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# Biocontrol activity of *Bacillus nakamurai* BDI-IS1 and investigation of its mechanisms against Northern corn leaf blight

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## Scientific communications

Two scientific papers included in this thesis were published, while two others are in preparation and will be submitted shortly. I have also participated in various scientific conferences, presenting two posters and three oral presentations.

**Published manuscripts:**

**Gaspard Nihorimbere**, Virginie Korangi Alleluya, François Nimbeshaho, Venant Nihorimbere, Anne Legrève and Marc Ongena (2024). *Bacillus*-based biocontrol beyond chemical control in central Africa: the challenge of turning myth into reality. Front. Plant Sci. 15:1349357. doi:10.3389/fpls.2024.1349357.

François Nimbeshaho, **Gaspard Nihorimbere**, Anthony Argüelles Arias, Charlotte Lienard, Sébastien Steels, Anaclet Nibasumba, Venant Nihorimbere, Anne Legrève and Marc Ongena (2024). Unravelling the secondary metabolome and biocontrol potential of the recently described species *Bacillus nakamurai*. Microbiological Research, 288, 127841. doi: 10.1016/j.micres.2024.127841.

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# Abbreviations

AOS: Allene oxide synthase

DNA: Desoxyribonucleic acid

CAT: Catalase

cDNA: Complementary DNA

CIMMYT: Centro Internacional de Mejoramiento de Maíz y Trigo (International Maize and Wheat Improvement Center)

CLSM: Confocal laser scanning microscopy

Cq: Quantification cycle

COGERCO: Compagnie de Gérence du Coton

CTAB: Cetyltrimethylammonium bromide

ET: Ethylene

FAO : Food and Agriculture Organisation

GDP : Gross Domestic Product

Ht : *Helminthosporium turcicum*

IAA: Indole-3-acetic acid

IFAD : International Fund for Agriculture Development

ISABU : Institut des Sciences Agronomiques du Burundi

ISR : Induced systemic resistance

JA: Jasmonic acid

LBA: Luria broth agar

MAT: Mating type

MINAGRI: Ministère de l'Agriculture et de l'Elevage

MINEAGRI: Ministère de l'Environnement, de l'Agriculture et de l'Elevage

MYC: Myelocytomatosis

NA: Nutrient agar

NASECO: Nalweyo Seed Company

NCBI: National Center for Biotechnology Information

NLB: Northern corn leaf blight

PAL: Phenylalanine ammonia-lyase

PGPR: Plant Growth Promoting Rhizobacteria

PRDAIGL:Projet Régional de Développmenet Agricole Integré dans les Grands Lacs

qPCR: Quantitative polymerase chain reaction

ROS: Reactive Oxygen species

RT-qPCR: Reverse Transcription qPCR

RNA: Ribonucleic acid

OAP : Organisation d'appui à l'autopromotion

ODECA : Office pour le Développement du café au Burundi

OHP : Office de l'Huile de Palme au Burundi

OPVs : Open pollinated varieties

OTB : Office du Thé du Burundi

SA: Salicylic acid

TEB: Tomato early blight

UB: Université du Burundi

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# Table of contents

Acknowledgements ..... i
Scientific communications ..... v
Credit ..... ix
Table of contents ..... xi
List of Figures ..... xv
List of Tables ..... xvii
GENERAL INTRODUCTION ..... 1
1. Problem statement ..... 1
2. Thesis outline ..... 3
LITERATURE REVIEW ..... 7
CHAPTER I. CONTEXT OF AGRICULTURE AND PHYTOSANITARY CONSTRAINTS IN BURUNDI ..... 9
1.1. Context of agriculture ..... 9
1.2. Phytosanitary constraints ..... 13
CHAPTER II. IMPORTANCE AND PHYTOSANITARY CONSTRAINTS OF MAIZE ..... 17
2.1. Description and cultivation of maize ..... 17
2.2. Importance of maize in Burundi ..... 19
2.3. Diseases and pests of maize ..... 20
2.4. Northern corn leaf blight ..... 21
CHAPTER III. INTERACTION BETWEEN PLANTS AND MICROORGANISMS ..... 39
3.1. Plant as a holobiont ..... 39
3.2. Plant-pathogen interaction ..... 40
3.3. Plant resistance and defenses against pathogens ..... 41

CHAPTER IV. BACILLUS-BASED BIOCONTROL BEYOND CHEMICAL CONTROL IN CENTRAL AFRICA: THE CHALLENGE OF TURNING MYTH INTO REALITY .....45
4.1. Abstract .....46
4.2. Introduction .....47
4.3. Diversity of phytopathogens and their control .....48
4.4. Limitations in local regulation of phytosanitary products and associated risks .....61
4.5. Non-biological alternatives to chemical pesticides in GLCCA .....65
4.6. Implementation of biological control .....66
4.7. Conclusions .....82
OBJECTIVES AND RESEARCH STRATEGIES.....91
RESULTS .....93
CHAPTER V. DISTRIBUTION OF NORTHERN CORN LEAF BLIGHT AND ASSESSMENT OF FARMERS' KNOWLEDGE AND SKILLS REGARDING THE DISEASE IN BURUNDI .....95
5.1. Abstract .....95
5.2. Introduction .....95
5.3. Materials and methods.....98
5.4. Results .....100
5.5. Discussion .....106
5.6. Conclusion.....108
CHAPTER VI. UNRAVELLING THE SECONDARY METABOLOME AND BIOCONTROL POTENTIAL OF THE RECENTLY DESCRIBED SPECIES *BACILLUS NAKAMURAI* ...111
6.1. Abstract .....112
6.2. Introduction .....113
6.3. Materials and Methods .....116

6.4. Results ..... 126
6.5. Discussion ..... 138
6.6. Conclusions ..... 142
CHAPTER VII. BIOCONTROL OF NORTHERN CORN LEAF BLIGHT BY *BACILLUS NAKAMURAI* BDI-IS1 THROUGH DIVERSE MODES OF ACTION ..... 157
7.1. Abstract ..... 157
7.2. Introduction ..... 158
7.3. Materials and methods ..... 160
7.4. Results ..... 169
7.5. Discussion ..... 180
7.6. Conclusion ..... 188
GENERAL DISCUSSION AND CONCLUSION ..... 197
CHAPTER VIII. GENERAL DISCUSSION ..... 199
8.1. Changing agricultural practices: embracing agroecology to reduce chemical pesticides in agriculture ..... 199
8.2. BDI-IS1 controls tomato early blight and Northern corn leaf blight ..... 201
8.3. BDI-IS1 acts by direct antagonism and by stimulating the plant defense ..... 204
8.4. Bioformulation of BDI-IS1 on bioresources substrates ..... 207
8.5. Perspectives ..... 209
CHAPTER IX. GENERAL CONCLUSION ..... 215
REFERENCES ..... 217

# List of Figures

Figure 1-1: Agroecological regions of Burundi. .....12
Figure 2-1: The area under maize cultivation and total maize production from 2010 to 2022. .....19
Figure 2-2: Northern corn leaf blight symptoms and conidia. .....23
Figure 2-3: Structure of monocerin, a non-host specific toxin produced by *Exserohilum turcicum* (sourced from Cuq et al., 1993). .....26
Figure 2-4: *Exserohilum turcicum* lifecycle.....28
Figure 5-1: Symptoms of Northern corn leaf blight. .....101
Figure 5-2: Prevalence and distribution of the Percent Severity Index (PSI) of Northern Corn Leaf Blight (NLB) in the Surveyed Areas of Burundi.....102
Figure 5-3: Variation in NLB severity according to sowing date (A) and field location in relation to hill position (B). .....103
Figure 5-4: Percentage of farmers recognizing Northern corn leaf blight (NLB). .....105
Figure 6-1: The Burundi isolated strain BDI-IS1 retains a great antagonistic potential against phytopathogens.....128
Figure 6-2: Predicted biosynthetic gene clusters (BGCs) in BDI-IS1 genome sequence. .....132
Figure 6-3: Secondary metabolome of BDI-IS1 and comparative genome mining profile of all the *B. nakamurai* strains.....133
Figure 6-4: Antagonistic activities of BDI-IS1 mutants against selected bacterial and fungal phytopathogens.....135
Figure 6-5: Biocontrol efficacy of BDI-IS1 against tomato early blight (TEB) and Northern corn leaf blight (NLB). .....137
Figure 6-A1: Scale developed for the evaluation of tomato early blight (A) and maize northern leaf blight (B).....151
Figure 6-A2: Black-brown pigment developed Burundi isolate BDI-IS1. .....151
Figure 6-A3: MS2 patterns of surfactin (A), iturin A (B), and plantazolicin .....152
Figure 6-A4: Biocontrol efficacy of QST713 against tomato early blight (TEB) and Northern corn leaf blight (NLB). .....153

Figure 6-A5: Biocontrol efficacy of BDI-IS1 and QST713 against tomato early blight (TEB) and Northern corn leaf blight (NLB).....154
Figure 6-A6: Comparison of protection provided by BDI-IS1 and QST713 against TEB and NLB .....155
Figure 7-1: Number of necrotic lesions and disease severity recorded in two field trials performed in season A (A&B) and in season B (C&D). .....171
Figure 7-2: Progression of NLB on maize plants treated with bioproducts.....172
Figure 7-3: Biocontrol efficacy of a BDI-IS1-based bioproduct obtained on bean residues against NLB.....173
Figure 7-4: Biocontrol efficacy of BDI-IS1 and defective mutants in the production of secondary metabolites (ΔituA: iturin A, ΔsrfAA: surfactin) against maize Northern corn leaf blight.....175
Figure 7-5: Evaluation of NLB on maize treated or not treated with BDI-IS1.....176
Figure 7-6: Differential expression of defense genes in maize plants treated or not with BDI-IS1 and inoculated or not with *E. turcicum*178
Figure 7-7: Localization of BDI-IS1 on the maize plant using confocal microscopy. .....180
Figure 7-A1: Number of colonies from BDI-IS1 grown on lignocellulosic substrates. .....191
Figure 7-A2: Expression profile of housekeeping genes based on Cq value obtained from qPCR assay. .....193
Figure 7-A3: Assessment of biocontrol efficacy of *Bacillus nakamurai* BDI-IS1 impeded in production of secondary metabolites (iturin A, surfactin and bacillibactin) against Northern corn leaf blight.....194
Figure 7-A4: Differential expression of defense genes in maize plants treated or not with BDI-IS1 and inoculated or not with *E. turcicum*. .....195

# List of Tables

Table 2-1: Origin and characteristics of maize varieties including open pollinated varieties (OPV) and hybrids, grown in Burundi. .....18
Table 2-2: Distribution of *E. turcicum* races in different countries grouped by continent. .....30
Table 2-3: Maize resistance genes to Northern corn leaf blight: their origins and phenotypic reactions in plant hosts carrying these genes.33
Table 4-1: Microbial pathogens affecting crop production in the GLCCA region and chemical pesticides used for their control .....51
Table 4-2: Status of some EU not authorized chemical pesticides in GLCCA. .....62
Table 4-3: Some global success stories of *Bacillus* isolates (subtilis group) in biocontrol of important crop pathogens reported in the GLCCA .....77
Table 4-A1: Registered pesticides in the GLCCA countries. .....84
Table 6-A1: Primers utilized in the BDI-IS1 mutant strains construction .....144
Table 6-A2: Antibacterial activity (mm of inhibition diameter ± SE) of the isolated bacterial strains from Burundian soils against 6 plant pathogenic bacteria .....146
Table 6-A3: Antifungal activity (mm of inhibition radius ± SE) of the isolated bacterial strain BDI-IS1 against nine phytopathogenic fungi .....147
Table 6-A4: Analysis of the daily variation (DAI) in the severity of early blight in tomato. .....147
Table 6-A5: Analysis of the daily variation in the severity of the Northern corn leaf blight disease. .....149
Table 6-A6: Area under the disease progression curve (AUDPC) and protection index (PI) of BDI-IS1 and QST713 against Northern corn leaf blight and tomato early blight. .....150
Table 6-A7: Temperature and relative humidity in greenhouse during the experimental period.....150
Table 7-A1: Primers used to quantify defense genes by RT-qPCR..189

Table 7-A2: Coefficient of variation and stability of housekeeping genes at different tested time points.....190
Table 7-A3: Daily analysis of NLB severity obtained in trial 1&2 carried out under field at Mparamba research center.....190
Table 7-A4: Variation of disease severity per day between inoculated substrates.....192

# GENERAL INTRODUCTION

## 1. Problem statement

Plant diseases and pests are increasingly threatening crop production in Burundi (Ochilo et al., 2022; Okonya et al., 2019b). This high prevalence of diseases and pests is exacerbated by a poorly managed seed system, insufficient knowledge among farmers and extension agents in disease and pest management and limited access to plant protection measures.

The Burundian seed system is highly dependent on imports from neighboring countries. The crop varieties currently cultivated are imported from organizations such as CIMMYT (for maize and wheat), CIP (for potato and sweet potato), and IITA (for banana and cassava). The hybrid maize seeds being used in Burundi are sourced from neighboring countries such as Zambia, Kenya, or Uganda. Additionally, farmers living near the borders unofficially introduce other crop varieties. These seeds are introduced along with their seed-borne pathogens, which are further spread throughout the country through official channels or from farmer to farmer. Consequently, several diseases and pests reported in neighboring countries are later discovered in Burundi, causing significant damage to agricultural production. This has been observed with the emergence of several viral, bacterial, and fungal diseases, including cassava mosaic disease, maize lethal necrotic virus, banana *Xanthomonas* wilt, and stem rust strain U99. These diseases affect cassava, maize, banana, and wheat, leading to significant losses in agricultural production in Burundi.

Additionally, the prevalence of disease and pests is exacerbated by the lack of knowledge and skills in disease and pest management among farmers and agricultural extensionists. This is evident in certain agricultural practices, such as the monoculture system widely adopted in Burundi, particularly for maize, where a single hybrid variety is

grown across the agro-ecological region, contributing to the proliferation of virulent pathogen strains. This could explain the high prevalence of Northern corn leaf blight observed on newly introduced varieties, which are affecting even tolerant varieties.

In Burundi, over 90% of the population is engaged in agriculture, earning less than $2 a day. Farmers have limited access to chemical pesticides, which are primarily used to control plant diseases and pests. Consequently, they face difficult choices in protecting their crops; diseases and pests affecting cash crops are usually prioritized for control, while those impacting staple crops are often neglected, ultimately limiting food production. The cash crops grown in Burundi, including coffee, tomatoes, potatoes and fruits, receive protection due to their high market value. However, chemical pesticides are applied at higher rates and more frequently than recommended, leading to significant pesticide exposure for farmers and consumers, either directly or through chemical residues in food. This situation is further exacerbated by the high level of illiteracy among farmers, who may struggle to understand product labels, resulting in confusion regarding chemical usage for instance, using insecticides instead of fungicides, or vice versa.

Of the chemical pesticides used in Burundi, more than 33% are not authorized in European countries due to their high toxicity (Nihorimbere et al., 2024). Numerous studies have documented the negative impact of these highly toxic chemical pesticides on human, animal, and environmental health. They disrupt the microbial communities associated with plants, which are crucial for growth and protection benefits. Furthermore, the prolonged use of chemical pesticides can lead to the development of pathogen resistance, complicating their management (Delnat et al., 2019; Hawkins et al., 2019; Siddiqui et al., 2023). In addition, the growing demand for pesticide-residue-free food is prompting researchers and stakeholders to rethink agricultural practices and promote agroecological methods that leverage the natural benefits of microbial-host interactions. These

methods present promising and safe alternatives to chemical pesticides for controlling plant diseases and pests. Some plant growth-promoting rhizobacteria (PGPR), which are widely distributed in the environment, have shown effectiveness in controlling plant diseases and may replace or supplement chemical pesticides in certain pathosystems. However, these naturally occurring microorganisms are rarely utilized in Burundi, with the exception of *Bacillus thuringiensis*, which is officially approved and registered as an insecticide. The use of biological control agents offers several advantages, including reducing the quantity of chemical pesticides applied, promoting biodiversity and protecting the environment, making them a viable option for managing diseases and pests where chemical pesticides have proven ineffective.

This thesis is part of the global context of searching effective biological control agents for managing the major plant diseases prevalent in Burundi.

## 2. Thesis outline

The first part of the thesis is a literature review that introduces the various areas of study covered in the thesis. We first present the context of agriculture and phytosanitary constraints in Burundi (Chapter I). This chapter describes the crops grown in all agro-ecological regions and the main phytosanitary constraints limiting crop production in the country. Given the importance of maize in Burundi, we detail its significance alongside its main diseases and pests (Chapter II). Among the diseases affecting maize, we focus on our analysis of Northern Corn Leaf Blight (NLB), describing its causal agent, epidemiology, and control methods.

In the next chapter (III), we emphasize the importance of plant-microbe interactions. In this section, plants are described as holobionts, where microbes and plants coexist and can establish either pathogenic or mutualistic interactions. In chapter IV, we present *Bacillus* as a promising alternative to chemical pesticides. This topic is integrated in a published review article that first presents the main diseases and pests occurring in the Great Lakes region, including Burundi, Rwanda, and

Congo, and outlines the current control measures implemented by each country. Then, we highlight success stories reported using *Bacillus* species to manage prevalent diseases and pests in the region. In a separate section, we present the specific objectives of this thesis and describe the research strategies used to address the research questions.

The second part of this thesis is the experimental section, which comprises three research chapters (V, VI, and VII). In chapter V, we demonstrate the significance of Northern Corn Leaf Blight (NLB) by assessing its distribution and evaluating farmers' skills and knowledge in managing this disease. This section includes a map illustrating NLB distribution based on disease severity across all agro-ecological zones in Burundi. Then, we showcase the efficacy of *Bacillus nakamurai* BDI-IS1 in controlling Tomato Early Blight (TEB) and NLB (Chapter VI). This chapter characterizes BDI-IS1 and presents its metabolomic profile. Additionally, we highlight the antagonistic activity of BDI-IS1 against various phytopathogenic bacteria and fungi observed through i*n vitro* assays, as well as its biocontrol efficacy against TEB and NLB under greenhouse conditions.

In chapter VII, we present the efficacy of *Bacillus nakamurai* BDI-IS1 in controlling Northern Corn Leaf Blight (NLB) under field conditions and discuss its modes of action in protecting maize against NLB. Two approaches were employed: the use of mutant bacteria to study the biocontrol efficacy of secondary metabolites and the quantification of the relative expression of defense genes. These methods aim to elucidate the mechanisms by which BDI-IS1 protects maize from NLB.

The third part of this thesis consists of the general discussion and conclusion. In general discussion (Chapter VIII), we mainly emphasize the importance of biological control as a promising alternative to chemical methods. We also discuss the role of *Bacillus* in biological control, highlighting the consistent biocontrol efficacy of BDI-IS1 against TEB and NLB, observed in both controlled and field conditions. Furthermore, we explore the potential applications of BDI-IS1 in managing plant diseases in Burundi, detailing its modes of action and

implications for future application methods under field conditions. In this chapter, we present several perspectives, including the need to assess the impact of BDI-IS1 on soil and plant microbial communities, to extend experiments to other pathosystems under field conditions, and to explore the potential for enhancing the efficacy of BDI-IS1 through integrated pest management approaches.

We conclude (Chapter IX) this thesis by highlighting the main results, including the biocontrol efficacy of BDI-IS1 and its multiple modes of action. Finally, we emphasize the strength of the methodology employed in this thesis, which combines various approaches to assess the efficacy of biological control agents, and we identify potential future research areas in this field.

# LITERATURE REVIEW

# CHAPTER I.

# CONTEXT OF AGRICULTURE AND PHYTOSANITARY CONSTRAINTS IN BURUNDI

## 1.1. Context of agriculture

Burundi is a tropical country located in East and Central Africa, covering an area of 27,834 km², with 23,500 km² of potential agricultural land. Of this, 36% are occupied by crops, while the remain is covered by forests (MINEAGRI, 2018). Agriculture is the main contributor to the economy, accounting for 40% of Gross Domestic Product (GDP) (PND, 2018). It supplies 95% of the country's food requirements and provides raw materials for the food industry (MINEAGRI, 2018). Additionally, agriculture is the main source of income, contributing more than 90% of foreign exchange earnings (Ochilo et al., 2022).

Agriculture in Burundi is performed during three distinct seasons: season A from September to February, season B from February to June, and season C from June to September. Seasons A and B both coincide with the rainy season, while season C takes place in the wetlands during the dry season.

Several export and staple crops are grown in Burundi. The main export crops are coffee and tea, which generate the foreign exchange needed for importing other goods. As for local production, staple crops include bananas and plantains, root and tuber crops (such as sweet potato, cassava, taro, and potato), legumes (beans and peas), cereals (maize, rice, wheat, and sorghum), vegetables, fruits, and oil crops (such as palm oil, groundnut, soybean, and sunflower) (MINEAGRI, 2018).

These crops are cultivated in various morpho-physical zones, including highlands, midlands, and lowlands. Within these zones, five agro-ecological regions have been identified from west to east, based on their

distinct climatic characteristics such as temperature, rainfall, vegetation, and crops grown. These regions include the western plains of Imbo, the steep slopes of Mumirwa, the Congo-Nile ridge, the humid and dry plateaus, and the depressions of Moso in the east and Bugesera in the northeast (Baramburiye et al., 2013) (Figure 1-1).

The Imbo Plain is located in the lowlands along Lake Tanganyika, with an altitude ranging from 774 to 1,000 meters. The region has an average annual temperature of 23°C and receives 800 to 1,000 mm of rainfall. It is the most fertile area, with alluvial soil formed by erosion from the fertile slopes of the Mumirwa region. However, the Imbo Plain frequently experiences water shortages, as it is the driest region, with a prolonged dry season lasting five to seven months, necessitating irrigation for water-intensive crops such as vegetables, maize, and rice. Agriculture is practiced during seasons A, B, and C, due to the rapid growth of crops and the resulting shortened vegetative cycle. The main staple crops grown in this area include rice, maize, and tomatoes, with beans, sorghum, tobacco, cassava, bananas, vegetables, and fruits such as mangoes and citrus grown to a lesser extent. Oil palm and cotton are the most widely cultivated industrial crops, supplying local industries with oil and cotton wool for cloth production.

The Mumirwa region is a highland area with steeply sloping mountains, characterized by severe erosion and significant soil loss, reaching up to 100 tons per hectare per year (Beun, 2022) . The altitude ranges from 1,000 to 1,750 meters, with temperatures between 18°C and 21°C, and annual rainfall ranging from 1,000 to 1,700 mm. Agriculture is undertaken in the mountains during seasons A and B, and in small swamps during season C. The region's moderate temperature and humidity supports the cultivation of various staple crops typical of both lowland and highland areas, including bananas, cassava, beans, maize, sweet potatoes, taro, and some coffee.

The Congo-Nile Ridge, located in the highlands, serves as a natural border between the eastern and western basins. This region contains several high mountains with altitudes ranging from 1,900 to 2,670

meters. The average temperature is around 15°C, and the area receives approximately 2,000 mm of rainfall annually. Agriculture is carried on the mountains during seasons A and B, while the wet swamps are cultivated in season C. The region is well-suited for growing staple crops such as wheat, peas, and potatoes, with maize, sweet potatoes, and beans grown to a lesser extent. Tea is the primary export crop of this region.

The humid and dry plateaus in the central part of the country feature moderate mountains with altitudes ranging from 1,500 to 1,800 meters. This region experiences moderate temperatures of 18 to 20°C and receives 1,200 to 1,300 mm of rainfall annually. In addition to the plateaus, where agriculture is practiced during seasons A and B, the region contains vast swamplands used for agriculture in season C. The main staple crops grown in this area include sweet potato, banana, and bean, with maize, cassava, cowpea, and peanut grown at minimal level. Coffee is widely cultivated as the primary export crop.

The Bugesera and Moso depressions are situated between the low and medium plains and contain fertile areas at altitudes ranging from 1,000 to 1,200 meters. The average annual temperature ranges from 14 to 24°C, with approximately 1,100 mm of rainfall. The main agricultural seasons are A and B, which correspond to the rainy seasons. The season C is a dry season, and some crops are cultivated under irrigation. This region is suitable for growing a variety of staple crops, including rice, maize, bean, peanut, pea, banana, cassava, sweet potato, sugarcane, and cotton.

