNA was amplified using 27F Forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R Reverse (5′-CGGCTACCTTGTTACGACTT-3′) primers of 16S rRNA gene [21]. The PCR was performed in a 40 µl mixture containing Master mix, PCR water, primers, and template DNA. Cycling conditions included enzyme activation at 96°C for 10 minutes, followed by 40 cycles of denaturation at 95°C, annealing at 52°C, and elongation at 72°C. The final extension was at 72°C for 10 minutes. Amplicon size were confirmed on agarose gel and sequenced using Thermofisher Applied Biosystems Sequencer (India).

2.1.5. In vitro Inhibition of P. infestans using B. velezensis

Bacillus velezensis living cells (LCs) were incubated on the same liquid medium for 24 hours at 37ºC with agitation (200 rpm), and the final concentration was adjusted to 1×10^8 CFU/ml with distilled water. Cellulose acetate membranes (0.22 µm) were used to filter cell suspension (CS) and to obtain a cell-free supernatant.

Phytophthora infestans mycelium was agitated at 10ºC for 3 hours to release sporangium followed by a zoospore formation. Zoospore suspension having 1×10^7 colony-forming unit in 1 ml culture was used to find inhibitory effects of *B. velezensis*. *Phytophthora infestans* mycelium growth was evaluated using *B. velezensis* living cells and CS through the dual-culture plate method as described by Wang *et al.* [22]. After coincubation at 25ºC for 5 days, the inhibitory zones were identified and inhibition rates were observed under a compound microscope (Magnus Compound Microscope). The inhibition rate (%) was determined using the following formula: (C - T)/C × 100, where T is the radius of the treatment group and C represents the radius of the colony of the control group.

Table 1. Ingredients of co-formulants.

Co- Formulation 1	
Ingredients	**Volume (ml)**
Tween 20	5
Paraffin oil	2.5
Soya oil	1.5
Neem oil	1
Xanthan gum	0.2
Sodium metabisulphite	0.3
Co- Formulation 2	
Ingredients	**Volume (ml)**
Tween 80	3.5
Paraafin oil	2.5
Soya oil	2.5
Neem oil	1.5
Xanthan gum	0.3
Sodium metabisulphite	0.5

Table 2. Details of bioformulations and coformulations used to inhibit *P. infestans* mycelium under laboratory conditions.

Bioformulations	Coformulations	Iturin concentration (mg per ml)	Percent inhibition of *P. infestans* mycelium
RPB1	1	5	40.5
RPB2	1	10	58.5$
RPB3	1	15	87.14#
RPB4	1	20	100*
RPB5	1	25	100*
RPB6	1	30	100*
RPB7	2	5	23.5
RPB8	2	10	32.45
RPB9	2	15	51.11$
RPB10	2	20	62.75
RPB11	2	25	71.25
RPB12	2	30	80.45#

Note: $p > 0.05$ for values with same symbols.

2.2. Extraction of iturin A

To extract iturin A from a culture of *B. velezensis* cells (1×10^8 CFU/ml) CS was inoculated into 500 ml of medium within a 1l flask. The flask was then incubated at 37°C for a period of 72–96 hours with shaking at 200 rpm until the culture reached the stationary phase of growth. It was followed by the centrifugation of bacterial culture at 8,000 rpm for 10 minutes to pellet the cells, after which the supernatant was carefully decanted. The decanted supernatant was then combined with an equal volume of ethyl acetate in a separatory funnel. After vigorous shaking for 1 hour, the mixture was allowed to settle, facilitating the separation of layers. Subsequently, the upper organic layer, containing the extracted CLPs was collected. This organic extract was then dried using a rotary evaporator operating at 40°C under reduced pressure, resulting in the isolation of crude CLPs [22].