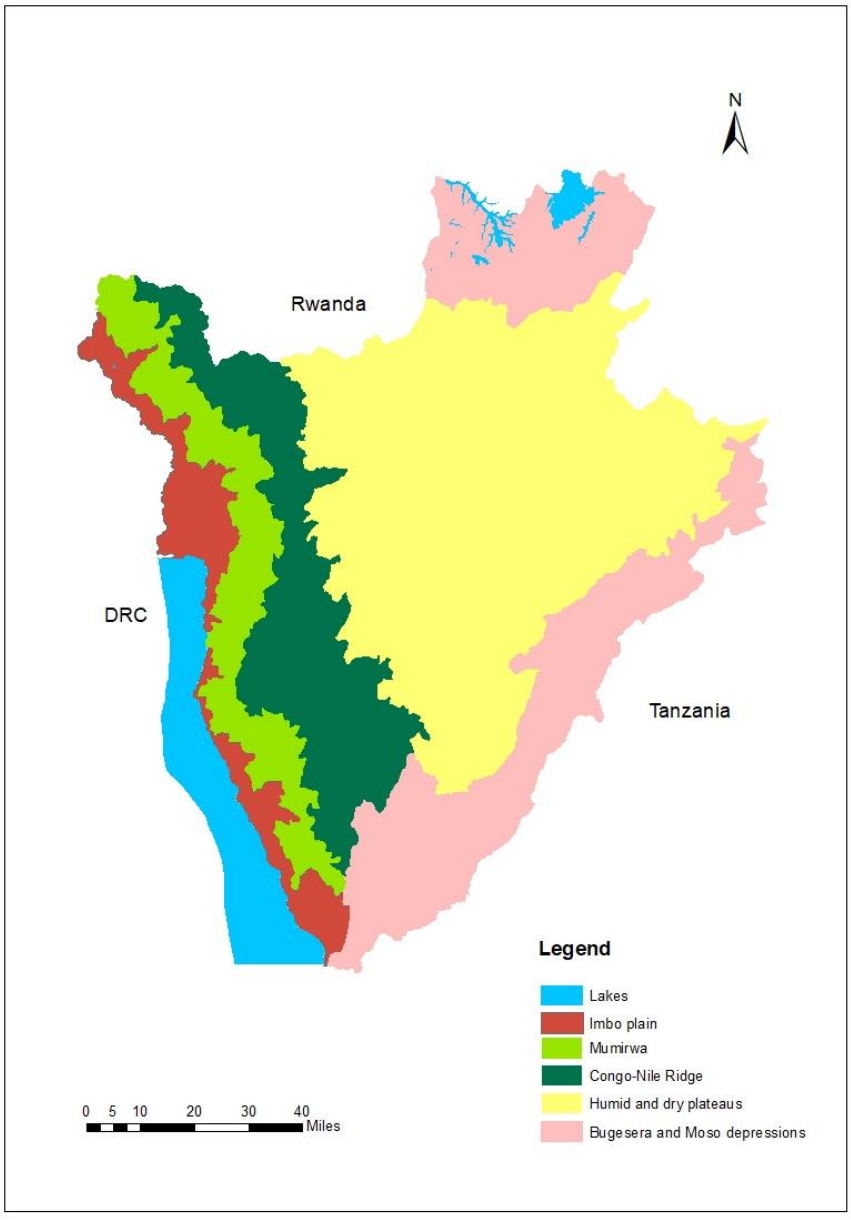


Figure 1-1: Agroecological regions of Burundi.
*From Western to Eastern region: Imbo plain, Mumirwa, Congo Nile Ridge, Humid and dry plateaus, Bugesera and Moso depressions. The map was established in this study by using ArcG software.*

Within the above agro-ecological regions, agriculture is carried out on an average of 0.5 hectare per household and is mainly consisting in subsistence farming (MINEAGRI, 2018). Burundi benefits from sufficient rainfall, distributed from September to May, to meet the water requirements for various crops. Additionally, the country has abundant water sources, which offer potential for irrigation system development, enabling year-round crop cultivation. Several irrigation projects, funded by IFAD, the World Bank, and the Bank of Africa, have expanded the cultivation of lowland areas, increasing the land under cultivation and boosting the production of crops such as maize, potato, and bean.

The production of certain crops, such as coffee, tea, palm oil, and, to a lesser extent, rice, maize, and cotton, is organized into a value chain involving companies like ODECA (Office pour le Développement du Café au Burundi), OTB (Office du Thé du Burundi), OHP (Office de l'Huile de Palme), and COGERCO (Compagnie de Gérence du Cotton), which oversee production, processing, and marketing of the final products. For coffee, tea, palm oil, and cotton, both state and private farms coexist, supplying raw materials to these companies. For other crops, farmers receive less support, and their production has continuously decreased due to several constraints, including low soil fertility, and pests and diseases affecting crops both in the field and in storage. In the following section, we will focus on plant diseases and pest-related challenges, as these issues are prevalent across all agro-ecological zones and impact all crops grown in Burundi.

## 1.2. Phytosanitary constraints

Burundi's tropical climate fosters the development of diseases and pests that can adversely affect crop production. The high prevalence of diseases and pests is attributed to several factors characterizing agriculture in Burundi, including a poorly managed seed system, insufficient knowledge among farmers and extension agents in disease management and institutional challenges.

Seed production is organized into prebasic, basic, and certified seeds (Baramburiye, 2010). Prebasic seeds are produced by the national agriculture research institute (ISABU), while basic and certified seeds are produced by private seed multipliers across different agro-ecological zones. Additionally, improved seeds for several crops are either scarce or unaffordable, with only 0.8% of farmers using certified seeds (MINAGRI, 2015). Most farmers rely on their own seeds from crop species grown several years ago, which results in the continuous transmission of seed-borne diseases. The lack of a well-organized seed production sector particularly affects vegetatively propagated plants where pests and diseases are especially transmitted through the exchange of infected planting materials, a common practice in Burundi. This has been demonstrated by spread of historical diseases such as cassava mosaic, banana bunchy top, and banana *Xanthomonas* wilt, which have been particularly devastating in Burundi, contributing to food shortages in 2000, 2010, and 2011, respectively (Bouwmeester et al., 2012; Ndayihanzamaso et al., 2016; Niyongere et al., 2013). Consequently, the prevalence and impact of these diseases on agricultural production have been exacerbated by inadequate disease management strategies and a lack of awareness among farmers and extension workers (Bigirimana et al., 2004). This is demonstrated by current agricultural practices, which often involve cultivating the same crop or even the same varieties across entire regions. The widespread use of a single crop variety increases the incidence and spread of epidemics while reducing field-level diversity. Field diversity is crucial for reducing inoculum and is ultimately an effective strategy for disease management (He et al., 2019; King and Lively, 2012).

The management of plant diseases and pests is further constrained by institutional challenges. At the institutional level, the Plant Protection Department of the Ministry of Environment, Agriculture, and Livestock oversees the control of plant diseases and pests. This department is responsible for ensuring the phytosanitary health of imported plant materials and conducting disease surveillance, with regional phytosanitary inspectors stationed at entry points into the country (PS,

2021). However, the effectiveness of this department is severely limited due to several issues, including inadequate laboratory facilities and the country's high permeability. Plant diseases identified in neighboring countries often spread rapidly into Burundi, causing significant damage to agricultural production.

The identification of plant diseases and pests is supported by the phytopathology laboratory of ISABU, which has played a significant role in identifying the major diseases and pests in Burundi. Recently, the laboratory received support from the Dutch Embassy in Burundi and Nuffic to implement a CABI-led plant protection program aimed at helping farmers reduce losses caused by plant diseases and pests. The project sought to establish an early warning system for pests and diseases in Burundi and to strengthen the capacity of farmers' organizations in pest management. It has established over one hundred crop clinics and trained more than 230 crop doctors (Ochilo et al., 2022). These clinics are managed by trained extension agronomists and operate under the coordination of the ISABU, where phytopathologists assist with diagnosing diseases and suggesting possible control measures. However, these clinics still lack essential equipment such as microscopes, growth chambers, and media for identifying plant pathogens.

# CHAPTER II.

# IMPORTANCE AND PHYTOSANITARY CONSTRAINTS OF MAIZE

## 2.1. Description and cultivation of maize

Maize is an annual plant belonging to the Poaceae family and the species *Zea mays*. The root system consists of adventitious roots, which develop from the node below the soil surface, as maize does not have a tap root. The maize stem can grow up to 5 m tall, from the ground to the flag leaf attached to the stem. The leaves, which serve as photosynthetic organs, are alternately attached to the stem and can range from 8 to 30 per plant (Awata et al., 2019).

Maize is adapted to a wide range of climatic conditions, from 58°N to 30°S in the northern and southern hemispheres and can grow at altitudes ranging from 0 to 4000 m above sea level. It requires approximately 12.5 hours of sunlight, temperatures between 17°C and 33°C, and a relative humidity of 85% to 100%. As a C4 plant, maize efficiently uses carbon dioxide for photosynthesis, resulting in high relative yields. It also exhibits better water-use efficiency, requiring 450 to 700 mm of water per growing season (Awata et al., 2019; Sales et al., 2021).

In Burundi, maize is typically grown after legumes such as beans and peas, or after tuber and root crops. The maize varieties used are usually introduced by ISABU from the International Maize and Wheat Improvement Center (CIMMYT). These varieties are evaluated for yield and disease resistance before being released to the agro-ecological zones of the country (Nkurunziza et al., 2012). Currently, eleven maize varieties, including both open-pollinated varieties (OPVs) and hybrids, have been released and are cultivated by farmers across various agro-ecological zones (Table 2-1) (MINEAGRI, 2020). Hybrid maize

varieties are introduced by external seed companies such as PANNAR and NASECO.

Table 2-1: Origin and characteristics of maize varieties including open pollinated varieties (OPV) and hybrids, grown in Burundi.

| **Variety** | **Type of variety** | **Origin** | **Cultivation zone** | **Yield** | **Diseases (s=susceptible, t=tolerant, r= resistant)** |
|---|---|---|---|---|---|
| ISEGA | OPV | local | High land | 2-3t/ha | Maize streak virus (s), *Spodoptera frugiperda (s)*, Northern corn leaf blight (t) |
| MUGAMBA | OPV | CIMMYT-Mexico | High land | 2-3 t/ha | Maize streak virus (s), *Spodoptera frugiperda (s)*, Northern corn leaf blight (t) |
| ZM605 | OPV | CIMMYT-Zimbabwe | Middle land | 3,5-4,5 t/ha | Maize streak virus (r), Northern corn leaf blight (s), *Spodoptera frugiperda (s)* |
| ZM621 | OPV | CIMMYT-Zimbabwe | Middle land | 3-4 t/ha | Maize streak virus (r), Northern corn leaf blight (s), *Spodoptera frugiperda (s)* |
| ECAVEL | OPV | CIMMYT-Kenya | Lowland | 4-5t/ha | Maize streak virus (t), *S. frugiperda* (s) |
| MM3 | OPV | NARO-Uganda | Lowland | 5-12 t/ha | Maize streak virus (s) |
| LONG 4 | OPV | NARO-Uganda | Lowland | 6,8-9,3 t/ha | Maize streak virus (s) |
| PAN 53 | hybrid | PANNAR-South Africa | Low and middle land | 10-11T/ha | Grey leaf spot (r) and Northen corn leaf blight (r) |
| PAN691 | hybrid | PANNAR-South Africa | High land | 8,5 T/ha | Northenrn corn leaf blight (t), Grey leaf spot (t) |
| Bazooka | hybrid | NASEKO | Middle land | 7,4 T/ha | Maize Necrotic lesions (r) |

## 2.2. Importance of maize in Burundi

In Burundi, maize is primary used for household consumption and, to a lesser extent, for livestock feed (Nijimbere, 2022). It is grown across all agro-ecological zones during both season A, B, and C. Season A, characterized by high rainfall, is the primary maize-growing season, representing 67% of the total annual maize produced across the country (Nijimbere, 2022). Season B is characterized by low rain intensity and short rain periods, during which maize is grown only in the lowlands, with high temperatures and a short growing season ranging from three to four months. In Season C, maize is grown in swamps, where wetlands are available during the dry season.

The area under maize cultivation and the quantity of maize grain produced vary significantly from year to year (Figure 2-1). Notably, from 2014 to 2022, the area under maize cultivation increased significantly, from 97,242 to 180,000 ha, and maize production doubled, from 127,829 to 280,000 tons (FAOSTAT, accessed on 14 August 2024).

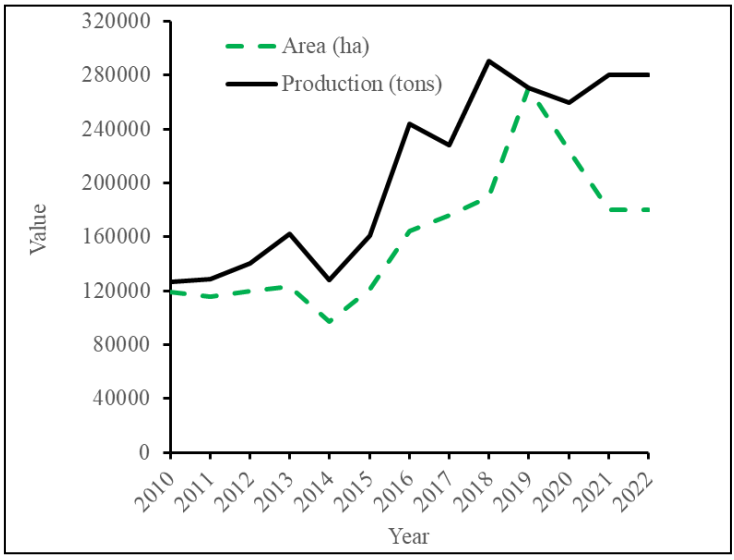


Figure 2-1: The area under maize cultivation and total maize production from 2010 to 2022. *The data used to create this graph was sourced from FAOSTAT.*

The increase in maize production can be attributed to the promotion and intensification of maize cultivation, as the government qualified maize as one of five national priority crops, alongside rice, cassava, potato, and banana. This designation led to a national commitment to enhance maize production in both swampy and mountainous areas.

The increase in maize production can also be attributed to the introduction of hybrid maize varieties, which have high yields ranging from 7 to 8 tons per hectare, compared to open-pollinated varieties (OPVs), which rarely exceed 3 tons per hectare. These hybrid varieties are being promoted by the government and financial partners to subsidize seed programs and boost maize production. Several organizations, including the World Bank through the Projet Régional de Développement Agricole Intégré dans les Grands Lacs (PRDAIGL), Tubura, World Vision, IFAD, and Food for the Hungry, have played a significant role in promoting maize seed and training various stakeholders in the maize value chain. Despite these efforts, maize production remains low due to several constraints, including a lack of improved seeds, insufficient irrigation, climate change, post-harvest losses, and the impact of diseases and pests.

## 2.3. Diseases and pests of maize

Maize is affected by several diseases and pests both in the field and in storage. Various insects, viruses, bacteria, and fungi have been reported as major constraints to maize production in Burundi. Among the most damaging insects, the fall armyworm, caused by *Spodoptera frugiperda*, is a significant pest affecting maize and other Poaceae crops in Burundi (Nagoshi et al., 2018; Paudel Timilsena et al., 2022). This insect is present in all agro-ecological zones, with particularly high incidence in the lowlands. Other pests affecting maize production include *Busseola fusca*, *Sesamia calamistis*, *Eldana saccharina*, *Rhopalosiphum padi*, *R. maidis*, *Sitophilus oryzae*, *S. zeamais*, and *Prostephanus truncatus*. Most of these pests are not controlled, except *S. frugiperda*, which is managed through various control methods,

including physical and chemical controls. The chemical pesticides used to control *S. frugiperda* include Orthene 75 SP (acephate), Decis 25EC (deltamethrin), Cypermetrin 25EC (cypermethrin), Dursban 4E and Dursban 5G (chlorpyrifos), as well as Dudu Fenos 44EC (profenofos 40% and cypermethrin 4%) (Durocher-Granger et al., 2018; Ndayiragije, 2019).

Maize is also affected by several microbial diseases, including maize lethal necrosis virus, maize streak virus, Northern corn leaf blight caused by *Exserohilum turcicum*, rusts caused by *Puccinia* spp., stem, root and ear rot caused by several *Fusarium* species, and corn smut caused by *Ustilago maydis* (Nkurunziza et al., 2012). Bacterial disease caused by *Pseudomonas fuscovaginae* was also reported on maize and was similar to that observed on upland rice introduced from Japan (Duveiller et al., 1989; Nkurunziza et al., 2012). These diseases are primarily managed through the use of resistant or tolerant varieties, which are sometimes unavailable for certain pathogens. Among these diseases, Northern corn leaf blight is widespread across all agro-ecological zones, damaging maize leaves and potentially causing significant yield losses (personal observation). This disease is increasingly observed in maize varieties already under cultivation, with particularly high severity seen in newly introduced varieties.

## 2.4. Northern corn leaf blight

### 2.4.1. Causal agent, classification, symptoms and distribution

Northern corn leaf blight (NLB) or Helminthosporiose [in French] is a global disease caused by *Exserohilum turcicum* (Pass) K.J. Leonard and E.G. Suggs., a filamentous fungus that produces different types of fruiting structures depending on whether it is in its anamorphic or teleomorphic phase. The asexual cycle produces isolated or clustered conidiophores that measure approximately 300 µm in length and 8-9 µm in thickness. At the apex of the conidiophore, the hilum structure produces conidia that are prominently visible, with a straight or slightly

curved shape, ellipsoid to obclavate (Nsibo et al., 2024). Conidia are pale to medium straw-colored, smooth, and possess 4-9 pseudosepta, measuring 50-144 µm in length and 20-24 µm in width at their widest point. The sexual cycle produces black, globose pseudothecia on the surface of the corn leaf. The pseudothecia are ellipsoidal, measuring 40-56 x 12-15 µm, with clearly visible septa surrounding the ostiole. These structures consist of cylindrical, clavate, short, pedicellate, and bitunicate asci that contain 1-8 ascospores (Bunkoed et al.2014).

*E. turcicum* was first described in Italy in 1876 by Passerini. Since then, the taxonomy of this fungus has evolved through several classifications: from *Helminthosporium turcicum* Pass. 1876, *Helminthosporium inconspicuum* Cooke & Ellis 1878, *Bipolaris turcica* (Pass.) Shaemaker (1959), *Kleissleriella turcica* (Luttr.) Arx *(1970), Luttrellia turcica (*Pass.) Khokhr (1978), and *Drechslera turcica (*Pass.) Subram. & Jain (1966) to *Exserohilum turcicum (Pass.) K.J. Leonard and E.G. Suggs (1974).* The teleomorphic state of the fungus was discovered by Luttrell as *Trichometasphaeria turcica* (Luttr.) in 1958 and later revised by Leonard & Suggs to *Setosphaeria turcica (*Luttr.) K.J. Leonard & Suggs (1974). This fungus belongs to the phylum Ascomycota, subphylum Pezizomycotina, class Dothideomycetes, subclass Pleosporomycetidae, order Pleosporales, family Pleosporaceae, genus *Setosphaeria* (teleomorph), and species *Setosphaeria turcica* (Ahangar et al., 2022; Welz and Geiger, 2000).

The center of origin of *E. turcicum* has been reported in Mesoamerica, with some sources also pointing to East Africa (Borchardt et al., 1998; Martin, 2011). It has since spread to Europe, North and South America, Australia, Asia, and Africa (Cao et al., 2020), affecting maize (*Zea mays*), *Sorghum* spp., and other grasses, including *Pennisetum* species (Ahangar et al., 2022). The disease is a major constraint to maize production in tropical areas where the climate is favorable for the development and reproduction of the pathogen (Ahangar et al., 2022; De Rossi et al., 2022). The main characteristic symptoms include necrotic lesions that have a cigar or elliptical shape and are parallel to

the leaf margin (Figure 2-2). The disease begins with small, oval, grayish-green, water-soaked spots that develop into irregular or elliptical shapes, sometimes appearing linear, with a dark gray coloration. The sporulated lesions can be olive, dark gray, or black (Ahangar et al., 2022; Kotze et al., 2019; Levy and Pataky, 1992). The necrotic lesions measure approximately 12 mm in width and 2.5 to 15 cm in length. As these lesions expand and coalesce, they lead to leaf blight and eventually plant death. Under high infection pressure, symptoms can spread to the upper parts of the plant, resulting in ear and crown rot as well as seedling infections.

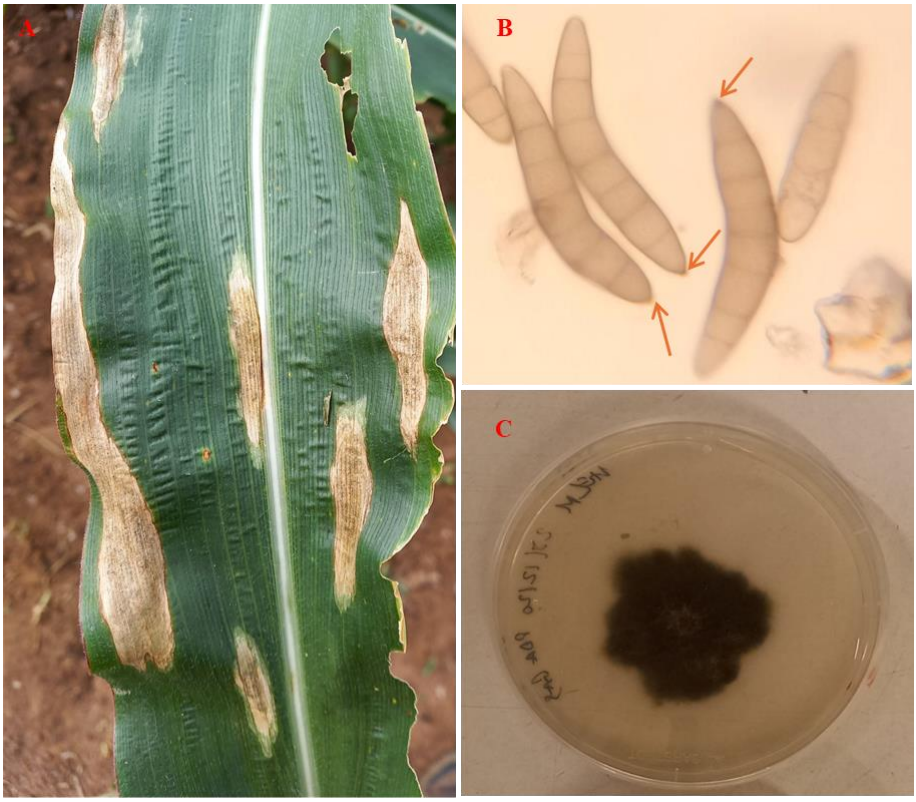


Figure 2-2: Northern corn leaf blight symptoms and conidia.
*A) Gray lesions measuring 12mm in width and 2.5 to 15cm in length, with an elliptical shape; B) Conidia with 20-24µm in width and 50-144µm in length, with protruding hilum (indicated by arrows); C) Mycelium of* Exserohilum turcicum *grown on PDA media at 27°C, two weeks old. Photo A was taken in the field during a trial conducted at the Mparambo Research Center. Photos B and C represent the strain of* E. turcicum *isolated from samples collected at Mwakiro commune of Burundi.*

### 2.4.2. Economic impact

NLB is a significant disease of maize that affects the leaves, reducing photosynthetic activity and causing yield losses ranging from 28% to 90%, throughout the world (Ahangar et al., 2022; De Rossi et al., 2022). Yield loss depends on several factors, including the growth stage at which infection occurs, host resistance, pathogen virulence, and the position of the infected leaves on the plant (De Rossi et al., 2022; Jakhar et al., 2023; Levy and Pataky, 1992). A substantial yield reduction is observed when infection occurs early, while late infection results in moderate yield losses. A yield reduction of 40% was noted when NLB occurred prior to silking, whereas minimal yield loss was observed when the fungus affected the plant six to eight weeks after silking.

In addition, NLB readily develops on susceptible host cultivars, leading to significant losses, while the disease rarely occurs on resistant cultivars. As previously shown, yield losses exceed 60% in susceptible varieties, whereas losses of 40% and 17% are observed in polygenic (quantitative) and monogenic (qualitative) resistant varieties, respectively (Jakhar et al., 2023; Levy and Pataky, 1992). NLB affects maize from the lower to the upper leaves, with high severity observed when symptoms reach the upper two-thirds of the maize plant due to increased inoculum pressure. Additionally, *E. turcicum* adversely affects seed quality by reducing sugar content and germination rates (Ahangar et al., 2022).

NLB is among the ten most damaging crop diseases in the USA and Canada. In the state of Illinois, yield losses due to NLB infection in maize have ranged from 31% to 72% (Mueller et al., 2020). This disease is also the most devastating maize disease in Africa, with countries such as Uganda experiencing epidemic periods resulting in 100% maize yield losses (Ramathani et al., 2011). More recently, average yield losses of 60% have been reported in Kenya, Uganda, Ethiopia, South Africa, and Zambia (Ahangar et al., 2022).