2.3. High-performance liquid chromatography (HPLC) Purification of iturin A

HPLC system (Shimadzu Analytical Pvt. Ltd., India) with a reverse-phase C18 column was used for the study. Standard iturin A (Sigma-Aldrich) and extract solution (10 mg/l) were run separately on an HPLC system. The solutions were then introduced into the system, where elution was carried out using a gradient of water and methanol. The rate of injection was 1 ml/minute and the eluate was monitored at 280 nm to detect the peaks corresponding to iturin A. Once identified, these peaks were collected for further processing.

2.4. Electrospray ionization mass spectrometry

Purified lipopeptides, at a concentration of 1 mg/ml (obtained from HPLC peaks), were analyzed using ESI-MS in collision-induced dissociation mode to determine the amino acid sequences of CLP iturin A. An optimal collision energy ranging from 35 to 50 eV was chosen based on the precursor targeted ions. The molecular mass was determined, and molecular structure was confirmed.

2.5. Nuclear magnetic resonance spectroscopy

The purified iturin A was dissolved in deuterated chloroform ($CDCl_3$). Subsequently, 1H and 13C NMR spectroscopy were performed to determine the structure in detail, with a specific focus on the peptide and lipid components.

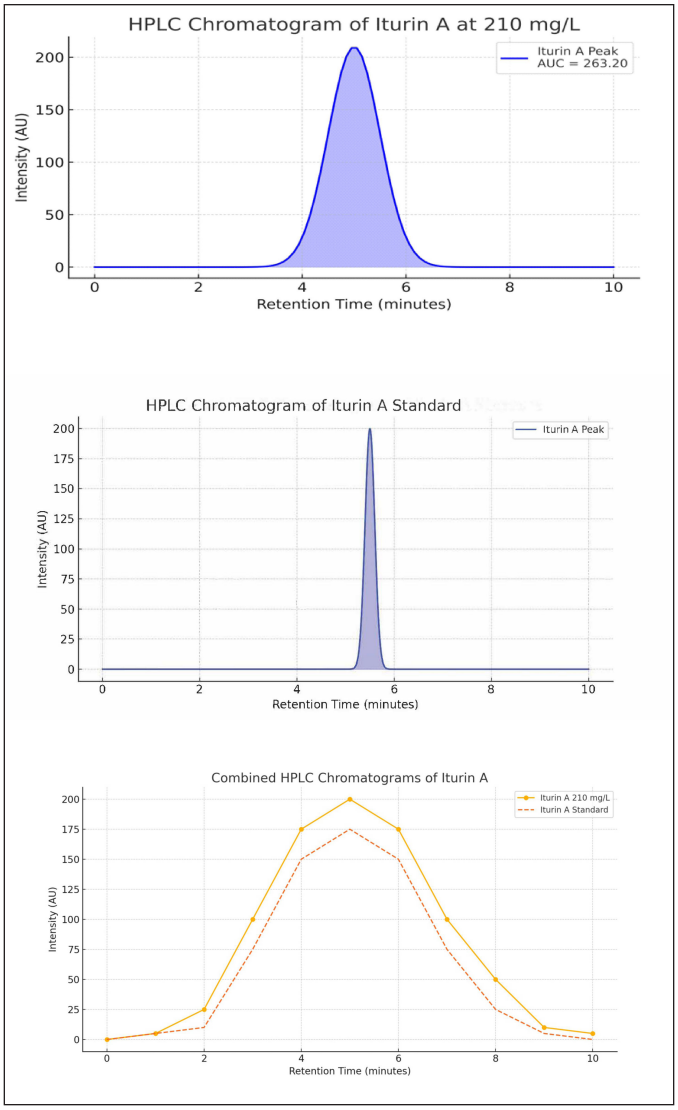

Figure 1. HPLC chromatogram of iturin A.
1a. iturin A chromatogram from *B. velezensis* Sample
1b: iturin A chromatogram of standard
1c. Combined HPLC chromatogram of iturin A

2.6. Preparation of bioformulation using iturin A

Two different compositions of co-formulant (Table 1) were prepared and iturin A was dissolved in it to make the final concentration of iturin A 5, 10, 15, and 20 mg/ml. Twelve bioformulations (Table 2) were explored for their potential against pathogen *P. infestans* and compared with metalaxyl fungicide which is commonly used to control Oomycete fungi diseases in plants.

2.7. Effect of bioformulation on mycelium growth of *P. infestans*

Bioformulation (RPB1- RPB12) at different doses (5%, 10%, 15%, 20%, 25%, and 30%) were added PDA media fortified with tetracycline (10 mg/l). To assess antagonism against mycelial growth, dual culture assays were conducted. In this setup, 10 days old *P. infestans* mycelial of size 7-mm-diameter was placed at the

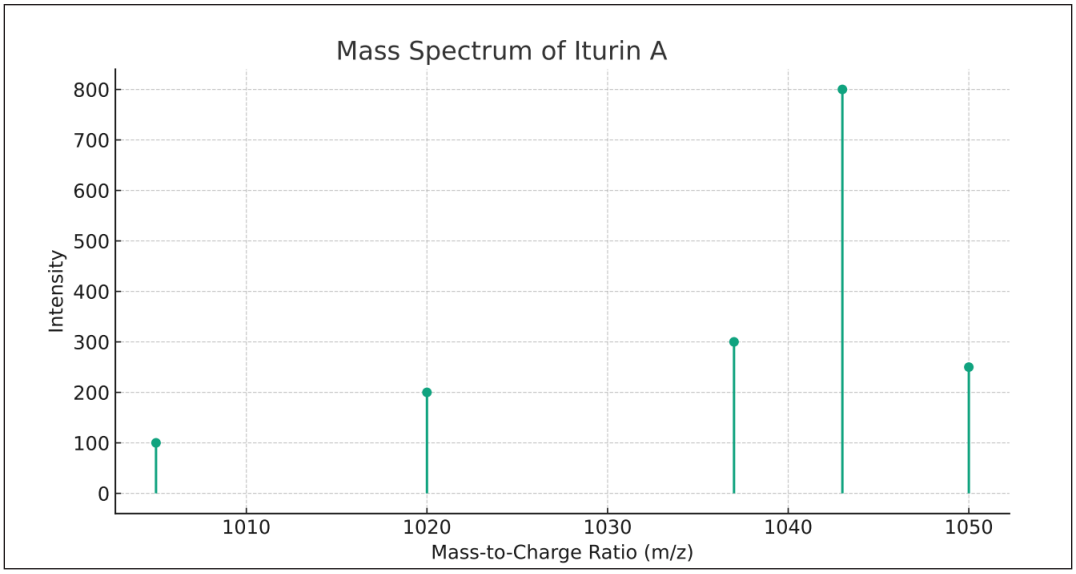

Figure 2. ESI-Mass Spectrum of iturin A.