In Burundi, NLB affects maize in all agroecological zones, with yield losses previously estimated at 50% (Nkurunziza et al., 2012). Recently, an increase in disease severity has been observed across all released maize varieties, with significant yield losses reported in hybrids introduced from neighboring countries. The introduction of new maize seeds may carry new strains of *E. turcicum*, which could spread to existing cultivated maize varieties and cause substantial damage to production. Furthermore, these new varieties may also encounter different strains of *E. turcicum* present in various agro-ecological maize-growing zones, leading to heightened susceptibility and subsequent yield losses. During our field visit, some introduced maize varieties from CIMMYT were completely devastated and excluded from the next selection trials. Early infections and high inoculum pressure are currently being observed in the released maize varieties, suggesting the potential emergence of new strains that could significantly increase yield losses in major production areas.

### 2.4.3. Lifestyle of *E. turcicum*

*E. turcicum* is a hemibiotroph that exhibits a dual lifestyle, beginning as a biotroph and transitioning to a necrotrophic phase, during which it kills host tissues to acquire nutrients. The boundary between these phases is not well defined. However, the biotrophic phase is characterized by the suppression of plant defense mechanisms and successful pathogen establishment, achieved through the production of pathogen effectors such as Ecp6, AVRHt1, Six13, and Six5 (Human et al., 2020). Following the biotrophic phase, the pathogen adopts a necrotrophic lifestyle, characterized by the general necrosis of plant tissues. This phase is enhanced by the production of various cell wall-degrading enzymes that break down host tissues and membranes into monomeric forms of sugars, amino acids, and fatty acids, which the pathogen can easily absorb (Human et al., 2020). The necrotrophic phase of *E. turcicum* is also marked by the production of toxins such as monocerin (Figure 2-3), a non-host specific toxin that inhibits chlorophyll synthesis, reduces root growth, and induces necrosis

symptoms, particularly in susceptible maize cultivars as well as in other grasses (Cuq et al., 1993; Human et al., 2020; Robeson and Strobel, 1982).

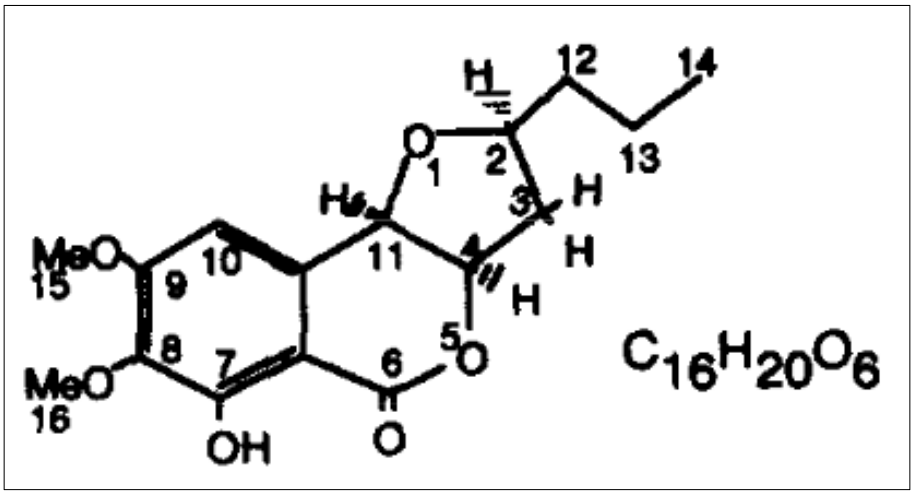


Figure 2-3: Structure of monocerin, a non-host specific toxin produced by *Exserohilum turcicum (sourced from Cuq et al., 1993).*

The fungus produces also HT-toxins, host specific toxins involved in inhibition of chlorophyll and chlorosis extension in maize. Furthermore, the fungus produces several enzymes and secondary metabolites, including xylanases, which degrade arabinoxylans responsible for cuticle integrity, and catalases, which are involved in conidial germination (Cuq et al., 1993).

### 2.4.4. Life cycle and diversity of *E. turcicum*

*E. turcicum* is a heterothallic fungus that overwinters in harsh conditions as conidia, dormant mycelia on host plant debris or maize seeds, or as chlamydospores in the soil. The fungal conidia or mycelia are spread by wind or rain splash onto new plant leaves (Nsibo et al., 2024). Germinated conidia can penetrate directly through the cuticle or epidermis of the host, though they rarely enter plant tissues through stomata or trichomes. Once inside, the pathogen grows within or

between epidermal cells and progresses slowly through adjacent mesophyll cells, with some hyphae moving toward xylem vessels. *E. turcicum* hyphae colonize the xylem, leading to initial symptoms, including light chlorotic spots (Kotze et al., 2019).

In susceptible plant hosts, fungal hyphae multiply rapidly within the xylem vessels, blocking veins and intensifying blight symptoms. Moreover, the fungus spreads from the xylem into adjacent mesophyll cells, causing widespread tissue necrosis, collapse of neighboring cells, and subsequent fungal growth near the stomata, where conidiophores produce new conidia. This reproductive cycle is typically completed within 18 days after infection (Kotze et al., 2019). NLB is thus a polycyclic disease that primarily affects the lower leaves and progressively spread toward upper leaves and to other plants in the field (Figure 2-4). In resistant cultivars, *E. turcicum* is rarely found in the xylem vessels, and small lesions form on leaves without sporulation, limiting the pathogen's spread to other tissues and plants (Jakhar et al., 2023; Navarro et al., 2020).

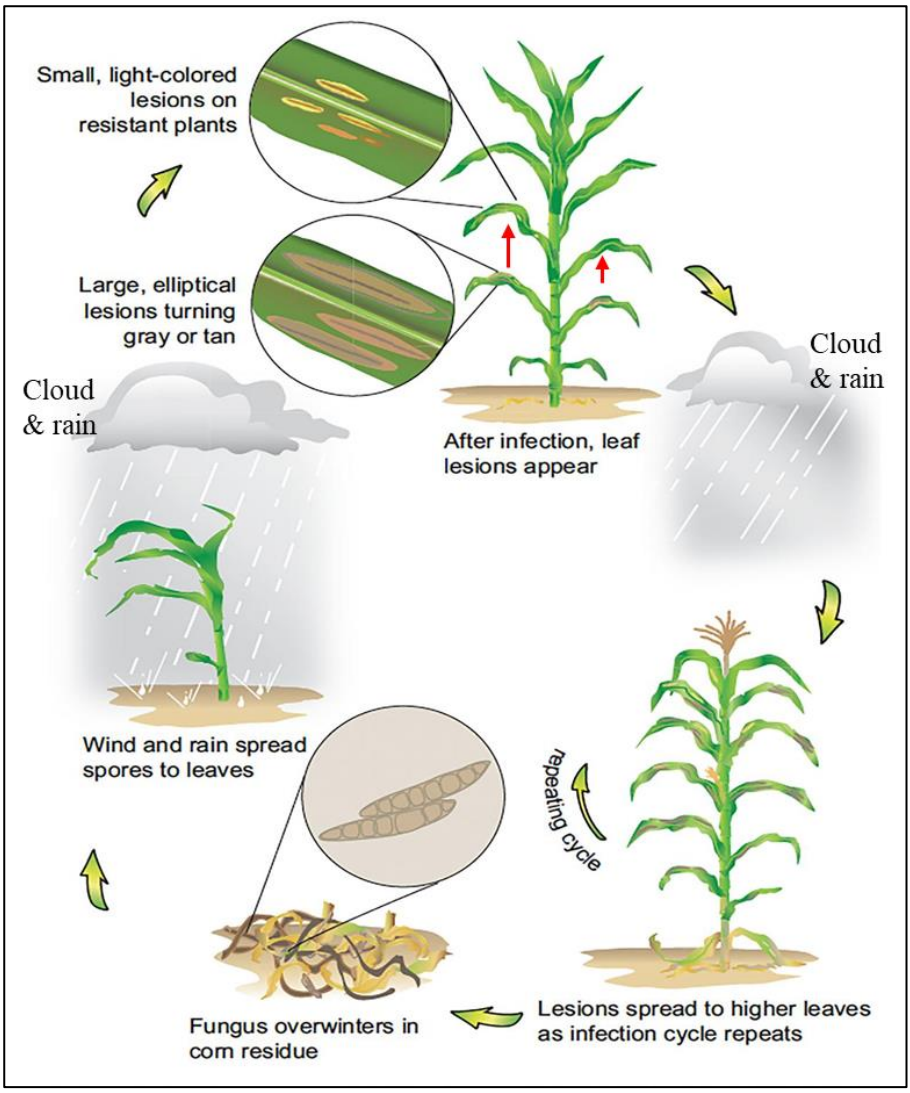


Figure 2-4: *Exserohilum turcicum* lifecycle.
*The red arrows indicate the spread of the fungi from lower to the upper leaves of maize plant. This lifecycle was adapted from (https://extension.sdstate.edu/northern-corn-leaf-blight-developing-corn).*

Under favorable conditions, *E. turcicum* grows and completes its life cycle through either asexual or sexual reproduction. Asexual reproduction is commonly observed in nature and involves significant mitotic growth of mycelia, followed by the formation of conidiophores,

which give rise to conidia. This mode of reproduction leads to the production of genetically homogeneous individuals and is associated with the frequent historical epidemics observed worldwide.

*E. turcicum* can also reproduce sexually, as it is heterothallic, with three reported mating types: "A", "Aa", and "a" (Bunkoed et al., 2014; Fan et al., 2007). Sexual reproduction has been observed in laboratory conditions but occurs rarely in nature, with the exception of Thailand, where pseudothecia containing mating types "A" and "a" were found on heavily infected maize residues from different regions (Bunkoed et al., 2014). Sexual reproduction is associated with high recombination rates between different mating types, contributing to the development of multiple pathogen races (Nsibo et al., 2024). Currently, 29 physiological races of *E. turcicum* are reported worldwide, with significant diversity in China and the USA (Table 2-2) (Jakhar et al., 2023; Nsibo et al., 2024). The distribution and diversity of *E. turcicum* races vary considerably between continents, with higher diversity recorded among tropical isolates compared to temperate isolates (Nsibo et al., 2024). The proportion of isolates carrying the MAT1 and MAT2 genes, which control mating types "A" and "a," respectively, is approximately equal in tropical regions such as Kenya, Mexico, and southern China. In contrast, in temperate regions, MAT2 has been found in France, while both MAT1 and MAT2 genes are unevenly distributed in Germany and Switzerland (Borchardt et al., 1998; Fan et al., 2007).

Table 2-2: Distribution of *E. turcicum* races in different countries grouped by continent.
*The races of the fungus are named based on the plant defense genes they overcome, with each fungal race carrying specific virulent gene(s) that enable this process. *: no defense gene.*

| Country | Races/Ht genes names | References |
|---|---|---|
| Kenya | 0/*, 1/Ht1, 2/Ht2, 3/Ht3, 12/Ht1 & Ht2, 13/Ht1&Ht3, 23/Ht2 1 Ht3, 123/Ht1&Ht2&Ht3, N/HtN1, 3N/Ht3&HtN1, 13N/Ht1&Ht3, 23N/Ht2&Ht3&HtN1 | Muiru et al. (2010) |
| Uganda | 0/*, 1/Ht1, 2/Ht2, N/HtN1, 23N/Ht2&Ht3&HtN1 | Welz et al. (1993) |
| Zambia | 0/*, 23/Ht2&Ht3, 23N/Ht2&Ht3&HtN1 | Welz et al. (1993) |
| Canada | 0/*, 1/Ht1, 2/Ht2, 3/Ht3, M/HtM1, N/HtN1, 12/Ht1&Ht2, 1M/Ht1&HtM1, 1N/Ht1&HTN1, 3M/Ht3&HtM1, 13M/Ht1&Ht3&HtM1, 12N/Ht1&Ht2&HtN1, 13N/Ht1&Ht3&HtN1, 1MN/Ht1&HtM1&HtN1, 12MN/Ht1&Ht2&HtM1&HtN1, 13MN/Ht1&Ht3&HtM1&HtN1, 123MN/Ht1&Ht2&Ht3/HtM1&HtN1 | Jindal et al. (2019) |
| United States | 0/*, 1/Ht1, 2/Ht2, M/HtM1, N/HtN1, 12/Ht1&Ht2,13/Ht1&Ht3, 23/Ht2&Ht3, 1M/Ht1&HtM, 1N/Ht1&HtN1, MN/HtM1&HtN1, 123/Ht1&Ht2&Ht3, 1MN/Ht1&HtM1&HtN1, 2MN/Ht2&HtM1&HtN1, 23M/Ht2&Ht3&HtM1, 23N/Ht2&Ht3&HtN1, 12MN/Ht1&Ht2&Ht3/HtM1&HtN1, 23MN/Ht2&Ht3&HtM1&HtN1, 123M/Ht1&Ht2&Ht3/HtM1, 123MN/Ht1&Ht2&Ht3/HtM1&HtN1 | Weems and Bradley (2018) |
| Argentina | 0/*, 1/Ht1, 2/Ht2, 3/Ht3, 3N/Ht3&HtN1, 13N/Ht1&Ht3&HtN1, 23N/Ht2&Ht3&HtN1 | Navarro et al. (2021b) |
| Brasil | 0/*, 1/Ht1, 2/Ht2, 1N/Ht1&HtN1, 12N/Ht1&Ht2&HtN1, 23N/Ht2&Ht3&HtN1, 123N/Ht1&Ht2&Ht3&HtN1 | Gianasi et al. (1996); Navarro et al. (2021b) |
| Mexico | 23N/Ht2&Ht3&HtN1,23/Ht2&Ht3, 2N/Ht2&HtN | Welz et al. (1993) |
| Australia | 0/*, 1/Ht1, 2/Ht2 | Muiru et al. (2010) |
| China | 0/*, 1/Ht1, 2/Ht2, 3/Ht3, N/HtN1, 12/Ht1&Ht2, 13/Ht1&Ht3, 23/Ht2&Ht3, N/HtN1, 1N/Ht1&HtN1, 2N/Ht2&HtN1, 3N/Ht3&HtN1, 12N/Ht1&Ht2&HtN1, 13N/Ht1&Ht3&HtN1, 23N/Ht2&Ht3&HtN1, 123N/Ht1&Ht2&Ht3&HtN1 | Dong et al. (2008); Ma et al. (2020) |
| Germany | 0/*, 1/Ht1, 2/Ht2, 12/Ht1&Ht2, 13/Ht1&Ht3, 23/Ht2&Ht3, 23N/Ht2&Ht3&HtN1 | Muiru et al. (2010) |
| Turkey | 0/*, 1/Ht1, 2/Ht2, 123/Ht1&Ht2&Ht3, N/HtN1, 1N/Ht1&HtN1, 3N/Ht3&HtN1, 12N/Ht1&Ht2&HtN1 | Turgay et al. (2020) |

### 2.4.5. Epidemiology of Northern corn leaf blight

Environmental factors strongly influence the survival and spread of *E. turcicum*. The survival time of *E. turcicum* mycelia and conidia ranges from 1 to 12 years, depending on temperature and relative humidity. For instance, conidia viability was lost after 1 year at 25°C and 77% relative humidity. In contrast, conidia were still able to germinate after 12 years when stored in freezer at 0°C and 49% of relative humidity (Levy and Pataky, 1992). Under moist conditions, *E. turcicum* conidia germinate on maize tissue within 1 hour at 25°C, followed by the formation of appressoria and infection pegs within 3-4 hours at 15°C and 20°C, respectively. The optimal temperature range for fungal infection is 15-30°C, with an optimum of 20°C for 5 hours under humid conditions (Bunkoed et al., 2014; Levy and Pataky, 1992; Navarro et al., 2020).

Sporulation of *E. turcicum* is primarily favored at night under high humidity, with an average temperature of 26°C during 14 hours dew period. Conidiophores develop optimally at 20°C. The number of lesions generally increases with longer dew periods and higher inoculum concentrations during the infection period. Light intensity and wavelength also affect conidial germination. Specifically, a photon flux density of 150 $\mu E \cdot s^{-1} \cdot m^{-2}$ and exposure to blue light have been shown to inhibit conidial germination. Additionally, *E. turcicum* is resistant to desiccation, except during the conidial germination phase, which is negatively impacted by desiccation and may limit further colonization (Levy and Pataky, 1992).

### 2.4.6. Management of Northern corn leaf blight

NLB is a foliar disease that significantly impacts maize production and threatens food security. Several control measures can reduce fungal inoculum and include the use of resistant varieties, good agricultural practices such as crop rotation, field sanitation, deep plowing, and appropriate fertilization, as well as the application of chemical pesticides. These strategies create unfavorable environmental

conditions for the fungus and limit the development and fitness of the pathogen. Additionally, some biological control agents have been effectively tested and have shown to reduce NLB both *in vitro* and *in planta*. The various strategies are described below.

#### 2.4.6.1. Resistant varieties

Control of NLB through the use of resistant varieties is recommended, particularly in developing countries, due to its low cost and environmental safety (Jakhar et al., 2023). Two types of resistance, namely qualitative and quantitative, have been developed in maize germplasm. Qualitative resistance, also known as monogenic resistance, is based on the gene-for-gene interaction between the pathogen and the host. The resistance genes, including Ht1, Ht2, Ht3, HtN, Htm1, HtP, HtNB, ht4, and rt, have been identified and utilized in breeding programs worldwide (Jakhar et al., 2023). These genes have been discovered in certain maize germplasms and related grasses and are associated with different phenotypic responses, as shown in Table 2-3. Cultivars carrying these genes can be either resistant or susceptible to strains of *E. turcicum* with corresponding avirulent or virulent genes. For example, plants carrying the Ht1, Ht2, and Ht3 genes are susceptible to *E. turcicum* races with virulent genes 1, 2, and 3, respectively. However, these plants exhibit a resistance response with few chlorotic lesions when infected with other avirulent races of *E. turcicum*. Plants carrying the HtN gene are susceptible to *E. turcicum* races with the corresponding N virulent gene. When infected with other avirulent races of *E. turcicum*, these plants show a resistance response characterized by prolonged incubation and latent periods of the fungus, resulting in a subsequent delay in disease development (Jakhar et al., 2023, 2017; Navarro et al., 2021, 2020). The delay in disease development is an important strategy for disease management, as it allows certain phenological stages of the plant to be achieved in the absence or with low intensity of the pathogen population. This leads to a reduced impact of disease on maize production. The introgression of

these qualitative genes into maize germplasm has significantly limited the evolution of NLB in a short period, while also contributing to the selection of more virulent races of the pathogen.

A more sustainable type of resistance, known as partial or polygenic resistance, has also been employed to control NLB. This type of resistance is based on poly- or oligogenes with small individual effects on disease reduction. These genes work together to provide moderate protection by delaying the pathogen's penetration time, reducing infection frequency, slowing lesion expansion rate, or extending the latent period of *E. turcicum*. However, several challenges exist in utilizing polygenic genes to develop plant resistance. They are present in multiple cultivars and related crops, and their introgression is often limited and time-consuming. Additionally, the expression of these genes is dependent on variation in environmental conditions, which can restrict their efficacy (De Rossi et al., 2022).

Table 2-3: Maize resistance genes to Northern corn leaf blight: their origins and phenotypic reactions in plant hosts carrying these genes.

| Resistant genes | Origin | Phenotype reaction | References |
|---|---|---|---|
| Ht1 | Ladyfinger popcorn and GE449 (Peru & USA) | Chlorosis lesions, delay in necrosis, inhibition of sporulation | (Navarro et al., 2021, 2020) |
| Ht2 | NN14B (Australia) | Chlorosis lesions | (Navarro et al., 2021, 2020) |
| Ht3 | *Tripsacum floridanum* | Chlorosis lesions | (Navarro et al., 2021, 2020) |
| HtN | Pepitilla (Mexico) | Long latent period | (Navarro et al., 2021, 2020) |
| Htm1 | Myorbela (Puerto Rico) | Chlorosis lesions | (Navarro et al., 2021, 2021) |
| HtP | L30R (Brazil) | Chlorotic lesions or fully resistance | (Navarro et al., 2021) |
| HtNB | Bramadi (Indonesia) | Fully resistance | (Navarro et al., 2021) |
| ht4 | BS19 (USA) | Chlorotic halo | (Navarro et al., 2021; Welz and Geiger, 2000) |
| rt | L40 (Brazil) | Chlorosis or full resistance | (Navarro et al., 2021) |

In Burundi, the most widely grown open-pollinated maize varieties (OPVs), including Isega, Mugamba, ZM605, ZM621, and Ecavel, are considered tolerant to NLB (Nkurunziza et al., 2012). However, these varieties have shown high disease severity in greenhouse trials, with some plants of varieties completely dying 23 days post-inoculation with a local strain of *E. turcicum* (Tasiaux, 2024). The moderate tolerance observed in the field may be attributed to the polygenic nature of their resistance, which was insufficient to withstand the high inoculum pressure of $4.5 \times 10^4$ conidia/ml applied to the leaves of plants in the greenhouse. These varieties remain at high risk, as their resistance could be compromised under increased pathogen pressure due to changing climatic conditions and the potential introduction of new strains through germplasm exchange. The high prevalence of Northern corn leaf blight in Burundi could also be attributed to its tropical environmental conditions, as the consistently high temperatures (above 22°C) in this region promote increased diversity and virulence of *E. turcicum* (Navarro et al., 2021).

#### 2.4.6.2. Good agriculture practices

Good agricultural practices related to soil management, seed selection, crop management, and fertilization are known to reduce pathogen pressure. For managing foliar diseases such as NLB, practices including non-host crop rotation and field sanitation have proven effective in limiting *E. turcicum* populations (Castellano et al., 2021; Shimada et al., 2021; Walters, 2013). Crop rotation with non-host crops is a vital control measure because it helps limit the availability of nutrients to the pathogen, leading to a reduction in the primary inoculum.

In Burundi, crop rotation is practiced by seed multipliers, who grow maize after legumes such as beans and peas, or root and tuber crops like potatoes, sweet potatoes, and cassava (Niyuhire et al., 2017). However, smallholder farmers producing maize for consumption often misuse or neglect crop rotation, typically planting maize after maize, wheat, or

sorghum, which all belong to the Poaceae family and share similar pathogens. This misuse of crop rotation increases the pathogen population and limits maize production at the household level.
Crop sanitation is also employed to manage foliar plant diseases such as NLB by reducing the source of inoculum and moderating environmental conditions favorable for pathogen development. This practice involves removing plant debris before planting and eliminating potential grasses that have emerged within the cultivated corn plot. The removed debris or grasses can be burned to prevent pathogen emergence and infection of the corn plants.

Other agricultural practices, such as avoiding late planting, no-till farming, excessive fertilization, and high sowing density, should be minimized, as these practices can increase fungal inoculum and disease damages. For example, the variety TZBR released in Burundi is completely damaged by NLB when sown later in the growing season (personal observation, 2024). Deep soil tillage is another effective agricultural practice that reduces *E. turcicum* inoculum. However, it is rarely used in Burundi, where maize is typically sown in a hole without plowing the surrounding land.

Fertilization provides essential nutrients to plants, promoting their growth and development. Consequently, specific stages of plant growth may evade pathogen damage, allowing plants to produce efficiently, even while the pathogens are still colonizing the plant. However, excessive nitrogen fertilization can lead to vigorous vegetation growth, creating a microclimate with elevated relative humidity that is potentially favorable for *E. turcicum* development and increasing the severity of NLB damage. To mitigate subsequent NLB damage, it is advisable to apply nitrogen fertilization at levels that have a reduced impact on NLB development. In Burundi, a recommendation of 100 kg/ha of urea, which contains nitrogen, is suggested for maize production.

#### 2.4.6.3. Chemical pesticides

NLB is controlled by various chemical fungicides, including quinone outside inhibitors, such as strobilurins (azoxystrobin, picoxystrobin, fluoxastrobin, pyraclostrobin, trifloxystrobin), demethylation inhibitors (flutriafol, propiconazole, prothioconazole, tebuconazole, metaconazole, mefentrifluconazole, tetraconazole), and succinate dehydrogenase inhibitors (benzovindoflupyr, bixafen, fluxapyroxad, fluopyram, pydiflumetofen) (CPN, 2024; Nsibo et al., 2024). Triazoles and strobilurins are the primary classes of fungicides widely used in North and South America (USA and Canada, Brazil and Argentina), Europe (France, Italy, and Germany), Asia (China and India), and Africa (South Africa and Kenya). These fungicides can be used alternately or in combination to effectively control NLB. They should be applied at specific stages of the crop, at recommended doses, to protect the upper leaves, which are primarily involved in photosynthesis. For example, the combination of trifloxystrobin and tebuconazole, which belong to the strobilurin and triazole families, respectively, has been successfully used to control NLB on the moderately susceptible cultivar DK72-10 in a low disease pressure environment (Carpane et al., 2020). This fungicide can be applied between the phenological stages V10 and V14, which correspond to the vegetative growth stage - from the first fully emerged leaf (V10) to the fourth deployed leaf (V14) - and coincide with the onset of NLB disease (Carpane et al., 2020). Despite the efficacy of these fungicides in controlling NLB, their prolonged use or failure to maintain recommended dosages and associated safety measures could lead to the development of fungal resistance and have harmful effects on human, animal, and environmental health. Additionally, the use of fungicides is often expensive and severely limited in developing countries, where crop losses due to disease are increasingly reported. An alternative to chemical pesticides for controlling plant diseases, such as biological control, is being promoted in developed countries. Promising results in managing NLB are already being reported, and these methods can be adopted by less developed countries.