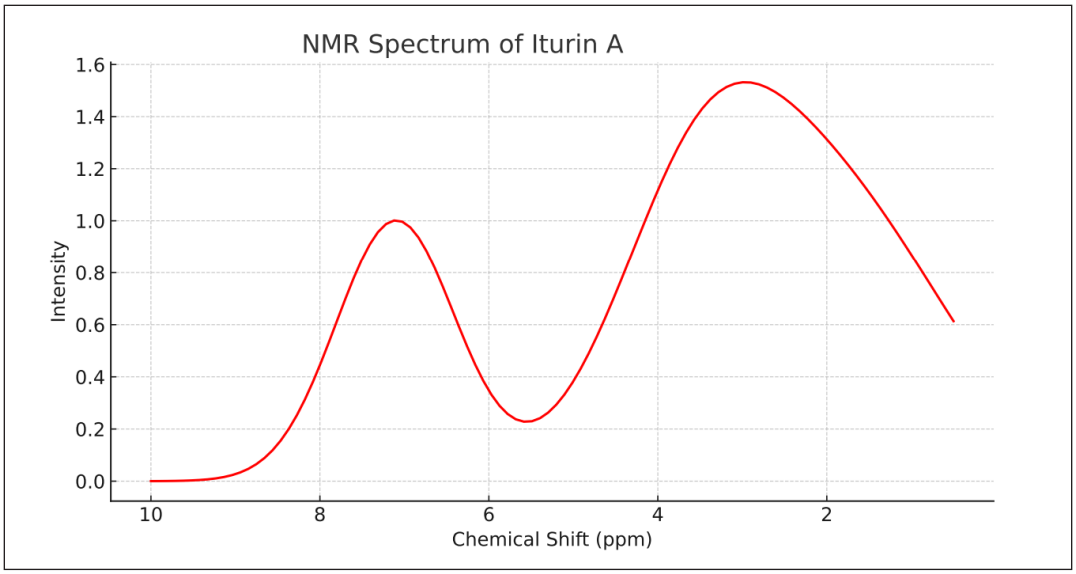

Figure 3. NMR spectrum of iturin A.

center of a 90 mm PDA medium and medium containing different doses of bioformulation. Media without Bioformulation was taken as negative control and media with metalaxyl (10%) was taken as positive control. Each composition was taken in 10 petri plates in triplicate. After 7 days of dark growth at 20°C, *P. infestans* colony diameter was measured using the cross method with a ruler, and the inhibition rate was calculated.

2.8. *In vivo* antagonism tests of pathogenicity in tomato plant

Solanum lycopersicum hybrid Variety Saaho (TO-3251) was purchased from Syngenta, India. Plug trays filled with sterile cocopeat were used to sow the seeds. Germination started after 6 days followed by seedling formation. After 24 days, seedlings were transferred to pot containing sterile red soil containing 50 g of farmyard manure per kg of soil. The experiment was performed after 25 days when tomato plants achieved 25–40 cm height and 4–5 leaves. 5 ml of *P. infestans* culture broth at a concentration of 10^8 CFU/ml was mixed with 100 ml water and applied to both the adaxial and abaxial leaf surfaces using a hand sprayer (Oriley Handheld, India). 100 µl of this suspension was applied per 600 cm^2 leaf surface. A day after fungal spray, bioformulation RPB 3, RPB4, and RPB 12 were sprayed on tomato leaves. 100 µl of the bioformulation composition was applied per 600 cm^2 leaf surface. Disease assessment was performed after 2, 4, and 6 days.

2.9. Assessment of disease severity

According to Romanazzi *et al.* [23], the percentage of leaf surface covered by late blight symptoms was used to assign the disease severity to 11 levels, ranging from 0 (healthy leaf) to 10 (over 90% of leaf surface exhibiting symptoms) [23]. Percent disease incidence on leaves and disease severity index (%) were calculated for control, iturin A formulation, and standard metalaxyl (10%). The efficacy of formulation in reducing the disease incidence (%) was calculated by the formula: Disease incidence of control- Disease incidence of formulation/Disease incidence of control X 100 [24].

2.10. Statistical analysis

All data obtained was subjected to ANOVA analysis using SPSS 26.0 Software. *p*-value was calculated for each dataset. $p = <0.05\%$ was considered a significant difference.

3. RESULTS

3.1. In vitro inhibition of *P. infestans* by *B. velezensis*

3.1.1. Sample collection and isolation of P. infestans

Infected leaf samples showing amorphous, dark brown, or tan spots were collected from the field. *P. infestans* was isolated from the PDA culture medium and identified through molecular characterization of *ITS* sequences. Sequences were deposited at NCBI with accession numbers OR677916. Spraying of 1 ml of *P. infestans* culture (10 ml inoculum/l) on 1,000cm^3 resulted in black/brown lesions followed by up to 80% wilting of leaves and fruits within 7 days of spray. It confirmed the isolated fungi was *P. infestans*.