#### 2.4.6.4. Biological control

Biological control, utilizing natural microorganisms or their derivatives, has proven effective in managing various plant diseases. The most used biological control agents for managing plant diseases belong to the genera *Bacillus*, *Pseudomonas*, and *Trichoderma* (Basso Valeria, 2022; Collinge et al., 2022; Lahlali et al., 2022; Sarrocco, 2023). The significance of *Bacillus* spp. has gained considerable attention and will be discussed in Chapter IV, where the advantages and mechanisms of action of *Bacillus* species are detailed. Here, we highlight the main biocontrol agents that have demonstrated efficacy against NLB. Only a few biocontrol agents, including bacterial and fungal species, have been reported to reduce the severity and incidence of NLB, primarily in greenhouse settings and, to a lesser extent, under field conditions. For example, NLB caused by *E. turcicum* has been successfully controlled by *Curtobacterium* sp., *Pantoea* sp., and *Bacillus* sp. (Sartori et al., 2020). These authors reported a significant inhibition of mycelial growth of *E. turcicum* by a specific strain of *Bacillus subtilis*, through the production of hydrolytic enzymes, volatile organic compounds, and antibiotics. This strain of *Bacillus subtilis* was also effective in controlling *E. turcicum* in the field across different agro-ecological zones in Argentina. The bacterial culture of *Bacillus subtilis* was sprayed before the grain-filling growth stage of maize, leading to a reduction in the incidence of NLB and an increase in grain production. A double application of this bacterial culture suspension resulted in a significant grain yield increase of 65% compared to untreated plants.

Additionally, some fungal strains have demonstrated bioactivity against NLB. For instance, the strain LB-2 of *Chaetomium* sp. was reported to inhibit more than 62% of the mycelial growth of *E. turcicum*. Furthermore, the crude butanol extract from this LB-2 strain inhibited over 89% of mycelial growth of *E. turcicum* and reduced NLB severity by more than 53% in greenhouse conditions (Liu and Chang, 2018). Many *Trichoderma* spp. have also been successfully employed to

control NLB *in vitro*, in greenhouses, and in the field. These strains protect maize against NLB by competing with other pathogens and producing antibiotic compounds (Limdolthamand et al., 2023).

# CHAPTER III.

# INTERACTION BETWEEN PLANTS AND MICROORGANISMS

## 3.1. Plant as a holobiont

Plants coexist with a diverse array of microorganisms, including bacteria, fungi, viruses, protists, and nematodes. These microorganisms occupy different niches within the plant and establish various interactions that benefit both the plant and the microorganisms. Microorganisms can preferentially colonize inside or on the surface of plant tissues; those residing inside are known as endophytes, while those on the surface are referred to as epiphytes. Other microorganisms colonize the rhizosphere, the region surrounding plant roots, or the phyllosphere, which includes the aerial parts of the plant (Lengrand et al., 2024; Nadarajah and Abdul Rahman, 2021). Some microorganisms in the phyllosphere also colonize flowers, fruits, and seeds (Sharma et al., 2021).

Together, the plant and its associated microbial community form a holobiont, a biological complex where various interactions occur to facilitate survival and reproduction (Hassani et al., 2018; Lengrand et al., 2024; Lyu et al., 2021; Mesny et al., 2023). Notably, the plant itself does not exist in isolation; certain plant organelles, such as mitochondria and chloroplasts, are derived from bacteria (Lyu et al., 2021). In addition to the existing microbial community in nature, other microorganisms can be recruited from the environment or vertically transferred from the seed (Lengrand et al., 2024). The interactions between plants and their associated microorganisms can yield various effects from parasitic to mutualistic effects (Lugtenberg et al., 2002; Sharma et al., 2021; Thoms et al., 2021). In the following sections, we will describe these main types of interactions, emphasizing their potential benefits or detriments to plant health and production.

## 3.2. Plant-pathogen interaction

Some microorganisms constantly harm plants due to the failure of plant defenses to limit their colonization and disease development. Fungi and bacteria are the most significant microorganisms responsible for plant diseases, followed by nematodes, protozoa, and phytoplasmas (Hariharan and Prasannath, 2021; Nazarov et al., 2020). These microorganisms are introduced to plants by rain, wind, insects, animals, or humans. They have evolved specialized features, such as adhesins in bacteria and mucilaginous substances in fungi, which facilitate their adherence to plant surfaces (Rossi et al., 2019).

These microorganisms can penetrate plant cells either mechanically or through biochemical processes (Ghosh et al., 2019). Only certain fungi and nematodes can mechanically penetrate the plant cell epidermis, while others produce enzymes and compounds that degrade the cell membrane via biochemical reactions (Agrios, 2005).

Nematodes have developed a stylet structure to extract nutrients from host plants. Bacteria penetrate plant cells through natural wounds, hydathodes, or stomata and colonize the apoplast. Viruses, transmitted by vectors, integrate into the plant genome, disrupting its metabolism (Du et al., 2024; Faulkner and Robatzek, 2012; Pfeilmeier et al., 2016). Fungal propagules, such as conidia or mycelia, germinate on the plant surface and produce appressoria, from which a peg structure forms, allowing the fungus to penetrate the plant cell membrane directly or through stomata. This direct fungal penetration is associated with the secretion of enzymes like cutinase and cellulase, which play a key role in softening the cell membrane and facilitating fungal entry. Furthermore, fungus produces pectinases and polygalacturonases enzymes used to degrade the pectin content of the plant cell wall, and get inside the plant cell (Agrios, 2005). Some fungi develop specialized structures, such as haustoria, to extract nutrients from the plant cytoplasm (Faulkner and Robatzek, 2012; Keane, 2012; Sharma and Gautam, 2019).

Except for viruses, plant pathogens produce various enzymes, toxins, growth regulators, polysaccharides, and defense suppressors to ensure successful colonization and disease development (Horbach et al., 2011; Y. Wang et al., 2022). These compounds may be produced constitutively or induced by plant substrates and can be produced at different stages of pathogen colonization (Ghannoum, 2000; Santi et al., 2010). For example, pathogen enzymes degrade cell membranes, break down host nutrients, and interfere with cell function. Toxins produced by pathogens can act directly on the protoplast, altering its permeability and disrupting plant cell functions. Growth regulators such as indole-3-acetic acid (IAA) can manipulate plant defenses, making them more susceptible to disease (Kunkel and Johnson, 2021; Zhao and Li, 2021). Polysaccharides secreted by vascular pathogens interfere with water transport in the host's vascular system. Pathogens can suppress plant defenses by producing effectors that alter the plant's immune responses, enabling pathogen growth and survival (Kalita et al., 2024; Todd et al., 2022). The importance of these compounds varies depending on the type of disease, with enzymes being critical in soft rot diseases and growth regulators in crown gall disease (Agrios, 2005).

## 3.3. Plant resistance and defenses against pathogens

Plants have been reported to evade pathogen attacks through constitutive and induced resistance. Constitutive resistance includes physical barriers and other secreted molecules or proteins produced by plants in the absence of inducers. These pre-existing structures include the waxy epidermal layer and cuticle, the composition of the cell wall, and the size, position, and shape of stomata and lenticels, all of which limit pathogen progression within plant tissues. Additionally, constitutive biochemical compounds known as phytoanticipins such as phenolic compounds, tannins and some fatty-acid compounds, are secreted by plants into the environment or on the surface of their roots and aerial parts (stems or leaves), prior to infection and serve as inhibitors of hydrolytic enzymes produced by pathogens (Belete, 2021; Kumar Mahawer et al., 2022).

Some pathogens are limited by constitutive defense mechanisms, while others are hindered by the plant's innate defense mechanisms (Boots and Best, 2018; Maschietto et al., 2016). The innate defense of the plant is activated upon recognition of the pathogen or its derivatives through Pathogen/Microbe-Associated Molecular Patterns (PAMPs/MAMPs), which are conserved molecules recognized by plants via Pattern Recognition Receptors (PRRs) (Ghosh et al., 2019; Kaur et al., 2022; Keane, 2012; Yuan et al., 2021). Several PAMP molecules have been reported in the literature, including bacterial flagellin, elongation factors, fungal chitin, peptides, proteins, glycoproteins, and lipids for bacteria, as well as oligogalacturonides and endopolygalacturonases for fungi (S. Ali et al., 2024; Jian et al., 2023; Trivedi et al., 2020; Zhang and Kong, 2022).

Plants can also induce their innate defense mechanisms upon perceiving Damage-Associated Molecular Patterns (DAMPs), which are oligopolysaccharides or polysaccharides resulting from the degradation of plant cell membranes following pathogen injury. Both PAMPs and DAMPs are recognized by plant receptors (PRRs) located on the surface or in the transmembrane space of plant cells, leading to PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Ngou et al., 2022).

In addition to PTI, plants have also another line of defense triggered by effectors secreted by pathogens and known as Effector triggered immunity (ETI) mediated by nucleotide-binding leucine-rich repeat (NB-LRR) located inside the plant cell. Moreover, both PTI or ETI separate or simultaneous activation lead to increased rapid calcium influx, the production of reactive oxygen species (ROS), mitogen-activated protein kinase (MAPK) phosphorylation cascades, cell wall degradation, and the expression of defense genes (Ngou et al., 2021; Nishad et al., 2020; Yu et al., 2024). ROS, which include hydroxyl radicals, superoxide anions, and hydroperoxides, are produced by injured plants and serve to degrade the pathogen's cell wall, strengthen the plant's cell wall, and signal the presence of pathogen invaders. In case of pathogen attack, a high $Ca^{2+}$ influx inside the plant cell either in

PTI and ETI activation, leading to extracellular alkalinization. Thus, $Ca^{2+}$ plays a key role in signaling of plant defense pathways (Lecourieux et al., 2006; Naz et al., 2024).

Mitogen-activated protein kinase (MAPK) plays a critical role in the early plant defense response by phosphorylating several transcription factors, leading to the expression of signaling and biosynthetic defense genes associated with salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Jones and Dangl, 2006; Ngou et al., 2022; Nishad et al., 2020). Furthermore, the activation of ETI is also associated with the hypersensitive response (HR), which involves programmed cell death that limits pathogen progression and colonization in plant tissues (Nishad et al., 2020; Yu et al., 2022; Yuan et al., 2021).

For example, the molecular resistance of maize against *E. turcicum* was reported to be mediated by many metabolomic pathways including benzoxazine, jasmonic acid, phenylpropanoid and glutathione-S-transferase proteins family. Benzoxazine mediate callose deposition and innate immunity and therefore limit the infection by *E. turcicum* (Ijaz and Fan, 2024). The other pathways comprise various difference genes and production of secondary metabolites involved in inhibiting the *E. turcicum* infection.

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# CHAPTER IV.

# BACILLUS-BASED BIOCONTROL BEYOND CHEMICAL CONTROL IN CENTRAL AFRICA: THE CHALLENGE OF TURNING MYTH INTO REALITY

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**Foreword**

This chapter was adapted from a collaborative manuscript published in *Frontiers in Plant Science*, with Nihorimbere Gaspard, Korangi Alleluya Virginie, and François Nimbeshaho as co-first authors, and Professors Anne Legrève and Marc Ongena as co-last authors. Nihorimbere Gaspard and François Nimbeshaho collected phytosanitary data from Burundi. Korangi Alleluya Virginie gathered data from the Democratic Republic of Congo (DRC). Data from Rwanda were primarily sourced from websites and publications. The manuscript was collaboratively written by the co-first authors and revised by Professors Venant Nihorimbere, Anne Legrève and Marc Ongena.

## 4.1. Abstract

Agricultural productivity in the Great Lakes Countries of Central Africa, including Burundi, Rwanda, and the Democratic Republic of Congo, is affected by a wide range of diseases and pests which are mainly controlled by chemical pesticides. However, more than 30% of the pesticides used in the region are not authorized in European Union due to their high toxicity. Globally available safe and eco-friendly biological alternatives to chemicals are virtually non-existent in the region. *Bacillus* PGPR-based biocontrol products are the most dominant in the market and have proven their efficacy in controlling major plant diseases reported in the region. With this review, we present the current situation of disease and pest management and urge the need to utilize *Bacillus*-based control as a possible sustainable alternative to chemical pesticides. A repertoire of strains from the *Bacillus subtilis* group that have shown great potential to antagonize local pathogens is provided, and efforts to promote their use, as well as the search for indigenous and more adapted *Bacillus* strains to local agro-ecological conditions, should be undertaken to make sustainable agriculture a reality in the region.

## 4.2. Introduction

The importance of agriculture for the development of societies has been demonstrated for several centuries and it employs about 43% of the world's working population (Roser, 2023). In the Great Lakes Countries of Central Africa (GLCCA), referred to in this report as Burundi, Rwanda, and the Democratic Republic of Congo (DRC), agriculture is the main activity employing more than 94%, 46% and 60% of the working population, while contributing to 40%, 30% and 36% of gross domestic product (GDP) in Burundi, Rwanda, and DRC respectively (FAAPA, 2021; Lokuruka, 2020; PND Burundi, 2018). However, this sector faces several constraints including low soil fertility and high incidence of diseases and pests leading to food insecurity (Bjornlund et al., 2020). For example, up to 52%, 43% and 38% of children under five in Burundi, DRC and Rwanda respectively are malnourished (Lokuruka, 2020; WFP, 2021). In the GLCCA, plant diseases and pests are generally controlled by chemical pesticides, especially those affecting cash crops such as coffee, cotton, tomatoes, potatoes, vegetables, and fruits (Muliele et al., 2018; Niyongere et al., 2015; Okonya et al., 2019b). However, some phytopathogens are inefficiently managed by traditional practices, such as the use of plant extracts, while others are not controlled (Korangi Alleluya et al., 2021; MINAGRI-BURUNDI, 2018; Rutikanga, 2015).

Biocontrol products are the most promoted tools within the Integrated Pest Management (IPM) framework, as they are generally recognized as safe (GRAS) compared to chemical pesticides (Raveau et al., 2020). Biocontrol products include biochemicals (semiochemicals, plant extracts, plant growth regulators and organic acids), macroorganisms (insects, mites, and nematodes) and microorganisms (beneficial bacteria, fungi, protozoa, viruses, yeasts) and their derivatives (cyclic lipopeptides, enzymes, chitosan oligopolysaccharides, etc) (Dunham Trimmer, 2023). Among microorganisms, plant growth-promoting rhizobacteria (PGPR), including species of the *Streptomyces*, *Paenibacillus*, *Pseudomonas* and *Bacillus* genera, are the most

commercially exploited for crop bioprotection, notably due to their efficient production of an arsenal of bioactive secondary metabolites (BSMs). These bacteria protect plants through direct antibiosis, competition with other pathogenic microbes for space and nutrients, and via their potential to induce plant systemic resistance (ISR) (Beneduzi et al., 2012; Dimkić et al., 2022; Wang et al., 2023). *Bacillus*-based products dominate the biocontrol market compared to their PGPR counterparts (Dunham Trimmer, 2023; Helepciuc and Todor, 2023) due to their ability to form resistant endospores, allowing stable formulations and ensuring long-term survival in the environment, especially in the current climate change context (Miljaković et al., 2020; Radhakrishnan et al., 2017). In this review, we provide a detailed topography of plant health and crop protection in the GLCCA, address constraints within the sector, and propose promising sustainable solutions. An overview of biological alternatives to chemical pesticides is discussed, with a focus on *Bacillus* PGPR for potential integration as biocontrol ingredients into local agricultural systems.

## 4.3. Diversity of phytopathogens and their control

We performed a comprehensive survey of the major diseases and causal agents affecting crop production in the GLCCA region by compiling data from published studies (articles and books) with those obtained from governmental and non-governmental agencies in the form of reports, newspapers, or online databases. Either in the field or in storage, agricultural crops are mainly affected by microbial pathogens consisting of fungi, oomycetes, bacteria, and viruses (Table 4-1). Among the most important fungal diseases are late blight of potato and tomato caused by *Phytophthora infestans*, angular leaf spot of bean caused by *Pseudocercospora griseola*, early blight of tomato caused by *Alternaria solani*, stem rot caused by *Sclerotium rolfsii*, late and early leaf spot caused by *Nothopassalora personata* and *Cercospora arachidicola* on peanut. These pathogens are most commonly controlled with the chemical mancozeb or its derivatives. Metalaxyl, benomyl, iprobenfos, and copper (II) chloride are other important

fungicides used in the region. One of the most common bacterial diseases prevailing in the region is *Xanthomonas* wilt of banana caused by *X. campestris* pv. *musacearum*, but unfortunately there is no effective pesticide available to control this pathogen (Table 4-1). Viral plant pathogens responsible for cassava mosaic disease, cassava brown streak disease, banana bunchy top disease and maize lethal necrosis, also cause serious threat to food security in the region but here again, no efficient chemical control is available.

Insects, nematodes, and weeds are also responsible for severe yield losses. Important insect pests affecting major crops in the region include fall armyworm (*Spodoptera frugiperda*), tomato leafminer (*Tuta absoluta*), coffee bugs (*Antestiopsis orbitalis ghesquierei*), whitefly (*Bemisia tabaci*), banana aphid (*Pentalonia nigronervosa*), and coton aphid (*Aphis gossypii*) (Cokola et al., 2021; Dushimirimana et al., 2016; MINAGRI-BURUNDI, 2018; Mukwa et al., 2021; Niassy et al., 2021; Niyibizi et al., 2019; Nyabyenda, 2005). The control of these insects relies heavily on the use of synthetic products such as acephate against *S. frugiperda* and *T. absoluta*, chlorpyrifos-ethyl against *A. orbitalis ghesquierei* and imidacloprid against aphids. Other major insecticides used in the region include lambda-cyhalothrin, cypermethrin, dimethoate, pirimiphos-methyl + permethrin, metaldehyde, abamectin and emamectin benzoate (ARECO-RWANDA NZIZA, 2020; MINAGRI-BURUNDI, 2018). Several species of nematodes also damage plants, including *Meloidogyne javanica* on various crops, *Pratylenchus goodeyi* and *Helicotylenchus multicinctus* on banana plants, and *Ditylenchus* spp. on potatoes (Coyne et al., 2018). These nematode-caused diseases are managed by using chemicals such as dazomet and terbufos. Parasitic plants or weeds that compete with crops such as sugarcane, rice, sorghum, and maize are also problematic and include among others *Striga* spp., *Cyperus* spp. and *Echinochloa* spp. Their control involves chemical herbicides like glyphosate, atrazine and dalapon (Nyabyenda, 2005; Rodenburg et al., 2016; Runo and Kuria, 2018).

The spread of some of these diseases and pests has led to historic famines in the GLCCA. For example, the Integrated Food Security Phase Classification (IPC) reported that nearly 13.1 million people in the DRC were acutely food insecure due to the spread of the fall armyworm on maize crops (IPC, 2021a; MINAGRI-RDC, 2018). In 2004-2005, the outbreak of cassava mosaic disease led to severe food shortages that threatened many families in the north-eastern provinces of Kirundo and Muyinga in Burundi. As a result, 100 famine-related deaths were reported (Legg et al., 2006).

Table 4-1: Microbial pathogens affecting crop production in the GLCCA region and chemical pesticides used for their control. *, **, ***: *slight, moderate and high impact, respectively; NI: no information*

| Pathogens and pests | Species | Host plant | Impact | Pesticides active ingredients | References |
|---|---|---|---|---|---|
| **Bacteria** | *Xanthomonas campestris* pv *musacearum* | Banana (*Musa* spp.) | *** | NI | (Ndayihanzamaso et al., 2016; Ndungo et al., 2006; Nyabyenda, 2006; Rietveld et al., 2020) |
| | *Pseudomonas syringae* pv. *phaseolicola*, *P. syringae* pv. *syringae* | Bean (*Phaseolus vulgaris*) | *** | Streptomycin sulphate | (Nyabyenda, 2005) |
| | *Xanthomonas campestris* pv. *phaseoli* | | *** | Streptomycin sulphate | (Nyabyenda, 2005) |
| | *Xanthomonas campestris pv manihotis* | Cassava (*Manihot esculenta*) | NI | NI | (Nyabyenda, 2005) |
| | *Ralstonia solanacearum* | Pepper (Chilli and sweet) (*Capsicum* sp.) | NI | NI | (Minengu et al., 2018; Nyabyenda, 2005) |
| | *Pseudomonas solanacearum* | Potato (*Solanum tuberosum*) | *** | NI | (Nyabyenda, 2005) |
| | *Ralstonia solanacearum* | | NI | NI | (Harahagazwe et al., 2007; Munyuli et al., 2017; Okonya et al., 2019a; Sharma et al., 2020) |
| | *Pseudomonas fuscovaginae* | Rice (*Oryza sativa*) | *** | Formol | (Nyabyenda, 2005) |

| Pathogens and pests | Species | Host plant | Impact | Pesticides active ingredients | References |
|---|---|---|---|---|---|
| | *Clavibacter xyli* | Sugar cane | *** | Methoxy-ethyl, mercury chloride | (Nyabyenda, 2006) |
| | *Xanthomonas vasculorum* | (*Saccharum officinarum*) | *** | Methoxy-ethyl, mercury chloride | (Nyabyenda, 2006) |
| | *Xanthomonas albilineans* | | * | NI | (Nyabyenda, 2006) |
| | *Ralstonia solanacearum* | Tomato | *** | Formol | (Nyabyenda, 2006) |
| | *Pseudomonas solanacearum* | (*Lycopersicon esculentum*) | *** | NI | (Nyabyenda, 2006) |
| | *Corynebacterium michiganense* | | *** | | (Nyabyenda, 2006) |
| **Fungi** | *Fusarium oxysporum* var f. sp. *cubense* | *Banana* | *** | NI | (Ndayihanzamaso et al., 2020; Nyabyenda, 2006) |
| | *Armillaria mellea, Cordona musae* | (*Musa* spp.) | * | NI | (Nyabyenda, 2006) |
| | *Cladosporium musae* | | * | NI | (Nyabyenda, 2006) |
| | *Gloesporium musarum* | | * | NI | (Nyabyenda, 2006) |
| | *Helminthosporium tolurosum* | | * | NI | (Nyabyenda, 2006) |
| | *Mycosphaerella musicola* | | * | NI | (Nyabyenda, 2006) |
| | *Stachylidium theobrome* | | * | NI | (Nyabyenda, 2006) |
| | *Ascochyta phaseolarum* | Bean | *** | Mancozeb, benomyl, benlate | (Nyabyenda, 2005) |

| Pathogens and pests | Species | Host plant | Impact | Pesticides active ingredients | References |
|---|---|---|---|---|---|
| | *Colletotrichum lindemuthianum* | (*Phaseolus vulgaris*) | *** | Benomyl, mancozeb, thiophanate-methyl, benlate | (Nyabyenda, 2005) |
| | *Fusarium solani* f. sp.*pv phaseoli* | | *** | NI | (Nyabyenda, 2005) |
| | *Mycovellosiella phaseoli* | | *** | Benomyl, mancozeb, thiophanate-methyl, benlate | (Nyabyenda, 2005) |
| | *Pseudocercospora griseola* | | *** | Benomyl, thiophanate-methyl, mancozeb | (Busogoro et al., 1999; Farrow and Muthoni-Andriatsitohaina, 2020; Kijana et al., 2017; Nyabyenda, 2005) |
| | *Pythium spp.* | | *** | NI | (Nyabyenda, 2005) |
| | *Rhizoctonia solani* | | *** | NI | (Nyabyenda, 2005) |
| | *Sclerotinia sclerotiorum* | | *** | NI | (Nyabyenda, 2005) |
| | *Thielaviopsis basicola* | | *** | NI | (Nyabyenda, 2005) |
| | *Uromyces appendiculatus* | | *** | Mancozeb, benlate | (Farrow and Muthoni-Andriatsitohaina, 2020; Nyabyenda, 2005) |
| | *Alternaria* spp. | Cabbage | *** | NI | (Nyabyenda, 2006) |
| | *Botrytis cinerea* | (*Brassica* spp.) | *** | NI | (Nyabyenda, 2006) |
| | *Peronospora* spp. | | *** | Mancozeb | (Nyabyenda, 2006) |

| Pathogens and pests | Species | Host plant | Impact | Pesticides active ingredients | References |
|---|---|---|---|---|---|
| | *Sclerotinia* spp. | Carrot (*Daucus carota*) | *** | NI | (Nyabyenda, 2006) |
| | *Cercospora hemingsii* | Cassava | NI | NI | (Nyabyenda, 2005) |
| | *Glomerella manihotis* | (*Manihot esculenta*) | NI | NI | (Nyabyenda, 2005) |
| | *Hemileia vastatrix* | Coffea | *** | Copper oxychloride 50%, dithianon, triadimefon | (Nyabyenda, 2006) |
| | *Ascochyta* sp., *Phoma* sp. | (*Coffea* spp.) | NI | NI | (Nyabyenda, 2006; MINAGRI-Burundi, 2018) |
| | *Cercospora coffeicola* | | NI | NI | (Nyabyenda, 2006; MINAGRI-Burundi, 2018) |
| | *Colletotrichum coffeanum* | | NI | Copper oxychloride 50%, dithianon, captafol, chlorotalonil | (Nyabyenda, 2006; MINAGRI-Burundi, 2018) |
| | *Rhizoctonia solani* | | NI | NI | (Nyabyenda, 2006; MINAGRI-Burundi, 2018) |
| | *Golovinomcyces spp.* | Eggplant | *** | NI | (Nyabyenda, 2006) |
| | *Leveillura* spp., | (*Solanum melongena*) | *** | NI | (Nyabyenda, 2006) |
| | *Peronospora* spp. | | *** | NI | (Nyabyenda, 2006) |
| | *Exserohilum turcicum, Helminthosporium. maydis* | Maize | ** | Thiram, thioral (thiram + mancozeb), benomyl | (Nyabyenda, 2005) |
| | *Puccina polysora* | (*Zea mays*) | * | NI | (Nyabyenda, 2005) |

| Pathogens and pests | Species | Host plant | Impact | Pesticides active ingredients | References |
|---|---|---|---|---|---|
| | *Ustilago zeae, Sphacelotheca reilina* | | * | NI | (Nyabyenda, 2005) |
| | *Puccinia* sp. | Morella (*Solanum aethiopicum*) | NI | NI | (Nyabyenda, 2005) |
| | *Alternaria* spp. | Onion (*Allium cepa*) | *** | Maneb, mancozeb | (Nyabyenda, 2005) |
| | *Peronospora* spp. | | *** | Maneb, mancozeb | (Nyabyenda, 2006) |
| | *Puccinia* spp. | | *** | Maneb, mancozeb | (Nyabyenda, 2006) |
| | *Nothopassalora personata (Syn. Cercospora personata), Cercospora arachidicola* | Peanut (*Arachis hypogaea*) | *** | NI | (Nyabyenda, 2005) |
| | *Macrophomina phaseolina* | | ** | NI | (Nyabyenda, 2005) |
| | *Puccinia arachidis* | | ** | NI | (Nyabyenda, 2005) |
| | *Sclerotium rolfsii* | | ** | NI | (Nyabyenda, 2005) |
| | *Colletotrichum nigrum, C. capsici* | Pepper (Chilli and sweet) (*Capsicum* sp.) | *** | NI | (Nyabyenda, 2005) |
| | *Rhizoctonia solani* | Potato (*Solanum tuberosum*) | *** | NI | (Nyabyenda, 2005; Harahagazwe et al., 2007) |
| | *Pyricularia oryzae* | Rice | *** | Benomyl, thiram, iprobenfos, isoprothiolane | (Kanyange et al., 2019; Liboga Oenabaiso et al., |

| Pathogens and pests | Species | Host plant | Impact | Pesticides active ingredients | References |
|---|---|---|---|---|---|
| | | | | | 2020; Nabahungu and Visser, 2013; REMA, 2011) |
| | *Cercospora oryzae* | (*Oryza sativa*) | ** | NI | (Nyabyenda, 2005) |
| | *Pythium bebaryanum* | | ** | NI | (Nyabyenda, 2005) |
| | *Rhizoctonia solani* | | ** | NI | (Nyabyenda, 2005) |
| | *Sclerotium rolfsii* | | ** | NI | (Nyabyenda, 2005) |
| | *Gerlachia oryzae* | | NI | NI | (Nyabyenda, 2005) |
| | *Helminthosporium oryzae* | | NI | NI | (Nyabyenda, 2005) |
| | *Sarocladium oryzae* | | NI | NI | (Nyabyenda, 2005) |
| | *Peronosclerospora sorghii* | Sorghum | *** | NI | (Nyabyenda, 2005) |
| | *Colletotrichum graminicola* | (*Sorghum bicolor*) | NI | NI | (Nyabyenda, 2005) |
| | *Exserohilum turcicum* | | NI | NI | (Nyabyenda, 2005) |
| | *Puccina purpurea* | | NI | NI | (Nyabyenda, 2005) |
| | *Sphacelotheca cruenta, S. reiliana, S. sorghi* | | NI | NI | (Nyabyenda, 2005) |
| | *Puccinia* sp. | Spinach | NI | Mancozeb | (Nyabyenda, 2006) |

| Pathogens and pests | Species | Host plant | Impact | Pesticides active ingredients | References |
|---|---|---|---|---|---|
| | | (*Basella alba*) | | | |
| | *Colletotrichum falactum* | Sugar cane | *** | NI | (Nyabyenda, 2006) |
| | *Fusarium moniliforme* | (*Saccharum officinarum*) | *** | NI | (Nyabyenda, 2006) |
| | *Ustilago scitaminea* | | *** | NI | (Nyabyenda, 2006) |
| | *Puccinia melanocephala* | | * | NI | (Nyabyenda, 2006) |
| | *Sclerospora sacchari* | | * | NI | (Nyabyenda, 2006) |
| | *Alternaria solani* | Sweet potato (*Ipomoea batatas*) | *** | NI | (Nyabyenda, 2006) |
| | *Armillaria mellea* | Tea | *** | NI | (Nyabyenda, 2006) |
| | *Rosellinia arcuate* | (*Camellia sinensis*) | *** | NI | (Nyabyenda, 2006) |
| | *Colletotrichum camelliae* | | * | NI | (Nyabyenda, 2006) |
| | *Corticium salmonicolor* | | * | NI | (Nyabyenda, 2006) |
| | *Pestalotiopsis theae* | | * | NI | (Nyabyenda, 2006) |
| | *Fusarium oxysporum* f. sp.*pv lycopersici* | Tomato | *** | NI | (Nyabyenda, 2006) |
| | *Cladosporium fulvum* | (*Lycopersicon esculentum*) | *** | NI | (Nyabyenda, 2006) |
| | *Alternaria solani* | | *** | Mancozeb, metalaxyl | (Nyabyenda, 2006) |

| Pathogens and pests | Species | Host plant | Impact | Pesticides active ingredients | References |
|---|---|---|---|---|---|
| **Oomyceta** | *Phytophthora infestans* | Potato (*Solanum tuberosum*) | *** | Mancozeb and metalaxyl | (Bararyenya et al., 2018; Biruma et al., 2007; Harahagazwe et al., 2007; Okonya et al., 2019b) |
| | *Phytophthora infestans* | Tomato | *** | Mancozeb and metalaxyl | (Nyabyenda, 2006) |
| | *Pythium* spp. | (*Lycopersicon esculentum*) | *** | NI | (Nyabyenda, 2006) |
| **Virus (and vector)** | African cassava mosaic virus "ACMV", East African cassava mosaic virus "EACMV", East African cassava mosaic virus-Uganda "EACMV-UG" (Vector: *Bemisia tabaci*) | Cassava (*Manihot esculenta*) | *** | NI | (Bigirimana et al., 2011, 2004; Nyabyenda, 2005b; Thresh and Cooter, 2005) |
| | Cassava brown streak virus "CBSV", Ugandan cassava brown streak virus "UCBSV", Cassava root necrosis disease "CRND" (Vector: *Bemisia tabaci*) | | *** | NI | (Bigirimana et al., 2011; Maruthi et al., 2017; Muhindo et al., 2020; Munganyinka et al., 2018; Nyabyenda, 2005; Okonya et al., 2019a) |
| | Banana bunchy top virus "BBTV" | Banana | *** | NI | (Boloy et al., 2014; Gaidashova et al., 2010; |

| Pathogens and pests | Species | Host plant | Impact | Pesticides active ingredients | References |
|---|---|---|---|---|---|
| | (Vector: *Pentalonia nigronervosa*) | (*Musa* spp.) | | | Mukwa et al., 2014; Niyongere et al., 2013; Okonya et al., 2019a; Ranade, 2004) |
| | Sweet potato chlorotic stunt virus "SPCSV" *(Vector: Bemisia tabaci)*, Sweet potato feathery mottle virus "SPFMV" (Vector: *Myzus persicae* and *Aphis gossypii*) | Sweet potato (*Ipomoea batatas*) | *** | NI | (Nyabyenda, 2005; Sheffield, 1957) |
| | Bean common mosaic virus "BCMV" (Vector: Aphid) | Bean (*Phaseolus vulgaris*) | *** | Dimethoate | (Nyabyenda, 2005) |
| | Virus A, Virus X, Virus S, Virus Y *(Vector: Aphid)* | Potato (*Solanum tuberosum*) | *** | NI | (Nyabyenda, 2005) |
| | Groundnut rosette virus "GRV" (Vector: *Aphis craccivora*) | Peanut (*Arachis hypogaea*) | *** | NI | (Nyabyenda, 2005) |
| | Maize chlorotic mottle virus "MCMV" | Maize | *** | NI | (Adams et al., 2014; Casinga et al., 2021; |

| Pathogens and pests | Species | Host plant | Impact | Pesticides active ingredients | References |
|---|---|---|---|---|---|
| | (Vectors: Thrips, root worms and leaf beetles), Maize streak virus "MSV" (Vector: *Cicadulina* spp.) Maize lethal necrosis "MLN" (Maize chlorotic mottle virus and Sugarcane mosaic virus) | (*Zea mays*) | | | Isabirye and Rwomushana, 2016; Lukanda et al., 2014; Mahuku et al., 2015; Nyabyenda, 2005; Redinbaugh et al., 2018) |
| | Pepper mild mottle virus (Vector: Thrips) | Pepper (Chilli and sweet) (*Capsicum* spp.) | *** | NI | (Nyabyenda, 2006) |

## 4.4. Limitations in local regulation of phytosanitary products and associated risks

Pesticides on the market must be regularly reconsidered by the relevant registration authorities and subsequently included in a list of approved or banned products. The aim is to ensure the quality, efficacy and safety of the chemicals used. However, there are some specific features of the management of pesticides in the GLCCA region that deserve special attention. First, many chemical pesticides not authorized in the European Union (EU Food Safety, 2023) are still officially registered in the GLCCA countries, representing up to 30% (Table 4-2) of the total number of products legally distributed (Table 4-A1). It includes the highly hazardous but most commonly used fungicide mancozeb and the insecticides imidacloprid, acephate and dimethoate not authorized in EU (Lewis et al., 2016). Furthermore, some ingredients such as endosulfan and dichlorodiphenyltrichloroethane, which have been officially discarded by governments in the GLCCA region, are still available in local markets (Bassily, 2022; Ngweme et al., 2019). Second, malpractices in the handling of chemical pesticides are common, either among traders or end-users such as farmers or industrial workers. These malpractices include the sale of adulterated products, failure to use personal protective equipment, incorrect dosage and selection of products, and application at inappropriate times (Mushagalusa Balasha et al., 2023; Okonya et al., 2019b). One of the consequences is that residues of pesticides have been found at toxic levels in crops and vegetables such as tomato and amaranth (Kavatsurwa et al., 2014; Ndisanze et al., 2022; Ngweme et al., 2021).

Consequently, some acute symptoms and even death of humans and animals due to pesticide poisoning have been reported in the region. Severe symptoms include reddened eyes, itchy skin, teary eyes, burning eyes, runny nose, headache, difficulty breathing, and heavy sweating (Mushagalusa Balasha et al., 2023; Ndayambaje et al., 2019; Okonya et al., 2019a). Chronic effects of pesticide exposure, including cancer,

infertility, and neurological problems (Mahmood et al., 2016), may be a reality in the region or may occur in the near future, although no official survey has been conducted to date. Several reasons may explain this chaotic situation in the region such as poverty, ignorance, and illiteracy among users (Muliele et al., 2018; Mushagalusa Balasha et al., 2023; Ndayambaje et al., 2019; Niyongere et al., 2015), inadequate governance in the pesticide sector, and possible socio-economic influence of agrochemical companies on officials (Gaberell and Viret, 2020).

Table 4-2: Status of some EU not authorized chemical pesticides in GLCCA. R: registered, NI: non-information, NA: not authorized, NAU: not authorized but used.

| Class of pesticides | Active ingredients not authorized in EU | Chemical family | Regulation status | | |
|---|---|---|---|---|---|
| | | | Burundi | DRC | Rwanda |
| Insecticides | Acephate | Organophosphorus | R | NI | NI |
| | Benfuracarb | Carbamates | R | NI | NI |
| | Carbosulfan | Carbamates | R | NI | NI |
| | Chlorfenapyr | Pyrroles | NI | NI | R |
| | Chlorpyrifos | Organophosphorus | R | R | R |
| | Cyfluthrin | Pyrethroids | R | NI | NI |
| | Diafenthiuron | Thioureas | NI | NI | R |
| | Diazinon | Organophosphorus | R | NA | NA |
| | Dichlorvos | Organophosphorus | R | NAU | NA |
| | Dimethoate | Organophosphorus | R | R | NA |
| | Endosulfan | Organochlorines | NA | NAU | NA |
| | Fenbutatin oxide | Organometallics | NI | NI | R |
| | Fenitrothion | Organophosphorus | R | R | NI |
| | Fenthion | Organophosphorus | R | NI | B |
| | Fenvalerate | Pyrethroids | R | NI | NI |
| | Fipronil | Phenylpyrazole | NI | R | R |
| | Flufenoxuron | Benzoylureas | NI | NI | R |
| | Imidacloprid | Neonicotinoid | R | R | R |

| Class of pesticides | Active ingredients not authorized in EU | Chemical family | Regulation status | | |
|---|---|---|---|---|---|
| | | | Burundi | DRC | Rwanda |
| | Isoxathion | Organophosphorus | R | NI | NI |
| | Omethoate | Organophosphorus | R | NI | NI |
| | Oxydemeton-methyl | Organophosphorus | R | NI | NI |
| | Permethrin | Pyrethroids | R | NI | R |
| | Profenofos | Organophosphorus | R | NI | R |
| | Pirimiphos-methyl | Organophosphorus | R | NI | NI |
| | Pymetrozine | Triazine | NI | NI | R |
| | Tetradifon | Diphenyl | NI | NI | R |
| | Tetramethrin | Pyrethroids | NI | R | NI |
| | Thiacloprid | Neonicotinoid | NI | NI | R |
| | Tralomethrin | Pyrethroids | R | NI | NI |
| | Triazophos | Organophosphorus | R | NI | NI |
| **Herbicides** | Atrazine | Triazine | R | NI | B |
| | Dalapon | Organochlorines | R | NI | R |
| | Hexazinone | Triazine | R | NI | NI |
| | Linuron | Methylureas | NI | NI | R |
| | Oxadiazon | Oxadiazole | NI | R | NI |
| | Paraquat | Pyridine | R | NAU | B |
| | Propanil | Anilide | NI | NI | R |
| | Terbutryn | Triazine | NI | NI | R |
| **Fungicides** | Benomyl | Carbamates | R | NI | R |
| | Bitertanol | Triazole | NI | NI | R |
| | Carbendazim | Carbamates | NI | NAU | R |
| | Chlorothalonil | Organochlorines | R | BU | R |
| | Cyproconazole | Triazole | NI | NI | R |
| | Epoxiconazole | Triazole | NI | NI | R |
| | Fenamidone | Imidazolinone | NI | NI | R |
| | Fenarimol | Pyrimidine | NI | NI | R |

| Class of pesticides | Active ingredients not authorized in EU | Chemical family | Regulation status | | |
|---|---|---|---|---|---|
| | | | Burundi | DRC | Rwanda |
| | Iprobenfos | Organophosphorus | R | NI | NI |
| | Iprodione | Dicarboximides | R | NI | R |
| | Mancozeb | Thiocarbamates | R | R | R |
| | Pencycuron | Phenylureas | NI | NI | R |
| | Propineb | Carbamates | NI | NI | R |
| | Triadimenol | Triazole | NI | R | NI |
| | Thiram | Thiocarbamate | R | R | R |
| **Rodenticides** | Bromadiolone | Coumarin | NI | R | NI |
| | Brodifacoum | Coumarin | R | NAU | R |
| | Chlorophacinone | Indandione | R | NI | NI |
| | Coumatetralyl | Coumarin | R | NI | R |
| | Diphacinone | Indandione | R | NI | R |
| **Insecticide-nematicides** | Carbofuran | Carbamates | R | NI | R |

## 4.5. Non-biological alternatives to chemical pesticides in GLCCA

The adverse effects of chemical pesticides have prompted the worldwide scientific community and policy makers to search for alternatives and promote the so-called IPM defined as a holistic approach that aims to control plant pests and diseases by mobilizing all existing methods while reducing reliance on chemicals (Stenberg, 2017). IPM promotes the development of non-biological strategies such as prevention, monitoring, and rational use of chemical phytoprotectants products, and the implementation of biological control methods, which will be discussed later.

Good agricultural practices are preventive measures consisting, for example, in the use of certified varieties, crop rotation, field sanitation and balanced fertilization. These practices have been adopted to some extent in the GLCCA region and promising results have been obtained. For example, cassava mosaic disease and maize streak disease have been controlled using resistant varieties (Legg et al., 2006; Nkurunziza et al., 2012). The banana bunchy top disease has recently been reduced via macro- and micro-propagation of healthy suckers (Niyongere et al., 2013; Paka et al., 2021; Tchatchambe et al., 2019). The incidence of banana *Xanthomonas* wilt and potato bacterial wilt caused by *Xanthomonas campestris* pv. Musacearum and *Ralstonia solanacearum, respectively,* has been limited via crop rotation, field sanitation, sterilization of farm tools, and avoidance of their exchange (Harahagazwe et al., 2007; Ndayihanzamaso et al., 2020; Uwamahoro et al., 2020). However, despite many attempts involving certified varieties or other agricultural practices, no substantial results have been achieved to control fungal diseases in the region using these approaches. Moreover, implementation of good agricultural practices is unfortunately hampered by several factors, including demographic pressure on arable land, the relative time required to develop new varieties, the lack of durability of resistance in developed varieties, and

the consequences of climate change in the agro-ecological context (Pandit et al., 2022).

Phytosanitary products must be used in a rational way, taking into account the epidemiological factors of the specific pathogens, which are usually obtained from accurate field monitoring. Where chemicals are irreplaceable, careful consideration must be given to the dose applied and the less toxic products must be favored. For example, flupyradifurone has been proposed to replace the insecticide imidacloprid (Maloney et al., 2020) and copper hydroxide in substitution of mancozeb (FPS Health, 2023) in EU. Unfortunately, this practice is less widespread in the region, where the choice of pesticide is made at random without prior information on the disease status and on recommended products for optimal efficiency.

## 4.6. Implementation of biological control

Biological control is a promising approach for plant protection based on the use of living organisms and/or their derivatives. These include macroorganisms (predatory insects, mites, and nematodes), microorganisms (beneficial bacteria, fungi, protozoa, viruses, yeasts) and biochemicals (semiochemicals, plant extracts, plant growth regulators and organic acids) (Dunham Trimmer, 2023). The biocontrol market accounts for 10% of the global pesticide market, with North America leading the way in promoting BCPs, while African share is very insignificant led by countries like Nigeria, South Africa, and Kenya (Marrone, 2023; Mordor Intelligence, 2024). Biological control is a new paradigm in GLCCA and only seven biocontrol products (3%) are registered up to now (Table 4-A1).

### 4.6.1. Macroorganisms

Macroorganisms are either predators or parasitoids of the same kind of pathogenic organisms (insects, mites, or nematodes). They are used in the form of eggs, larvae, pupae, or adults to kill or parasitize the target pest (Dunham Trimmer, 2023; Ramalakshmi et al., 2020). Insect

predators have been successfully used in the region to control some pests. For instance, *Epidinocarpis lopezi* isolated in South America, has been used to control the cassava mealy bug (*Phenacoccus Manihoti*) (Neuenschwander, 2001; Nyabyenda, 2005), while *Gyranusoidea tebygi* and *Anagyrus mangicola* have been developed to control the devastating mango mealy bug in Burundi (FAO, 2022). Other common predators could be introduced locally to control a range of pests. Indeed, the green lacewing (*Chrysoperla carnea*), the Malaysian ladybird beetle (*Chilocorus nigritus*) and the mealybug ladybird (*Cryptolaemus montrouzieri*) have been reported to be effective in controlling the cotton aphid jassid, peanut thrips, sugarcane scales and coffee and mango mealybugs respectively (Ramalakshmi et al., 2020).

### 4.6.2. Biochemicals

Biochemicals in the form of plant extracts appear to be the most widely used biocontrol agents in the region. These extracts are prepared from various plant species like *Azadirachta indica*, *Capsicum* spp., *Allium sativum*, *Tephrosia* spp., *Tithonia diversifolia* or *Ricinus communis* and are used as insecticides to control various pests such as *S. frugiperda* on maize, *Tuta absoluta* on tomato, *Ophiomya phaseoli*, *Aphis fabae* on beans, etc. (Korangi Alleluya et al., 2021; MINAGRI-Burundi, 2016; Rutikanga, 2015). In addition, two plant molecules azadirachtin and pyrethrin, and spinosad from actinomycetes have been registered as insecticides in Rwanda (Table 4-A1). However, the use of these extracts is still globally rudimentary and based on traditional and community-based knowledge. Moreover, the use of this type of product requires increased land for growing plants to prepare the extracts, which would inevitably compete with staple and cash crops for agricultural land.

### 4.6.3. Microorganisms

Several microorganisms encompassing bacteria, fungi, and yeasts are commercialized as biocontrol agents. Fungal based agents mainly include the genera *Trichoderma*, *Metarhizium* and *Beauveria*; while

yeasts include the genera *Pichia, Yarrowia* and *Saccharomyces*. The largest group of worldwide marketed microorganisms are bacteria (up to 75%), dominated by plant growth-promoting rhizobacteria (PGPR) of the genera *Bacillus, Pseudomonas* and *Streptomyces* (Bonaterra et al., 2022; Dimkić et al., 2022; Saeed et al., 2021). However, these globally adopted microbial-based alternatives are rarely used in the GLCCA region and mainly as bioinsecticides such as *B. thuringiensis* sold in the three countries and *B. bassiana* and *T. harzanium* only registered in Rwanda. In this review, an overview of the PGPR reported in literature with biocontrol activities and their mechanisms of action is presented for their possible integration into local agricultural systems in GLCCA.

#### 4.6.3.1. Plant growth-promoting rhizobacteria in crop protection

Plant growth-promoting rhizobacteria are a group of bacteria found mainly in the vicinity of plant roots, the rhizosphere. The plant provides them with nutrients in the form of root exudates, while these bacteria protect the host and promote its growth. These benefits are mediated through various pathways, including biofertilization by nitrogen fixation and phosphate solubilization, phytostimulation by the production of phytohormones (cytokinin, abscisic acid, auxin, gibberellins, and ethylene), stress tolerance and biological control (Anckaert et al., 2021; El-Saadony et al., 2022; Vejan et al., 2016). PGPR include several species belonging to different genera, namely *Rhizobium, Azotobacter, Streptomyces, Enterobacter, Klebsiella, Rhodococcus, Paenibacillus, Variovorax, Azosprillum, Bulkholderia, Serratia, Pseudomonas* and *Bacillus* (Caulier et al., 2018). Bacterial strains of the genera *Streptomyces, Paenibacillus, Pseudomonas* and *Bacillus* are the best described and have been shown to be effective biocontrol agents against various plant pathogens through their multiple mechanisms of action such as nutrient and niche competition, antibiosis, signal interference and induction of host resistance (Wang et al., 2021).