3.1.2. Isolation and characterization of B. velezensis

Three bacterial cultures were isolated from media M3A. DNA isolation and molecular characterization using 16S ribosomal RNA gene primers identified the sequences. BLAST similarity analysis of these sequences confirmed this strain as *B. velezensis*. The sequence was submitted to NCBI and GenBank accession number PP434602 was allotted.

3.1.3. In vitro Inhibition of P. infestans

Bacillus velezensis living cells and CS showed 85.4% ± 2.31% and 95.6% ± 4.34% inhibition rate against *P. infestans*.

3.2. Extraction and identification of iturin A

The extraction process resulted in 2.5 g/l dried powder of CLPs. HPLC analysis of this powder was compared with standard iturin A to know the concentration of iturin in the sample. Standard iturin A peaked at 5.5 minutes at 200 AU intensity. Purification of crude lipopeptide extract (CLE) from *B. velezensis* showed the chromatogram for the CLE peaked at 5.2 minutes at 263.20 AU intensity upon analytical ultracentrifugation. It was used against a calibration curve to determine the concentration of iturin (Fig. 1 a–c). The concentration of iturin A in the sample was noted as 210 mg/g of dried CLP.

Mass spectrometry showed the most abundant molecular weight at 1043 Da along with several smaller peaks at lower and higher *m/z* values representing fragment ions (Fig. 2). This ion peak at *m/z* 1,043 was considered iturin A. It confirmed the abundance of iturin A in *B. velezensis*. The NMR data show chemical shifts for hydrogen atoms from 2.70–6.90 ppm. Figure 3 showed signals of a methyl group at δ 2.80 ppm and protons of tyrosine at δ 6.81 ppm at C14 fatty acid side chain in iturin A.

3.3 Antagonism tests of iturin A against *P. infestans*

The effectiveness of different bioformulations and coformulations containing varying concentrations of iturin A against *P. infestans* mycelium was evaluated (Table 2). Full growth of *P. infestans* mycelium was observed after 4 days of inoculation (Fig. 4a). RPB1-RPB12 based on coformulations 1 and 2 had iturin A concentrations ranging from 5 mg/ml to 30 mg/ml. RPB1- RBP6 based on co-formulation 1 corresponded the percent inhibitions ranging from 40.5% to 100%. Notably, higher concentrations of iturin A (RPB4, RPB5, and RPB6) with concentrations of 20 mg/ml and above achieved complete inhibition (Fig. 4b). However, bioformulations with coformulation 2 were noted less effective in suppressing *P. infestans* mycelium growth (Fig. 4c). Petri plates containing metalaxyl (10%) showed 95% inhibition of *P. infestans* mycelium (Fig. 4d).

3.4. Evaluation of bioformulation on tomato under greenhouse condition

The bioformulations RPB3, RPB4, and RPB12 showed promising efficacy in reducing disease incidence and severity index in leaves. Specifically, RPB4 exhibits the highest efficacy among the bioformulations in reducing disease incidence and severity index, with values of 95.50% and 96.66%, respectively. Its impact was comparable with metalaxyl (10%), a chemical fungicide (Fig. 5a–e). RPB3 and RPB12 also demonstrate considerable efficacy, with disease incidence reduction rates of 81.50% and 77.27%, respectively, and disease severity index reduction rates of 84.25% and 80.5%, respectively (Table 3; Fig. 5b, d).

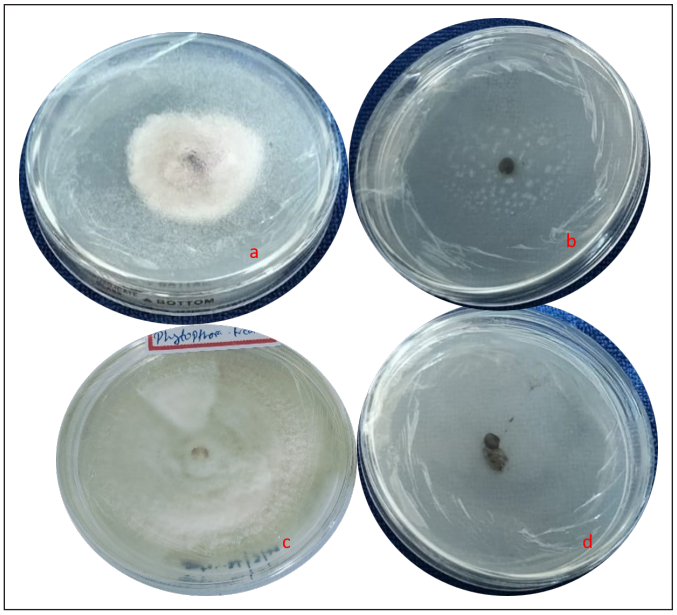

Figure 4. Antagonism tests of iturin A against *P. infestans*.
4a: *P. infestans; 4b: P. infestans* and RPB4; 4c. *P. infestans* and RPB8; 4d. *P. infestans* with metalaxyl (10%)

Table 3. Efficacy of bioformulations to control late blight caused by *P. infestans*.

Efficacy	Bioformulation RPB3	Bioformulation RPB4	Bioformulation RPB12	Metalaxyl (10%)
Efficacy of bioformulations to reduce disease incidence (%)	81.50**	95.50*	77.27**	95.00*
Efficacy of formulations to reduce disease severity index (%)	84.25***	96.66#	80.5***	93.33#

Note: $p > 0.05$ for values with the same symbols.

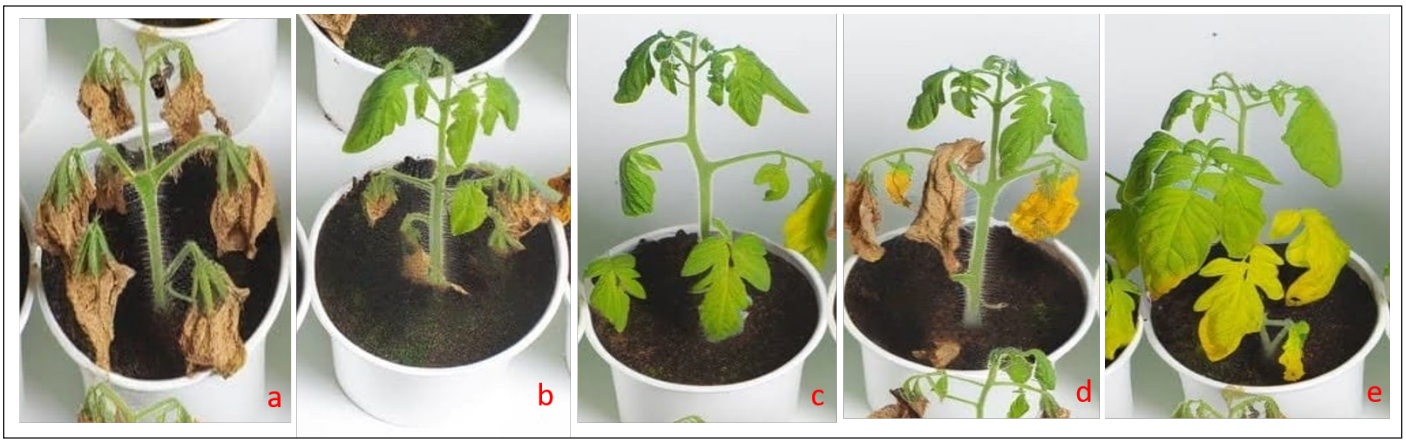

Figure 5. Study of bioformulations efficiency in controlling late blight caused by *P. infestans.*
5a: Tomato plant infected with *P. infestans; 5b:* Effect of the spray of RPB3 on infected plant; 5c. Effect of the spray of RPB4 on infected plant; 5d. Effect of the spray of RPB12 on infected plant; 5e: Effect of the spray of metalaxyl on the infected plant