### a. Competition

Competition for nutrients and niches between pathogens and beneficial microbes is an important factor in limiting disease incidence and severity (Bishnoi, 2015). Indeed, rhizosphere microorganisms compete for a variety of plant root exudates, including sugars, amino acids and organic acids. These exudates also act as chemoattractants for bacteria to plant roots. These beneficial bacteria colonize the root and can inhibit the growth of soil-borne phytopathogenic microbes by competing for nutrients such as glucose, asparagine, and iron (Dutta and Lee, 2022). Besides exudates, competition for iron is the most well-studied mechanism and involves several bacterial siderophores, such as hydroxamates (*Pseudomonas* spp.), carboxylates (*Rhizobium* spp.) and catecholates (*Pseudomonas* spp., *Bacillus* spp.), which are used to extract iron from insoluble forms and sequester it from other competitors by chelation (Arguelles-Arias et al., 2009; Mazumdar et al., 2020). On the other hand, rhizobacteria outcompete other microbes for space through their ability to form biofilms, which also act as shelter-like against pathogens (Flemming et al., 2016; Singh et al., 2022).

### b. Antibiosis

Antibiosis is a biological process in which antimicrobial substances synthesized by microorganisms inhibit or kill plant pathogens (Fira et al., 2018). These are toxins that interfere with the synthesis of the cell membrane or other metabolic processes of the pathogen and are variable depending on the PGPR species. For example, *Pseudomonas* spp. are known to produce 2,4-diacetylphloroglucinol, phenazine, pyoluteorin, pyrrolnitrin, hydrogen cyanide and a wide range of lipopeptides such as viscosin, amphisin, orfamide, bananamide and sessilin (Oni et al., 2022; Wang et al., 2021). *Bacillus* spp. produce various cyclic lipopeptides (CLPs), polyketides, dipeptides, RiPPs and volatile organic compounds (Anckaert et al., 2021; Arguelles Arias et al., 2013; Caulier et al., 2019). *Streptomyces* species are known producers of several antibiotics such as kasugamycin (antifungal and

antibacterial), polyoxin B and D, validamycin (antifungal) families (Barka et al., 2016), as well as cyclic lipopeptides such as lipopeptins recently discovered with antagonistic activity against *Fusarium oxysporum* (Wang et al., 2023). *Paenibacillus* spp. are another important PGPR, producing mainly polymyxin active against Gram-negative bacteria, fusaricidin with anti-Gram-positive bacteria and antifungal activities (Ali et al., 2021), pelgipeptins and tridecaptins with antagonistic activity against fungi and bacteria (Costa et al., 2022; Le et al., 2020). PGPR also produces lytic enzymes (the so-called cell wall degrading enzymes) such as chitinase, glucanase and proteases, which are known for their antagonistic activities against pathogens (Abdelaziz et al., 2023).

### c. Signal interference

Like other organisms, bacteria live in communities where they communicate to obtain nutrients and perform other metabolic functions. Cell-to-cell communication within bacteria is known as quorum sensing (QS), mediated by several molecules including N-acylhomoserine lactones, oligopeptides and LuxS/autoinducers (De Kievit, 2009; Rodríguez et al., 2020; Wang et al., 2021). QS is important for bacterial phenotype and virulence, biofilm formation and other physiological processes. QS-mediated metabolites from phytopathogenic bacteria can be chemically or enzymatically degraded by so-called quorum quenching (QQ) enzymes such as lactonase, acylase and oxidoreductase produced by some PGPR. This interferes with QS-regulated processes that are crucial for pathogen development (Sikdar and Elias, 2020) and some important bacterial diseases such as *Pectobacterium carotovorum*, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Erwinia amylovora* have been successfully managed by using this approach (Rodríguez et al., 2020).

### d. Induced systemic resistance

Induced systemic resistance (ISR) is the process by which plants treated with PGPR species or their secreted molecules induce defense genes and develop immunity to subsequent plausible pathogen attack. PGPR-induced ISR is phenotypically similar to the well-studied systemic acquired resistance (SAR), which is activated after a first infection by an incompatible or necrotizing pathogen (Pršić and Ongena, 2020). This process is regulated by the jasmonate or ethylene phytohormone pathways, whereas SAR is dependent on the salicylic acid regulation pathway. The main elicitors of ISR produced by PGPR are Acyl-Homoserine Lactones, cyclic lipopeptides, rhamnolipids, N-alkylated benzylamine derivative, siderophores, volatile compounds and other metabolites with antibiotic functions such as 2,4-diacetylphloroglucinol and phenazine (Pršić and Ongena, 2020; Wang et al., 2021; Zhu et al., 2023). These elicitors interact with plant cell membrane and lead to induction of plant defense, although their mechanisms of action are not fully known. However, for a successful symbiosis with plant roots, PGPR have evolved to evade or suppress the pattern triggered immunity developed by the plant as the first line of defense (Seth et al., 2023; Yu et al., 2022). This PGPR-induced plant immunity ultimately leads to defense mechanisms such as cell wall reinforcement via deposition of lignin or callose, enhanced production of defense-related proteins (lipoxygenase, glucanase, chitinase, phenylalanine ammonia-lyase) and accumulation of phytoalexins as small-size antimicrobial metabolites (Abdelaziz et al., 2023).

#### 4.6.3.2. *Bacillus* as successful PGPR for biological control

*Bacillus* is a rod-shaped, gram-positive and spore-forming bacterium that is widespread in a variety of ecological niches (Yin et al., 2023). Phylogenetically, the *Bacillus* genus can be broadly divided into two clades, namely the *Bacillus cereus* group, which includes the well-known and most commercialized bioinsecticide *B. thuringiensis*, and the *Bacillus subtilis* group, which includes most of *Bacillus* strains

marketed for the biocontrol of microbial pathogens (Dunlap, 2019; Nikolaidis et al., 2022). Strains in the *B. subtilis* group are generally recognized as safe and have therefore been used as alternatives to chemical pesticides in agriculture (Etesami et al., 2023a). For example, *B. licheniformis* SB3086 (Ecoguard®, Novozymes®, Biofungicide®, Green Relief®), *B. pumilus* GB34 (GB34®, Concentrated Biological Fungicide®, Ballad®), *B. velezensis* MBI600 (Subtilex®, Histick N/T®), *B. velezensis* GBO3 (Kodiak®, Companion®), *B. velezensis* QST713 (Serenade®, Rhapsody®) and *B. velezensis* FZB42 (Taegro®, Rhizovital®) are among the commercial biocontrol agents from the *B. subtilis* group (Lahlali et al., 2022; Yadav et al., 2022). These strains are continuously attracting researchers and industrials thanks to their intrinsic ability to form resistant endospores that allow stable formulations, relatively rapid growth on different substrates, and the secretion of a wealth of bioactive secondary metabolites retaining key biocontrol functions (Prasad et al., 2023). The biosynthesis of these biochemicals involves up to 10% of the bacterial genome and includes a huge diversity of compounds produced via non-ribosomal and ribosomal pathways (Borriss, 2020; Grubbs et al., 2017; Yin et al., 2023).

Cyclic lipopeptides are a group of compounds with a fatty acid and peptide moiety that are produced via the non-ribosomal pathway involving a multi-modular enzyme complex (Iqbal et al., 2023). This machinery gives rise to a large chemical diversity with different structures, based on their amino acid sequence or on the length of the fatty acid chain. These metabolites are classified into the heptapeptides surfactins (pumilacidins/lichenisins) and iturins (iturin A, mycosubtilin, bacillomycin) and the decapeptides fengycins (plipastatins); together with their homologues of different alkyl chain lengths ($C_{12}$ - $C_{17}$ for surfactins, $C_{13}$-$C_{17}$ for iturins and $C_{14}$-$C_{18}$ for fengycins) (Théatre et al., 2022). They are produced by members of the *Bacillus subtilis* group and are known to be involved in the induction of resistance in plants (Dimkić et al., 2022; Mahapatra et al., 2022; Pršić and Ongena, 2020)

and, thanks to their amphiphilic nature, exhibit antimicrobial activities against a wide range of pathogenic microorganisms by penetrating and disrupting the cell membrane of target organisms (Fira et al., 2018; Ngalimat et al., 2021; Puan et al., 2023).

Polyketides are bioactive metabolites synthetized via pathways catalyzed either by the polyketide synthases (difficidins and macrolactins) or by the hybrid polyketide synthases/non-ribosomal peptide synthetases (bacillaene) and are structurally composed of an alternation of polyenes and carbonyl groups (Iqbal et al., 2023). They retain consistent antibacterial activity against several phytopathogens either by interfering with protein synthesis or by damaging the bacterial cell wall (Miao et al., 2023; Rabbee and Baek, 2020; Zhang et al., 2023). The siderophore bacillibactin is a catecholate peptide also produced via the non-ribosomal pathway that retains a high affinity for iron. This property is very important in bacterial niche competition, helping bacilli to gain advantage over their competitors by chelating available iron in the environment (Yu et al., 2011). The dipeptide bacilysin is biosynthesized via a typical non-ribosomal pathway not shared by the lipopeptide/polyketide/siderophore trio. It exhibits toxicity against bacteria by blocking peptidoglycan biosynthesis (Borriss, 2015) and against fungi by probably inhibiting fungal glycans in a manner similar to the inhibition of bacterial peptidoglycan synthesis (Han et al., 2021).

Ribosomally produced and post-translationally modified peptides include bacteriocins and lantibiotics (such as amylocyclicin, amylolysin, plantazolicin, subtilin, pumilarin or LCI) known for their high inhibitory potential against bacterial pathogens, either by vascularization of the protoplasm or by pore formation or cell disruption (Sumi et al., 2015). In addition to these soluble metabolites, *Bacillus* volatile compounds (hydrocarbons nonane and tridecane, acetoin, 2,3-butanediol, benzaldehyde, 3-methylpropanoic acid, methylbenzene, benzothiazole, etc.) have been reported to inhibit the

growth of some fungi, bacteria, and nematodes by disrupting cell wall integrity (Caulier et al., 2019; Iqbal et al., 2023; Kai, 2020).

#### 4.6.3.3. The potential of *Bacillus*-based biocontrol in the GLCCA region, a myth or reality

The market for biocontrol agents based on strains of the *B. subtilis* group for the control of plant diseases is growing rapidly around the world but no product based on this bacterium is registered in the region. However, some promising preliminary studies using *B. velezensis* strains to control fungal diseases in Burundi and DRC have demonstrated the potential of these bacteria to adapt to local agro-ecological conditions, which could pave the way for their possible future dissemination and adoption by local farmers. *Bacillus velezensis* S499, isolated in Ituri/DRC in 1950s (Delcambe and Devignat, 1957), is effective against *Fusarium* sp. on tomato (65-70% of disease reduction) under field conditions in Burundi, and lipopeptide-mediated ISR is proposed as the main involved mechanism (Nihorimbere et al., 2010). Lipopeptides secreted by the same strain showed high *in vitro* antagonistic activity against pathogens isolated from maize cob such as *Rhizopus stolonifer, Penicillium variable, Fusarium verticillioides*. In the same study, S499 was able to efficiently reduce disease severity (39-67%) caused by these fungi in greenhouse and field conditions (Kulimushi et al., 2018). In addition, *B. velezensis* GA1 and its cyclic lipopeptides played an important role in the control of peanut stem rot disease caused by *Athelia rolfsii*, providing up to 60% of protection (Korangi Alleluya et al., 2023).

Furthermore, a number of studies have demonstrated the potential of *Bacillus* isolates in controlling a range of important and widespread phytopathogens reported in the GLCCA. For example, *B. altitudinis* and *B. velezensis* were shown to control rice blast caused by *Pyricularia oryzae* (15% and 25% of disease reduction, respectively) by direct antagonism and ISR involving iturin and fengycin (Lam et al., 2021). Volatiles from *B. amyloliquefaciens* NJN-6 reduce the mycelial growth

of the banana pathogen *F. oxysporum* f.sp. *cubense in vitro* by up to 30-40% (Yuan et al., 2012). *B. subtilis* AUBB20 inhibited the growth of the coffee pathogen *F. xylarioides* through its secreted lytic enzymes (Muleta et al., 2007). Antagonistic activities of *Bacillus subtilis* strains have also been reported against fungi and oomycetes, including *Alternaria linariae* (Da Silva Junior et al., 2023), *Sclerotinia sclerotiorum*, *Rhizoctonia solani* (Al-Mutar et al., 2023), *Colletotrichum lindemuthianum* (Martins et al., 2019) , *Mycosphaerella fijiensis* (Gutierrez-Monsalve et al., 2015), *Verticillium dahliae* (Dhouib et al., 2019), *Fusarium oxysporum* f. sp. *lycopersici* (Aydi Ben Abdallah et al., 2017), *Colletotrichum gloeosporioides* (Luna-Bulbarela et al., 2018) and *Phytophthora infestans* (Zhang et al., 2023).

The biocontrol activity of *Bacillus* has also been demonstrated against important bacterial phytopathogens. For example, the application of *B. subtilis* to potato plants infected with *R. solanacearum* reduced the incidence of wilt by about 50% (Elazouni et al., 2019). Tomato bacterial wilt caused by the same bacterium was efficiently controlled by *B. methylotrophicus* DR-08 due to its secreted metabolites oxydifficidin and difficidin (Im et al., 2020). *Bacillus megaterium* USB2103 efficiently controlled common bean bacterial wilt caused by *Xanthomonas axonopodis* pv. *phaseoli* through ISR mechanisms (Giorgio et al., 2016). Brown sheath blight of rice caused by *Pseudomonas fuscovaginae* was successfully controlled (76.6%) by *B. amyloliquefaciens* Bk7 (Kakar et al., 2014). Other examples of *Bacillus* spp. with potential applications in the control of fungal and bacterial diseases for crop protection in the GLCCA are shown in Table 4-3.

In addition to their efficacy against phytopathogenic fungi and bacteria, some *Bacillus* spp. have been reported for their potential to control nematodes, insects and weeds (Mnif and Ghribi, 2015). For instance, surfactins from *B. velezensis* S499 showed insecticidal activity against the fruit fly *Drosophila melanogaster* (Assié et al., 2002). The wheat rhizosphere *Bacillus altitudinis* D30202 was shown to have bioherbicidal potential on the grass *Avena fatua* L. (Ma et al., 2023).

*Bacillus* strains such as *B. altitudinis*, *B. pumilis*, *B. velezensis*, *B. mojavensis* and *B. megaterium* showed nematicidal activity against *Meloidogyne incognita* (El-Nagdi and Abd-El-Khair, 2019; Guimarães Pacifico et al., 2021; Huang et al., 2010; Padgham and Sikora, 2007; Xiang et al., 2017; Ye et al., 2022) and *Heterodera glycines*, which threatens soybean production (Dalvan Do Nascimento et al., 2022). Cell-free culture supernatants and volatiles of *B. amyloliquefaciens* BV03, PTA4838 and *B. velezensis* MBI600 killed more than 85% of *Helicotylenchus dihystera* infesting soybean (Camatti et al., 2023). So even if very few products have been developed to the market scale so far, the results already available are promising for the design of bionematicides, bioinsecticides and bioherbicides based on *Bacillus*.

Table 4-3: Some global success stories of *Bacillus* isolates (subtilis group) in biocontrol of important crop pathogens reported in the GLCCA. *ns: not specified.*

| ***Bacillus* strains** | **Plant** | **Pathogen** | **Mechanism involved** | **Experiment condition** | **Biocontrol potential** | **References** |
|---|---|---|---|---|---|---|
| ***B. velezensis* NJN-6** | Banana | *Fusarium oxysporum* f.sp. *cubense* | Antibiosis (VOCs) | *In vitro* | 30-40% of *Foc* inhibition compared to control | (Yuan et al., 2012) |
| ***B. velezensis* EB1** | | | Antibiosis and ISR | *In vitro, in greenhouse* | 75.43% Inhibition rate | (Xiang et al., 2023) |
| ***B. amyloliquefaciens* W19** | | | Antibiosis (CLPs and VOCs) | *In vitro, in greenhouse* | 21% Inihibition rate | (Wang et al., 2013) |
| ***B. siamensis* Gxun-6** | | | Antibiosis (probably), ISR (probably) | *In vitro, in greenhouse* | >68.8% Inhibition rate ; >88.26% Biocontrol efficacy | (Shen et al., 2022) |
| ***B. pumilus* CCIBP-C5** | | *Mycosphaerella fijiensis* | Antibiosis | *In vitro, in greenhouse* | 45% Inhibition rate ; 33.6% Biocontrol efficacy | (Di Francesco et al., 2017) |
| ***B. licheniformis, B. siamensis, B. subtilis* subsp. *Inaquosorum*** | | | ISR | *In greenhouse* | ns | (Marcano et al., 2016) |
| ***B. velezensis* QST713** | Bean | *Xanthomonas axonopodis* pv. *phaseoli* | Antibiosis (PKs) | *In vivo* | 52.26% Disease incidence reduction | (Belete et al., 2021) |
| ***B. pumilus*** | | *Sclerotium rolfsii* | ns | *In greenhouse* | 26% Disease incidence reduction | (Pleban et al., 1995) |

| ***Bacillus* strains** | **Plant** | **Pathogen** | **Mechanism involved** | **Experiment condition** | **Biocontrol potential** | **References** |
|---|---|---|---|---|---|---|
| ***B. velezensis* MBI600, *B. velezensis* FZB42, *B. velezensis* QST713** | | *Colletotrichum lindemuthianum* | ISR (probably) | *In greenhouse* | 21.9% ,15.2%, 10.9% Diseased plants respectively | (Tinivella et al., 2009) |
| ***B. velezensis* GBO3** | Cassava | *Fusarium solani* | Antibiosis | *In vitro, in greenhouse* | Approx. 10mm inhibition zone ; 0% Disease incidence (asymptomatic plants) | (Freitas et al., 2019) |
| ***B. subtilis* ME9** | | *Xanthomonas phaseoli pv. Manihotis* | Antibiosis | *In vitro, in greenhouse* | Approx. 14mm inhibition zone diameter | (Feng et al., 2023) |
| ***B. subtilis* AUBB20** | Coffea | *Fusarium xylarioides* | Antibiosis (lytic enzymes) | *In vitro* | Approx. 30-70 mm inhibition zone | (Muleta et al., 2007) |
| ***B. pumilus*** | Cotton | *Rhizoctonia solani* | ns | *In greenhouse* | 56% Disease incidence reduction | (Pleban et al., 1995) |
| ***B. velezensis* SQR9** | Cucumber | *Fusarium oxysporum* f.sp. *Cucumerinum* | Competition | *In vitro, in greenhouse* | 49-61% Disease reduction compared to control | (Cao et al., 2011) |
| ***B. subtilis Y*B-04** | | | Antibiosis (lytic enzymes), Competition for nutrients (siderophore) | *In vitro, in greenhouse* | >90% Control efficacy | (Xu et al., 2022) |
| ***B. subtilis* TM4** | Maize | *Fusarium verticolloïdes* | ns | *In field* | <9% Disease incidence | (Mirsam et al., 2021) |
| ***B. licheniformis*** | Mango | *Colletotrichum gloeosporioides* | Antibiosis | *In vivo* (on harvested fruits) | 30-40% Disease incidence | (Govender et al., 2005) |

| ***Bacillus* strains** | **Plant** | **Pathogen** | **Mechanism involved** | **Experiment condition** | **Biocontrol potential** | **References** |
|---|---|---|---|---|---|---|
| | | *Botryosphaeria spp.* | | | | |
| ***B. safensis* C3** | Mungo bean | *Xanthomonas axonopodis Pseudomonas syringae* | Antibiosis (Bacteriocin-like antimicrobial peptides) | *In vitro* | ns | (Romero-Severson et al., 2021) |
| ***B. subtilis* BsW4, Bs76, *B. amyloliquefaciens* Ba100** | Pea | *Ascochyta pinodes* | Antibiosis (CLPs) | *In vitro, in greenhouse* and *in field* | >65% Biocontrol efficacy | (Liu et al., 2016) |
| ***B. velezensis* TN-TB4, *B. amyloliquefaciens* TN-TB6** | Peanut | *Cercospora arachidicola* | Antibiosis | *In vitro* | >55% Inhibition rate | (Thanh and Yen, 2023) |
| ***B. subtilis* G1** | | *Macrophomina phaseolina* | Antibiosis and ISR (hypothesis) | *In greenhouse, in field* | 15-20% Disease incidence | (Shifa et al., 2018) |
| ***B. velezensis* LHSB1** | | *Sclerotium rolfsii* | Antibiosis (CLPs) | *In vitro, in greenhouse* | 93.8% Inhibition rate ; 62.6–70.8% Biocontrol efficacy | (Chen et al., 2019) |
| ***B. velezensis* 6-5** | Potato | *Phytophthora infestans* | Antibiosis (lytic enzymes) | *In vitro* | >90% Inhibition rate | (Zhang et al., 2023) |
| ***B. amyloliquefaciens* Bk7** | Rice | *Pseudomonas fuscovaginae* | Antibiosis | *In vitro, in greenhouse* | 93% Inhibition rate ; 76.6% | (Kakar et al., 2014) |
| ***B. subtilis* PTS-394** | Pepper | *Fusarium solani* | Antibiosis (CLPs) and ISR | *In vitro, in greenhouse* and *in field* | 69,63% Biocontrol efficacy; 74,43% Biocontrol efficacy | (Qiao et al., 2023) |
| ***B. subtilis* 168** | | *Ralstonia solanacearum* | ISR (VOCs) | *In vitro* | ns | (Yi et al., 2016) |

| ***Bacillus* strains** | **Plant** | **Pathogen** | **Mechanism involved** | **Experiment condition** | **Biocontrol potential** | **References** |
|---|---|---|---|---|---|---|
| ***Bacillus subtilis* HSY21** | Soybean | *Fusarium oxysporum* | ns | *In vitro, in greenhouse* and *in field* | 81.30 % Inhibition rate; 63.83% control effects; 57.07% control effects | (Han et al., 2021) |
| ***B. amyloliquefaciens* Q-426** | Spinach | *Fusarium oxysporum* f.sp *spinaciae* | Antibiosis (CLPs) | *In vitro* | Approx. 28 mm inhibition zone diameter | (Zhao et al., 2013) |
| ***B. subtilis* CAS15** | Sweet pepper | *Fusarium oxysporum* f.sp *capsici* | Competition for nutrients and ISR | *In greenhouse* | Reduction of the disease incidence by 12.5 to 56.9% | (Yu et al., 2011) |
| ***B. megaterium* TRS-4** | Tea | *Fomes lamaoensis, Sphaerostilbe repens, Poria hypobrumea, Sclerotium rolfsii* | Antibiosis and ISR | *In vitro, in greenhouse* | 55–84% Inhibition rate | (Chakraborty et al., 2006) |
| ***B. amyloliquefaciens* XJ5** | Tomato | *Alternaria solani* | Antibiosis (CLPs, lytic enzymes) | *In vitro* | 82.5% Inhibition rate | (Mu et al., 2023) |
| ***B. velezensis* K01** | | *Botrytis cinerea* | Antibiosis (CLPs, lytic enzymes) | *In vitro, in vivo* (on detached leaves and harvested fruits) | 84.1% Inhibition rate, >80% Disease reduction, >78% Disease reduction | (Xue et al., 2023) |

| ***Bacillus* strains** | **Plant** | **Pathogen** | **Mechanism involved** | **Experiment condition** | **Biocontrol potential** | **References** |
|---|---|---|---|---|---|---|
| ***B. subtilis* EPC016** | | *Fusarium oxysporum* f.sp. *lycopersici* | Antibiosis (VOCs, lytic enzymes), Competition for nutrients (siderophore) | *In vitro, in greenhouse* | 46.04% to 60.78% Inhibition rate | (Ramyabharathi and Raguchander, 2014) |
| ***B. velezensis* UQ9000N** | | *Fusarium oxysporum* f.sp. *lycopersici*, *Macrophomina phaseolina* | Antibiosis | *In vitro* | 52 % to 56% inhibition rate | (Arkhipov et al., 2023) |
| ***B. velezensis* FJAT-46737** | | *Ralstonia solanacearum* | Antibiosis | *In vitro, in greenhouse* | Approx. 82.0% Biocontrol efficiency | (Chen et al., 2020) |
| ***Bacillus amyloliquefaciens* FJAT-2349** | | | Antibiosis (CLPs) | *In greenhouse* | 97.6% Biocontrol efficiency | (Chen et al., 2019) |
| ***B. amyloliquefaciens* PKM16** | | *Sclerotinia sclerotiorum* | Antibiosis | *In vitro, in greenhouse* | 40.27% Inhibition rate | (Mattos et al., 2023) |
| ***B. subtilis* IJ10** | Turmeric | *Fusarium solani*, *Pythium aphanidermatum* | Antibiosis (VOCs, lytic enzymes) | *In vitro, in greenhouse* | >45% Inhibition rate ; >60% Biocontrol efficacy | (Kharshandi and Kayang, 2023) |

## 4.7. Conclusions

This review summarizes the current phytosanitary situation in the GLCCA region and the possibility of shifting to safer and more environmentally friendly alternatives to chemical pesticides for sustainable agriculture. Several diseases and pests are devastating many crops, leading to the use of chemical pesticides despite their known hazardous nature. Although pesticide regulations exist in the three countries, their implementation is problematic with one third of registered pesticides already banned from market in EU and numerous mishandling incidents. For the sake of the terrestrial and aquatic ecosystems, governments in the GLCCA need to take urgent action to mitigate the harmful effects of chemical pesticides. The regulatory systems should be reviewed and updated, and other less toxic pesticides or more sustainable control strategies should be promoted. Inspectors should be increased in number and motivation, and retailers and farmers should be made aware of pesticide regulations and the associated health and environmental risks.

Globally available alternatives inspired by integrated pest management (IPM), including good agricultural practices and biological control, are not widely used and/or in some cases non-existent. Although a limited number of biocontrol products are registered in the region, their use has yet to become a "reality". Moreover, all available products are used against insect pests but there is no biological solution that has been developed in practical terms to fight highly prevalent fungal and bacterial diseases. Some *Bacillus* isolates clearly represent promising tools for the control of these microbial diseases, and their inherent ability to form resistant endospores, to grow on poor substrates such as agro-industrial residues in semi-solid systems, and their potential to secrete a vast array of bioactive secondary metabolites (Bouassida et al., 2023; Kumar et al., 2021) would allow designing stable, rich, and affordable formulations of products suitable for the local socio-economic context. Unfortunately, although some preliminary studies have demonstrated efficacy in controlling some important local

pathogens, these *Bacillus*-based products are still a "myth" for local farmers. Efforts should be directed towards promoting the use of already available *Bacillus*-based products against endemic pathogens devastating crops in local farming systems. However, bioprospecting for indigenous *Bacillus* strains that are well-adapted to local agro-ecological conditions and retain strong biocontrol properties should be promoted to ensure sustainable plant disease management in the region. The great potential of indigenous *Bacillus* strains over the commercial ones has been shown, for example in the management of grapevine trunk disease (Langa-Lomba et al., 2023) and tropical fruit diseases (Reyes-Estebanez et al., 2020). Governments of the GLCCA countries should not only encourage the investment in such biocontrol products, which are virtually absent in the region, but also facilitate their registration and commercialization.

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## Appendix

Table 4-A1: Registered pesticides in the GLCCA countries. R, registered; NR, not registered; Names in red, EU not authorized pesticides.

| Class of pesticides | Active ingredients | Burundi | DRC | Rwanda |
|---|---|---|---|---|
| Insecticides + Acaricides | Abamectin | R | R | NR |
| | Acephate | R | NR | NR |
| | Acetamiprid | R | R | R |
| | Acrinathrin | NR | NR | R |
| | Alpha-cypermethrin | R | NR | R |
| | Aluminium phosphide | R | NR | R |
| | Azocyclotin | NR | NR | R |
| | Benfuracarb | R | NR | NR |
| | Beta-cyfluthrin | R | NR | R |
| | Bifenthrin | R | R | R |
| | Bromopropylate | NR | NR | R |
| | Buprofenzin | NR | NR | R |
| | Carbofuran | NR | NR | R |
| | Carbosulfan | NR | NR | R |
| | Chlorfenapyr | NR | NR | R |
| | Chlorpyriphos | R | NR | NR |
| | Chlorpyriphos-ethyl | R | R | NR |
| | Chlorpyriphos-methyl | R | NR | NR |
| | Clofentezin | NR | NR | R |
| | Copper oxychloride | NR | R | NR |
| | Cyfluthrin | R | NR | NR |
| | Cypermethrin | R | R | R |
| | Cyphenothrin | NR | R | NR |
| | Cyromazine | NR | NR | R |
| | Deltamethrin | R | R | R |
| | Diafenthiuron | NR | NR | R |
| | Diazinon | R | NR | NR |
| | Dichlorvos | R | NR | NR |
| | Diflubenzuron | NR | NR | R |
| | Dimethoate | R | R | NR |

| Class of pesticides | Active ingredients | Burundi | DRC | Rwanda |
|---|---|---|---|---|
| | Esfenvalerate | R | R | NR |
| | Fenamiphos | NR | NR | R |
| | Fenaxaquin | NR | NR | R |
| | Fenbutatin oxide | NR | NR | R |
| | Fenitrothion | R | R | NR |
| | Fenthion | R | NR | NR |
| | Fenvalerate | R | NR | NR |
| | Fipronil | R | R | R |
| | Flufenoxuron | NR | NR | R |
| | Flumethrin | NR | NR | R |
| | Imidacloprid | R | R | R |
| | Indoxacarb | NR | NR | R |
| | Isoxathion | R | NR | NR |
| | Lambda-cyhalothrin | R | R | R |
| | Lufenuron | R | NR | NR |
| | Magnesium Phosphide | R | NR | R |
| | Malathion | R | NR | R |
| | Methomyl | NR | NR | R |
| | Methoxyfenozide | NR | NR | R |
| | Nicosulfuron | NR | R | NR |
| | Novaluron | NR | NR | R |
| | Omethoate | R | NR | NR |
| | Oxydemeton-methyl | R | NR | NR |
| | Permethrin | NR | NR | R |
| | Piperonyl Butoxide (PBO) | NR | NR | R |
| | Pirimiphos | R | NR | R |
| | Pirimiphos-Methyl | NR | NR | R |
| | Profenofos | NR | NR | R |
| | Prosuler Oxamatrine | R | NR | NR |
| | Pymetrozine | R | NR | NR |
| | Pyridaphenthion | NR | R | NR |
| | Pyrimicarb | R | R | R |
| | Snake repellent | NR | NR | R |

| Class of pesticides | Active ingredients | Burundi | DRC | Rwanda |
|---|---|---|---|---|
| | Spiromesifen | NR | NR | R |
| | Tau-fluvalinat | NR | NR | R |
| | Teflubenzuron | NR | NR | R |
| | Tetradifon | NR | NR | R |
| | Tetramethrin | NR | R | NR |
| | Thiacloprid | NR | NR | R |
| | Thiamethoxam | NR | R | R |
| | Tralomethrin | R | NR | NR |
| | Triazophos | R | NR | NR |
| | Zeta-cypermethrin | R | NR | NR |
| **Fungicides** | Albesilate | NR | NR | R |
| | Azoxystrobin | R | NR | R |
| | Benalaxyl | NR | NR | R |
| | Benomyl | R | NR | R |
| | Bitertanol | NR | NR | R |
| | bromoxinyl-octanoate | NR | NR | R |
| | Bupimate | NR | NR | R |
| | Calcium Hypochlorite | NR | NR | R |
| | Captan | NR | NR | R |
| | Carbendazim | NR | NR | R |
| | Carboxin | NR | NR | R |
| | Chlorothalonil | R | NR | R |
| | Copper | NR | NR | R |
| | Copper ammonium acetate | NR | NR | R |
| | Copper hydroxide | R | R | R |
| | Copper oxychloride | R | R | R |
| | Cuprous Oxide | NR | NR | R |
| | Cymoxanil | NR | NR | R |
| | Cyproconazole | NR | NR | R |
| | Dichlofluanid | NR | NR | R |
| | Didecyldimethylammonium chloride | NR | NR | R |
| | Difenoconazole | NR | R | R |

| Class of pesticides | Active ingredients | Burundi | DRC | Rwanda |
|---|---|---|---|---|
| | Dimethomorphe | NR | NR | R |
| | Dithianon | NR | NR | R |
| | Dodemorph acetate | NR | NR | R |
| | Epoxiconazole | NR | NR | R |
| | Fenarimol | NR | NR | R |
| | Fenamidone | NR | NR | R |
| | Fenhexamid | NR | NR | R |
| | Flutriafol | NR | NR | R |
| | Fluzilazol | NR | NR | R |
| | Fosetyl - Aluminium | R | NR | R |
| | Hexaconazole | NR | R | R |
| | Imidachloriprid | NR | NR | R |
| | Iprobenfos | R | NR | R |
| | Iprodione | R | NR | R |
| | Iprovalicarb | NR | NR | R |
| | Isoxadifen-ethyl | NR | NR | R |
| | Kresoxim-methyl | NR | NR | R |
| | Mancozeb | R | R | R |
| | Maneb | R | NR | NR |
| | Mefenaxam | NR | NR | R |
| | Metalaxyl | R | R | R |
| | Metiram | NR | NR | R |
| | Micronised Sulphur | NR | NR | R |
| | Penconazole | NR | NR | R |
| | Pencycuron | NR | NR | R |
| | Prochloraz | R | NR | NR |
| | Propamocarb hydrochloride | NR | NR | R |
| | Propineb | NR | NR | R |
| | Pyrimethanil | NR | NR | R |
| | Spiroxamine | NR | NR | R |
| | Sulphur | NR | R | R |
| | Tebuconazole | R | NR | R |

| Class of pesticides | Active ingredients | Burundi | DRC | Rwanda |
|---|---|---|---|---|
| | Thiabendazole | NR | NR | R |
| | Thiophanate methyl | R | R | R |
| | Thiram | R | R | R |
| | Tolclofos Methyl | NR | NR | R |
| | Triadimefon | NR | R | NR |
| | Triadimenol | NR | R | NR |
| | Tricyclazole | NR | NR | R |
| | Trifloxystrobin | NR | NR | R |
| | Triforine | NR | NR | R |
| | Vinchlozoline | NR | NR | R |
| **Herbicides** | 2,4-D | R | NR | R |
| | 2,4-DB | R | NR | NR |
| | Ametryn | R | NR | NR |
| | Atrazine | R | R | NR |
| | Bispyribac sodium | NR | R | NR |
| | Clethodim | NR | NR | R |
| | Dalapon | R | NR | R |
| | Dimethamethryn | R | NR | NR |
| | Diuron | R | NR | R |
| | Fluazifop butyl | NR | R | NR |
| | Fluazifop-p-butyl | R | NR | NR |
| | Fluroxypyr | NR | R | NR |
| | Fluroxypyr ester-methylheptyl | NR | R | NR |
| | Glyphosate | R | R | R |
| | Hexazinone | R | NR | NR |
| | Isoproturon | R | NR | NR |
| | Linuron | NR | NR | R |
| | Methribuzin | NR | NR | R |
| | Metolachlor | R | NR | R |
| | Metribuzin | R | NR | NR |
| | MSMA | R | NR | NR |
| | Nicosulfuron | NR | R | NR |

| Class of pesticides | Active ingredients | Burundi | DRC | Rwanda |
|---|---|---|---|---|
| | Oxadiazon | NR | R | NR |
| | Oxyfluorfen | NR | NR | R |
| | Penoxsulam | NR | R | NR |
| | Promethrin | R | NR | NR |
| | Propanil | NR | NR | R |
| | Tembrotrione | NR | NR | R |
| | Terbutryn | NR | NR | R |
| | Thiobencarb | NR | NR | R |
| | Triclopyr | NR | R | NR |
| **Rodenticides** | Brodifacoum | R | NR | R |
| | Bromadiolone | R | R | R |
| | Chlorophacinone | R | NR | NR |
| | Coumatetralyl | R | NR | R |
| | Difenacoum | R | NR | R |
| | Diphacinone | R | NR | R |
| | Flocoumafen | R | NR | NR |
| **Nematicides** | Dazomet | R | NR | R |
| | Fenamiphos | NR | NR | R |
| | Oxamyl | NR | R | NR |
| | Terbufos | R | NR | NR |
| **Molluscicides** | Mercaptodimethur | NR | NR | R |
| | Metaldehyde | R | NR | R |
| **Growth regulators** | Serricornine | R | NR | NR |
| | Etherphon | NR | R | NR |
| | Daminozide | NR | NR | R |
| **Biopesticides** | Azadirachtin | NR | NR | R |
| | *Bacillus thuringiensis* | R | R | R |
| | *Beauveria bassiana* | NR | NR | R |
| | Pyrethrins | NR | NR | R |
| | Spinosad | NR | NR | R |
| | *Trichoderma harzianum* | NR | NR | R |
| | Emamectine benzoate | NR | R | NR |

# OBJECTIVES AND RESEARCH STRATEGIES

Chemical pesticides are widely used to manage plant diseases and pests, although some are known to have harmful effects on human health and the environment. The search for safe and sustainable alternatives to chemical pesticides has yielded promising results, including biological control, which plays a crucial role for sustaining crop production. Preliminary tests revealed that the bacterium *Bacillus nakamurai* BDI-IS1 isolated from Burundi exhibits promising biocontrol potential.

This study focused on biocontrol activity of BDI-IS1 against major diseases affecting crops in Burundi, and more specifically against Northern corn leaf blight (NLB). This thesis aims to (i) assess the distribution of NLB and farmers' knowledge of its management, (ii) evaluate the biocontrol efficacy of BDI-IS1 and its formulated products, and (iii) investigate the mechanisms underlying its biocontrol potential. Specifically, this thesis addresses the following questions:

1. What is the distribution of NLB, and what control measures are implemented by farmers in Burundi for its management?

2. Does BDI-IS1 effectively control NLB and tomato early blight, the major foliar diseases affecting maize and tomato, respectively?

3. What active metabolites are produced by BDI-IS1 that contribute to direct or indirect biocontrol activity against NLB?

4. Does BDI-IS1 induce systemic resistance in maize against the disease?

5. Which plant defense pathways in maize are activated by BDI-IS1 in response to infection by *Exserohilum turcicum*, the causal agent of NLB?

To address these questions, we first conducted a survey in farmers' fields to assess the severity of NLB and gather information on farmers'

knowledge and practices in its management. Secondly, the biocontrol efficacy of BDI-IS1 was evaluated through various trials, conducted both *in vitro* and *in planta* (in greenhouse and field conditions), assessing its antagonistic activity against several bacterial and fungal pathogens, including *E. turcicum* and *A. solani* strains isolated in Burundi. Additionally, we tested the production of BDI-IS1 on lignocellulosic substrates, which are widely available in Burundi. The resulting bioproduct was then tested under field conditions against NLB. Thirdly, *in vitro* and greenhouse bioassays combining the use of wild type and mutants of BDI-IS1, dynamic of gene expression and microscopy, were performed to investigate the role of secondary metabolites produced by BDI-IS1 in controlling NLB and their modes of actions.

# RESULTS

# CHAPTER V.

# DISTRIBUTION OF NORTHERN CORN LEAF BLIGHT AND ASSESSMENT OF FARMERS' KNOWLEDGE AND SKILLS REGARDING THE DISEASE IN BURUNDI

## 5.1. Abstract

Maize is affected by several diseases, including Northern corn leaf blight (NLB), which impacts the leaves and reduces production. The objective of this study was to determine the significance of NLB in Burundi. A structured questionnaire was developed to assess farmers' knowledge and skills in NLB management. Additionally, a field visit was conducted to evaluate the severity of NLB across all agro-ecological zones in Burundi. The severity of NLB was assessed by trained enumerators, and the disease scores were utilized to create a map of NLB distribution in the country. NLB was observed in all agro-ecological zones, with the highest severity reported in the lowlands, where temperature and humidity levels are elevated. Significant disease severity was also noted in newly introduced maize varieties, and an increase in NLB prevalence was reported in the visited areas. Furthermore, many farmers are unaware of NLB and do not implement any control measures, except for a few producers located near research centers who apply fungicides.

## 5.2. Introduction

Maize is an important global crop, with production estimated at 1,162 million tons in 2020. The main producing countries are the United States, China, Brazil, and Argentina, which together account for more than two-thirds of global production (Erenstein et al., 2022; McCormick, 2020). In Africa, maize production was approximately 75

million tons in 2018, representing 7.5% of global maize production. The leading maize-producing countries in Africa are Nigeria, which produces over 33 million tons annually, followed by South Africa, Egypt, and Ethiopia (Awata et al., 2019). In Burundi, maize is a vital staple crop, primarily used for human consumption and, to a lesser extent, for livestock. Between 2014 and 2022, maize production increased from 127,000 to 280,000 tons. However, this still does not meet the local demand, which is estimated at over 1 million tons per year (FAOST, accessed on 14 August 2024).

Maize production is constrained by several factors, including a lack of improved seeds, low soil fertility, and various diseases and pests that affect maize both in the field and during storage. Among the foliar diseases of maize, NLB is a prevalent disease impacting maize at various vegetative growth stages. It is caused by *Exserohilum turcicum*, with its teleomorph *Setosphaeria turcica*, a fungal species belonging to the phylum Ascomycota, subphylum Pezizomycotina, class Dothideomycetes, subclass Pleosporaceae, order Pleosporales, family Pleosporaceae. The teleomorphic phase is typically observed in laboratory and sometimes in tropical regions, while the anamorphic phase can be found worldwide under field conditions (Levy and Pataky, 1992).

This fungus can survive saprophytically as mycelia or conidia on maize residues and as chlamydospores in the soil and on other plant debris (Levy and Pataky, 1992). Under favorable conditions, conidia are dispersed over both short and long distances by wind or rain. The fungus propagules germinate on maize leaves or plant surfaces within 3 to 6 hours after inoculation. The fungus then penetrates directly into the epidermal cells or, less commonly, through stomata within 12 to 18 hours after inoculation. The disease begins as small, oval, grayish-green, water-soaked spots that develop into elongated, spindle-shaped necrotic lesions, leading to complete leaf blight. Lesions can reach up to 12 mm wide and 2.5 to 15 cm long, often parallel to the leaf margin (Singh, 2019).

The severity of NLB on maize increases when it occurs before or within 2 to 3 weeks after pollination, resulting in a grain yield reduction of 40 to 70%. Conversely, if NLB does not become severe until 6 to 8 weeks after pollination, yield is typically unaffected (Jakhar et al., 2017; Levy and Pataky, 1992). The incidence and severity of NLB depend on environmental conditions and can vary from year to year. High severity of NLB is often observed at moderate temperatures (20-28°C), high humidity (85-95%), and during prolonged wet periods. Additionally, the intensity of NLB is influenced by agricultural practices and the resistance level of the crop variety.

NLB can be controlled through prophylactic measures such as crop rotation, deep ploughing, field sanitation, and the use of resistant varieties, along with, to a lesser extent, chemical pesticides. These prophylactic measures are recognized as good agricultural practices that influence the growth and fitness of both the pathogen and the host plant. However, the effectiveness of these practices in controlling the disease is limited, although they can be combined with other control measures to enhance their performance.

Chemical fungicides, such as triazoles and strobilurins, are highly effective in controlling NLB (Carpane et al., 2020), yet they are not used in Burundi despite the high prevalence of NLB. Farmers are encouraged to utilize resistant varieties, which are regarded as a promising and rational strategy for managing crop diseases and pests in low-income countries like Burundi. Several resistance genes to *E. turcicum*, including Ht1, Ht2, Ht3, Htm1, Htn1, HtNN, ht4, and rt, have been identified in maize germplasm worldwide and successfully used in breeding programs. However, the widespread use of these genes has led to the selection in *E. turcicum* populations and the development of virulent effectors, thereby limiting the effectiveness of this control strategy (Jindal et al., 2019). These virulent strains of *E. turcicum* are randomly distributed globally. Accurately assessing the diversity of *E. turcicum* is a promising approach to designing sustainable control measures tailored to specific regions.

In Burundi, NLB is frequently observed. However, no surveys or studies have been conducted to elucidate its effects or identify the strains of the pathogen. Based on recent personal observations, NLB appeared even before flowering, damaging the lower leaves and progressively affecting the upper leaves of maize plants. In many cases, necrotic lesions varied with plant age and maize variety. Local cultivated maize varieties showed some tolerance, whereas newly introduced varieties appear to be more susceptible to NLB across all agro-ecological zones. To the best of our knowledge, the prevalence and susceptibility of cultivated maize varieties to NLB in Burundi remain not reported.

The objectives of this study are to (i) assess the severity of NLB in the main growing agro-ecological zones, (ii) map its geographical distribution in the study area, and (iii) evaluate farmers' knowledge and practices in managing NLB.

## 5.3. Materials and methods

### 5.3.1. Study area and sampling

A survey was conducted in 2022 during the main maize growing season (A) in Burundi. The survey targeted households with maize crops in two communes from each agro-ecological zone, including the lowlands (Rugomba and Buganda), the midlands (Mutaho and Mwakiro), and the highlands (Mugongomanga and Gisozi). The survey was carried out in collaboration with the research centers of Gisozi (highlands), Murongwe (midlands), Mparambo (lowlands), and Bukemba (eastern depression). In each community, 40 households were surveyed within approximately 1 km along the main road.

### 5.3.2. Questionnaire

To assess the distribution of NLB and farmers' knowledge and skills in managing the disease, a questionnaire was developed and transcribed into KoBocollect v2021.2.4 software installed on enumerators' phones.

The questionnaire consisted of two main sections: farmer-related questions and a field assessment of NLB severity conducted by trained enumerators.

The first section focused on household identification, where farmers were asked to locate their farms in terms of hill, zone, commune, and province. They were also asked to list the maize diseases and pests that occurred in their fields and to rank these diseases and pests according to the damage they caused to the crop. The symptoms of NLB and of the other diseases were described in detail to ensure that farmers recognized their prevalence and occurrence in the selected fields, based on the seasons and the location of the fields (hill or swamp). Additionally, farmers were asked for their opinions on the potential impact of NLB on maize production and food security. Finally, enumerators recorded the knowledge and practices used by farmers to control NLB. The questionnaire also included a specific section dedicated to the evaluation of NLB in the surveyed areas.

### 5.3.3. Field identification and assessment of Northern corn leaf blight

Field identification was conducted by recording the latitude, longitude, and altitude of each surveyed field using the Global Positioning System (GPS). The position of the field in relation to the hill (i.e., swamp, slope, and hilltop) was also documented. Additionally, the previous crop and the maize variety grown in each field were recorded.

The severity of NLB was assessed in each maize field by evaluating 20 plants along a diagonal line across the field. A scale of 1 to 5 was used for this assessment, where: **1** = very lightly infected (one or two limited lesions on lower leaves or traces); **2** = slight to moderate infection (a few scattered lesions on lower leaves); **3** = many lesions on lower leaves and a few on middle leaves; **4** = many lesions on lower and middle leaves, extending to upper leaves; **5** = many lesions on all leaves; the plant may die prematurely from blight. The individual scores for each

plant were then used to calculate the percent severity index (PSI) for each field using the following formula (Tajudin et al., 2018):

$$\text{PSI (\%)} = \frac{\text{sum of numerical rating}}{\text{Total number of plants observed X maximum rating}} \text{ X } 100.$$

### 5.3.4. Data analysis

SPSS 16.0 and Microsoft Excel softwares were used to analyze the intensity of NLB severity across sites and to determine the level of household knowledge regarding NLB management. A disease distribution map was generated using ArcGIS software, incorporating GPS points and calculated percent severity indices grouped into three classes: 0-29 (low severity), 30-49 (moderate severity), and 50-100 (high severity) (Nwanosike et al., 2015; Tajudin et al., 2018).

## 5.4. Results

### 5.4.1. Status of NLB in surveyed area

The severity of NLB was assessed in 273 fields located across 75 hills, 21 districts, and 8 provinces, including 11 in the province of Bubanza, 68 in Bujumbura, 24 in Bururi, 66 in Cibitoke, 40 in Gitega, 13 in Karusi, 71 in Mwaro, and 23 in Rutana. The surveyed sites represent the main agro-ecological zones of Burundi as well as areas surrounding the research stations. The disease severity recorded in each field was used to calculate the percent severity index (PSI), which is plotted on a map of Burundi's communes (Figure 5-1). NLB was observed in season A 2022 in all surveyed fields across the highlands, midlands, and lowlands (Figure 5-1), resulting in an incidence of 100%.




Figure 5-1: Symptoms of Northern corn leaf blight in Burundi.
*Symptoms observed at different locations: A, F, & G: Bukemba; B & C: Isare; D & E: Cibitoke, on maize plants of various ages: A: young plants, and B, C, D, E, F & G: post flowering stage. A newly introduced maize variety was completely damaged by NLB (G) at the Bukemba Research Station (photos taken by Gaspard Nihorimbere during field works).*

The PSI were moderate, ranging from 30% to 49% in several sites of the surveyed provinces, such as Karusi, Mwaro, and Bujumbura. In contrast, high disease severity, ranging from 50% to 100%, was recorded in lowland fields located in Cibitoke, Bubanza, Rutana, and Bukemba (Figure 5-2). The disease severity varied according to the age of the maize plants: fields sown in September were at the flowering stage during the survey (in March 2022) and exhibited a higher disease severity score of 3, while those sown in February were still young, with 3 to 4 leaves, and had an average disease severity score of 1.5 (Figure 5-3A). The mean disease severity exceeds the value of 2.5 regardless of the field location (800 m in low land at Cibitoke or 220 m in high land at Gisozi (swamp, hillside, or top of a hill) (Figure 5-3B).