4. DISCUSSION

Several species of *Bacillus* have emerged as promising biological control agents (BCAs) in recent years [25]. The present investigation confirms that the bacterium *B. velezensis*, isolated from soil in tomato fields, has emerged as a highly effective biological control agent (BCA) against *P. infestans*, the pathogen responsible for late blight disease in tomatoes. This study highlights the significant potential of *B. velezensis* strains in biocontrol applications, primarily through the secretion of enzymes such as proteases and cellulases, and the production of stable antagonistic compounds that lead to morphological deformation of *P. infestans* hyphae, achieving up to 85%–95% inhibition of mycelial growth [26]. Strain SDTB038 of *B. velezensis* notably reduced late blight incidence by 40.79%, primarily through the synthesis of lipopeptides that inhibit *P. infestans* infection [27]. Additionally, various strains of *B. velezensis*, including ND, UTB96, T701, ES2-4, and BR-01, have been reported to contain gene clusters encoding secondary metabolites such as iturin, difficidin, and bacilysin. These metabolites are crucial in downregulating genes associated with the pathogenicity and virulence of *P. infestans*, thereby enhancing biocontrol efficacy [28–32].

The present study also exhibited remarkable efficacy in inhibiting the *P. infestans* mycelium using iturin A extracted from *B. velezensis*. The extraction of iturin A from *B. velezensis* and using that to formulate a bioformulation to control *P. infestans* mark it as the first documented instance of such pronounced effectiveness. This underscores the significance of *Bacillus* species as prevalent and extensively researched biological control agents (BCAs), as highlighted in prior studies.

A key focus of this investigation is the use of iturin A, a secondary metabolite produced by *B. velezensis*. Recent findings indicate that strains capable of synthesizing iturin A, such as those mentioned above, can effectively inhibit the growth of *P. infestans* mycelium. The present study also exhibited remarkable efficacy in inhibiting the *P. infestans* mycelium using iturin A extracted from *B. velezensis*. The extraction of iturin A from *B. velezensis* and using that to formulate a bioformulation to control *P. infestans* mark it as the first documented instance of such pronounced effectiveness. This underscores the significance of *Bacillus* species as prevalent and extensively researched biological control agents (BCAs), as highlighted in prior studies [5,30,32]. Beyond its role in plant pathogen control, iturin A has diverse industry applications. It has been employed in the synthesis of silver nanoparticles and as a biosurfactant for enhanced oil recovery in the petroleum sector [33]. Furthermore, iturin-based bioformulations have shown potential against various pathogens, including bacteria and insects, and have applications in food packaging and water purification [34–35]. However, its use against plant pathogenic fungi, specifically in controlling late blight, remains underexplored. The development of the bioformulation RPB4, which leverages the potential of iturin A to combat *P. infestans*, represents a promising and innovative solution in sustainable agriculture. This study underscores the pivotal role of *Bacillus* species as BCAs, reinforcing their importance in ongoing and future research efforts aimed at managing plant diseases effectively and sustainably.

5. CONCLUSION

The present study has suggested that bioformulations, particularly RPB4, having 20 mg/l of iturin A can be an effective alternative to chemical fungicides like metalaxyl for managing diseases in leaves, offering potential benefits such as reduced environmental impact and improved sustainability in agriculture. Further field application of Bioformulation RPB4 could be explored to optimize the application methods and dosages. Additionally, optimizing the formulation parameters, such as concentration, application method, and timing, is crucial for maximizing efficacy while minimizing costs and environmental impact. This optimization process can be facilitated through field trials conducted under diverse environmental conditions to ensure the reliability and consistency of the results. Furthermore, assessing the long-term effects of these bioformulations on soil health, microbial communities, and non-target organisms is essential for evaluating their overall sustainability and safety. Comprehensive risk assessments will enable informed decision-making regarding their integration into agricultural practices on a larger scale

6. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval

of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

8. FUNDING

There is no funding to report.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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