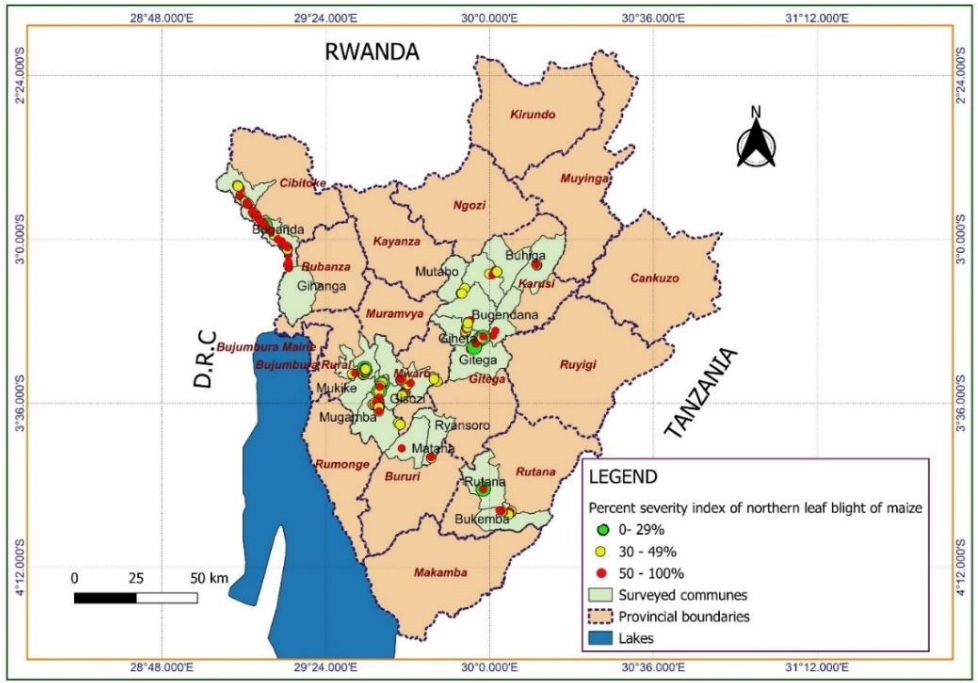


Figure 5-2: Prevalence and distribution of the Percent Severity Index (PSI) of Northern Corn Leaf Blight (NLB) in the surveyed Areas of Burundi.

*The severity of NLB was assessed in March 2022 (season B) in 273 fields and the percent severity index was calculated. The results are represented by circles color-coded as follows: green for low severity, yellow for moderate severity, and red for high severity.*

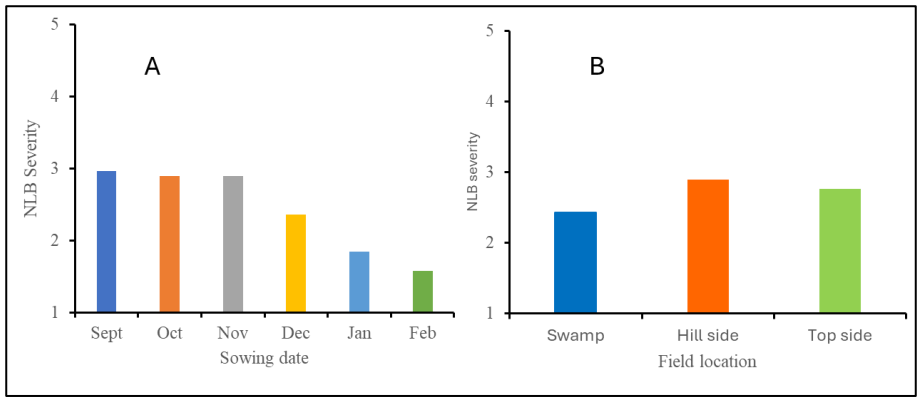


Figure 5-3: Variation in NLB severity according to sowing date (A) and field location in relation to hill position (B).
*The data presented are the means of disease severity assessed on 20 plants across 84 fields (out of 273) with known sowing dates. The fields were sown in September (12), October (45), November (15), December (7), January (3), and February (2). The surveyed fields were in swamps (17), on hillsides (61), and at the tops of hills (6). Disease severity was assessed using a scale from 1 to 5: 1 = very lightly infected (one or two limited lesions on lower leaves or traces); 2 = slight to moderate infection (a few scattered lesions on lower leaves); 3 = many lesions on lower leaves and a few on middle leaves; 4 = many lesions on lower and middle leaves, extending to upper leaves; 5 = many lesions on all leaves.*

### 5.4.2. Assessment of farmers' knowledge and skills in NLB management

Our findings revealed that less than 20% out of the 273 farmers surveyed recognized NLB affecting maize leaves (Figure 5-4A). From those recognizing NLB, only 30 to 35% reported experiencing it during season A (from September to January) and season B (from February to May) (Figure 5-4B). Notably, none of the surveyed farmers observed NLB during season C, the dry season.

Additionally, farmers reported that NLB affected every phenological growth stage of maize, including emergence, flowering, and post-flowering. However, over 80% of the surveyed farmers noted that NLB was most prevalent during the flowering stage, 67% reported its

occurrence during post-flowering and only 17% at emergence (Figure 5-4C).

The survey also revealed that the main diseases and pests commonly reported by farmers include NLB, fall armyworm, maize streak virus, rust, corn smut, and stem borer. More than 30% of farmers identified NLB as the most important maize disease, followed by fall armyworm and maize streak virus (Figure 5-4D). Over 98% of households recognized that NLB negatively affects maize production, while 100% acknowledged its detrimental impact on food security (Figure 5-4E). Despite NLB being a major disease in the surveyed areas, only 23% of farmers reported controlling it using chemical pesticides, and merely 2% employed alternative methods, such as local products or plant extracts (Figure 5-4F).

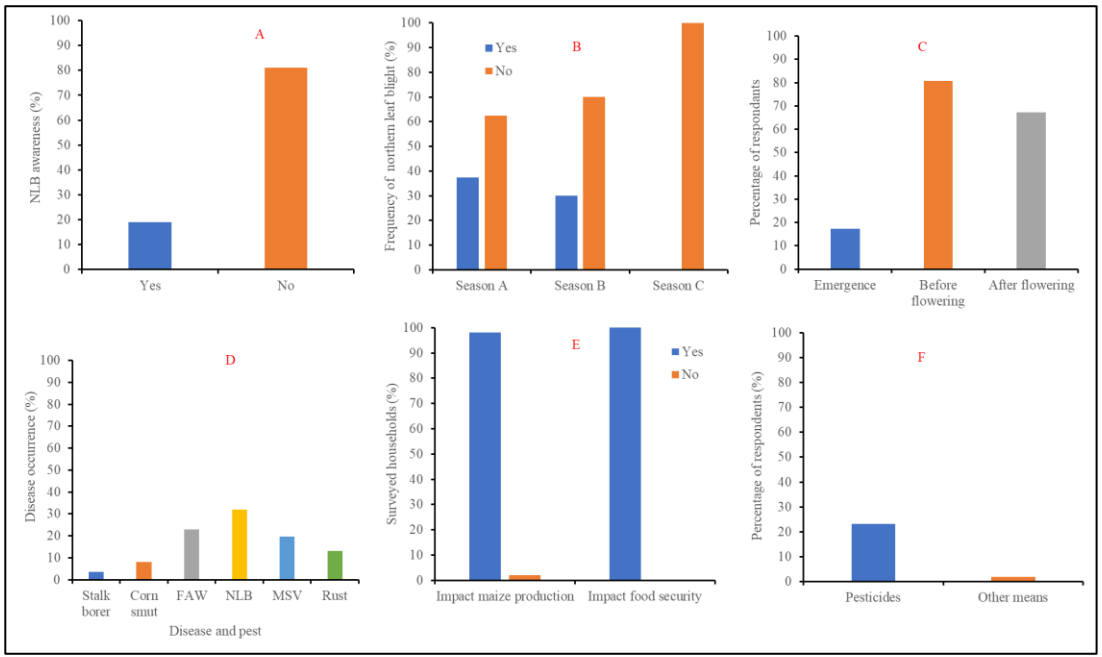


Figure 5-4: Percentage of farmers recognizing Northern corn leaf blight (NLB).
(*A), number of farmers experiencing NLB in their fields (B), percentage of farmers reporting NLB occurrences during emergence, flowering, or post-flowering (C), importance of various maize diseases (D), number of farmers acknowledging the negative impact of NLB on maize production and food security (E), and percentage of farmers controlling NLB using chemical pesticides or other methods (F). The data presented are sourced from 273 farmers from 8 provinces in Burundi (season A 2021 & B 2022).*

## 5.5. Discussion

NLB is a significant disease affecting maize across the surveyed regions of Burundi, including the lowlands, midlands, and highlands, with varying intensities. In the surveyed fields, the disease was most severe in the lowlands, where the weather is moderately warm and humid. These findings are consistent with those reported by Tajudin et al.(2018), who observed high incidence and percent severity indices of NLB in high-temperature zones (31-35 °C) and humid areas. Similarly, Nwanosike and Mabagala (2017) found that NLB was more severe in warm temperatures (31-35 °C) with high relative humidity (75-100%) in Tanzania. Given that Burundi is near Tanzania, the high temperatures and humidity levels are prevalent in the lowlands, including Cibitoke and Bubanza, which are the main maize-growing regions. In these areas, maize production is increasingly constrained by NLB, which could potentially constitute a real threat in the absence of control strategies.

Our analysis indicates that all surveyed fields were infected by NLB at relatively high intensity, as reported by the farmers. The negative impact of NLB on maize production is attributed to the necrotic lesions on maize leaves, which reduce the total photosynthetic area (De Rossi et al., 2022). The high prevalence of NLB may also threaten overall food security in Burundi, as maize is among the major staple crop. Numerous plant diseases and pests have been reported to affect crop production in Burundi, further limiting food security (Ochilo et al., 2022).

The high prevalence of plant diseases and pests in Burundi can likely be attributed to favorable temperatures and humidity that promote the development and spread of this disease (Jakhar et al., 2017; Singh et al., 2017). Additionally, the reliance on non-certified and traditional variety, rather than pathogen free seeds, contributes to the accumulation of pathogens and subsequent production losses. Plant diseases and pests, along with other abiotic stresses, have been reported to be

responsible of many recurrent episodes of food insecurity in Burundi, affecting more than 200,000 people (IPC, 2021b). The decline in maize production has also led to hunger and other social issues, including school dropouts and potential population displacement to neighboring countries (Chahed, 2019; USAID, 2020).

The significant impact of NLB is exacerbated by its ability to occur at all phenological stages of maize growth, from emergence to post-flowering. NLB was detected in all surveyed fields, including fields at the early stages of development (with an average severity score of 2 on a scale from 1 to 5 for plants aged 1 to 2 months), demonstrating that disease pressure is significant even at the young growth stage. Early infection by *E. turcicum* leads to increased inoculum production and restricts maize development, ultimately limiting overall yield. At the Bukemba Research Centre, some maize varieties such as TZBR and an introduced maize variety from CIMMYT, sown later in the season, exhibited high levels of infection by *E. turcicum*, with leaves becoming completely necrotic, leading to total production loss (personal observation, 2022). Consequently, these varieties were excluded from the seed system, despite their potentially beneficial traits.

Currently, NLB is particularly severe on newly introduced maize varieties, especially high-yield hybrids. These new maize lines may have been exposed to indigenous strains of *E. turcicum* in Burundi, to which they have become susceptible. Based on personal observations, complemented by interviews with researchers working on the maize program at ISABU, we observed a general trend of increasing NLB severity across all agro-ecological zones and on cultivated maize varieties, including those previously considered tolerant (data not presented).

Since *E. turcicum* is known to be a seed-borne pathogen, newly introduced maize varieties, from various origins (CIMMYT, IITA, neighbor countries) may be associated with new virulent strains that thrive under Burundi's environmental conditions, becoming virulent against cultivated maize varieties.

We found that farmers in Burundi were largely unfamiliar with NLB, despite recognizing its characteristic symptoms-cigar-shaped necrotic lesions that progress to full blight on maize leaves, after we illustrated them. Generally, farmers in Burundi have limited training and knowledge in diseases and pests diagnosis and management. As a result, pathogens often multiply unchecked in fields, leading to outbreaks. In some cases, extension services become aware too late, by which time the disease has already caused significant damage and become uncontrollable.

The high prevalence of NLB and other diseases in Burundi can be largely attributed to farmers' lack of knowledge in disease management. In the past, several outbreaks, such as banana *Xanthomonas* wilt (Ndayihanzamaso et al., 2016) and cassava mosaic virus (Bouwmeester et al., 2012), devastated staple crops like banana and cassava, leading to one of the most significant famines in Burundi's history. The failure to detect these plant diseases and pests in time allowed pathogens to proliferate and spread unchecked.

Our results indicated that only 23% of surveyed farmers use chemical pesticides to control NLB, while 2% use plant extracts and physical control measures. In other countries, NLB is controlled using several fungicides, including strobilurins and triazoles, which have demonstrated efficacy in managing NLB under field conditions (Carpane et al., 2020). However, these products are likely underutilized in Burundi due to their high cost and the uncertain return on investment for maize production.

## 5.6. Conclusion

NLB is a significant maize disease in Burundi. The disease is prevalent across all agro-ecological zones, with particularly high severity in the lowlands, where warm temperatures and high humidity prevail. Most farmers surveyed were unfamiliar with NLB, except for those located near research centers. In the surveyed areas, farmers implement few

control measures for NLB, leading to an increase in fungal inoculum and damage to maize production. The disease severely affects new maize varieties introduced from CIMMYT or neighboring countries and poses a major constraint to maize production and food security in Burundi. Some private seed producers use chemical fungicides to manage NLB, but these products are often unaffordable for low-income farmers. Further research is needed to explore the diversity of *E. turcicum* strains and the varietal susceptibility as well as to identify effective, accessible control measures.

# CHAPTER VI.

# UNRAVELLING THE SECONDARY METABOLOME AND BIOCONTROL POTENTIAL OF THE RECENTLY DESCRIBED SPECIES *BACILLUS NAKAMURAI*

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**Foreword**

This chapter was published in the *Microbiological Research* journal. It presents the molecular and metabolomic characterization of the bacterium *Bacillus nakamurai* BDI-IS1, as well as its biocontrol efficacy against tomato early blight and Northern corn leaf blight, the major foliar diseases affecting tomato and maize in Burundi, respectively. The samples used in this study were collected from farmers' fields in Burundi by Gaspard Nihorimbere (GN) and François Nimbeshaho (FN). FN isolated and characterized the bacterium, while GN isolated and identified the fungi used in this study. FN performed the molecular and metabolomic characterization of BDI-IS1, and GN assessed the biocontrol efficacy of this bacterium against *A. solani* and *E. turcicum* both *in vitro* and in the greenhouse. The conceptualization and protocols for characterizing the bacterium were developed by FN under the supervision of Marc Ongena (MO), while those related to biocontrol efficacy were established by GN under the supervision of Anne Legrève. Charlotte Lienard provided technical assistance for various greenhouse tests. Both FN and GN analyzed the data, drafted the manuscript, and are co-first authors. Marc Ongena and Anne Legrève provided guidance, reviewed the manuscript, and are co-last authors. Venant Nihorimbere, Anthony Argüelles Arias, and Anaclet Nibasumba contributed to the conceptualization and reviewed the manuscript. MO and VN secured funding for this work throughout its duration.

## 6.1. Abstract

In the prospect of use of natural products as safe and eco-friendly alternatives to chemical pesticides, the *Bacillus nakamurai* BDI-IS1 strain, derived from a Burundian soil sample, was selected among other bacterial isolates for its *in vitro* strong antimicrobial activities against a range of bacterial and fungal phytopathogens. Genome mining coupled with metabolomics revealed that it produces bioactive secondary metabolites including surfactins, iturins A, bacillaenes, bacillibactin,

bacilysin, plantazolicin, and potentially amylocyclicin, bacinapeptin and LCI. The antagonistic activity of some of these metabolites was demonstrated by testing mutant strains of BDI-IS1. Although this bacterium is not able to produce the large arsenal of secondary metabolites as the commercial strain *Bacillus velezensis* QST713, greenhouse experiments show that BDI-IS1 is able to protect tomato and maize plants against early blight and Northern corn leaf blight caused by *Alternaria solani* and *Exserohilum turcicum*, respectively, at levels similar to or better than QST713. The reduction of these foliar diseases, following root or leaf application of the bacterial suspension demonstrates that BDI-IS1 can act by direct antibiosis and by inducing plant defense mechanisms. These findings indicate that *B. nakamurai* BDI-IS1 can be considered as a good candidate for biocontrol of plant diseases prevailing in Burundi and in tropical regions, and encourage further research into its spectrum of activity, its requirements and the conditions needed to ensure its efficacy.

**Keywords:** *Bacillus nakamurai* BDI-IS1, antimicrobial potential, secondary metabolome, biocontrol, *Alternaria solani*, *Exserohilum turcicum*.

## 6.2. Introduction

The development of bacterial inoculants to control plant diseases is one of the most promising eco-friendly and efficient alternatives to the use of chemical pesticides in sustainable modern agriculture (Elnahal et al., 2022; Galli et al., 2024). This biocontrol strategy relies on the potential of selected beneficial species to reduce diseases caused by phytopathogens via two main often additive mechanisms which are direct inhibition of the infectious agent through antibiosis and stimulation of plant defenses leading to the so-called induced systemic resistance (ISR) phenomenon (Benaissa, 2023; Serrão et al., 2024). ISR is particularly interesting since beneficial bacteria inoculated to the roots enhances the defensive capacity of the entire plant against various

pathogens including those infecting the aerial parts (Salwan et al., 2023).

The biocontrol activity of these bacteria depends on the potential to produce bioactive secondary metabolites (BSMs) acting as antimicrobials and/or elicitors of host immunity (Abd-Elsalam and Mohamed, 2024; Lourenzi et al., 2022). However, other BSMs retain key ecological functions because they are involved in competition for nutrients (iron-chelating siderophores) or in root colonization (biofilm formation, motility) (Deb and Tatung, 2024; Liu et al., 2024). Several bacterial genera including *Pseudomonas, Streptomyces, Acetobacter, Azospirillum, Paenibacillus, Serratia, Burkholderia, Rhizobium and Bacillus* have been reported for their biocontrol activity (Backer et al., 2018) and in most cases these species are naturally living in close association with plants and were isolated from the rhizosphere. The potential of *Bacillus* to control plant diseases has been amply demonstrated from lab to field, especially species of the *Bacillus subtilis* group thanks to their ability of forming resistant endospores, colonizing quite efficiently the rhizosphere, secreting an arsenal of BSMs, and establishing a mutualistic relationship with plants (Dunlap et al., 2019; Miljaković et al., 2020; S.-Y. Wang et al., 2022). Bacilli dedicate up to 10-13% of their genome to the biosynthesis of BSMs (Borriss, 2020; Rabbee and Baek, 2020) including volatile organic compounds (VOCs) mediating chemical signalling and antifungal activities (Kai, 2020) but also a wide range of soluble metabolites of different types such as non-ribosomal (NR) cyclic lipopeptides, polyketides (PKs), oligopeptides, ribosomally produced and post-translationally modified peptides (RiPPs) encompassing bacteriocins and lantibiotics, as well as various lytic enzymes (Iqbal et al., 2023). From an ecological perspective, evolving such diversity of BSMs can also be considered as an adaptive trait to improve rhizosphere fitness (Anckaert et al., 2021; Etesami et al., 2023b). Additionally, the importance of low molecular weight organic compounds in communication among microorganisms and between microorganisms and plants is increasingly appreciated, extending their ecological roles

beyond microbial warfare, defense, and development (Mithöfer and Boland, 2016; Vaishnavi and Osborne, 2021; Xu et al., 2023).

Furthermore, as for other microbial species, the success of these *Bacillus*-based products as soil bioinoculants suffers from variable efficacy and inconsistency in real-world agricultural conditions (Marian and Shimizu, 2019). This is due to the high complexity of the ecological context and many factors may restrict proper invasion of the soil or rhizosphere niche by the introduced strain and hamper therefore its establishment and persistence within the crop-associated microbiome, at threshold populations necessary to provide the beneficial effects (Hossain et al., 2023; O'Callaghan et al., 2022). Successful invasion depends on the ability of bioinoculants to cope with specific abiotic soil factors including pH, temperature, water content, nutrient status but it is also driven by intricate interactions with the host plant and with the associated microbiome communities whose structure, functions and compositions are also dictated by the plant genotype and inherent soil physico-chemical conditions (Bonaterra et al., 2022; Hu et al., 2021; Santos and Olivares, 2021). This supports the importance of keeping mining soils to discover new bacterial isolates with biocontrol potential and well adapted to the specific agro-ecological conditions in which they will be used (Novello et al., 2023; Thakur et al., 2022).

In this work, we describe a new strain BDI-IS1, isolated in the highlands of tropical Africa (Burundi), belonging to the *Bacillus nakamurai* species and displaying a broad-spectrum antagonistic activity against bacterial and fungal phytopathogens. We combined genomics and metabolomics to provide a first characterization of the whole range of specialized metabolites formed by this understudied species. We identified some compounds that play a key role in the antimicrobial activity observed *in vitro*, but our data also suggest that this secondary metabolome may also contribute to the strong biocontrol potential of BDI-IS1 against diseases caused by *Alternaria solani* and

*Exserohilum turcicum*, responsible for tomato early blight and Northern corn leaf blight, respectively.

## 6.3. Materials and Methods

### 6.3.1. Sample collection, isolation of beneficial bacteria and phytopathogenic fungi from Burundi

Arable soil samples containing bean and potato roots were collected in Murwi (North-west of Burundi) and Isare (West of Burundi) communes, respectively, for isolation of beneficial bacteria. Samples of tomato, bean, and maize leaves showing typical necrotic lesions of early blight, angular leaf spot and Northern leaf blight, respectively, were collected in Mwakiro (North-East of Burundi), placed in paper bags, and taken to the laboratory for isolation of fungal pathogens.

For bacterial isolation, small roots were then selected from soil samples and mixed with peptone water + Tween 80 (2%) and glass beads in a falcon (15 mL). The mixture was vortexed (5 min), serially diluted and plated (100 µL) on LBA (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L and agar 15 g/L). After 16 h of incubation (30˚C), *Bacillus*-like colonies were selected based on their morphological traits and subcultured (three times) on LBA for purification. Pure isolates were cultured in LB broth at 30°C overnight and stored at -80°C in peptone water + 20% glycerol until use.

For isolation of pathogenic fungi, diseased leaf samples were cut into small pieces (<1 $cm^2$), superficially disinfected with sodium hypochlorite (1 %) for 30 seconds, rinsed three times in sterile water and placed in Petri dishes containing water agar (10 g/L) with chloramphenicol (0.2 g/L) as antibiotic. After 24 h incubation at 22˚C, pieces of freshly formed mycelia were taken from the edge of the colonies and transferred to potato dextrose agar (PDA) to obtain pure cultures of *A. solani*, *E. turcicum* and *Ascochyta rabiei*, which were then stored in a freezer at 4˚C for further use. Identification of the fungal species was based on morphology (microscopic observation of fungal

structures produced by *E. turcicum*, *A. solani* and *A. rabiei* cultured on PDA and V8, respectively) and molecular characterization of the nuclear ribosomal internal transcribed spacer (ITS) region. For this purpose, fungal mycelia grown on PDA were gently scraped with a sterile blade and used to extract DNA using the cetyltrimethylammonium bromide (CTAB) protocol as described by Lee and Taylor (1990). The purity and integrity of the DNA was checked by electrophoresis on 0.8 % agarose gel for 45 minutes at 130 volts. ITS4 (TCC-TCC-GCT-TAT-TGA-TAT-GC) and ITS5 (GGA-AGT-AAA-AGT-CGT-AAC-AAG-G) primers were used to amplify the ITS region for the isolated fungi as described by White et al.(1990). PCR products were run on 1.2 % agarose in 1X TAE buffer for 45 minutes at 130 volts and sequenced by Sanger method at Microsynth Seqlab GmbH (Germany). Both sequences of each isolated pathogen were analyzed using Bioedit software and multiple alignment was performed using the Muscle algorithm based on MEGA X software. The consensus sequences were then blasted against the sequences available at NCBI platform.

### 6.3.2. Direct antagonism assays

#### 6.3.2.1. Biological materials

Six plant pathogenic bacteria i.e. *Clavibacter michiganensis* subsp. *michiganensis*, *Rhodococcus fascians* D188, *Pectobacterium carotovorum* LMG6663, *Xanthomonas campestris* subsp. *Campestris* LMG582, *Pseudomonas cichorii* LMG2162, *Pseudomonas fuscovaginae LMG2158* and six phytopathogenic fungi i.e. *Fusarium oxysporum* MUCL31742, *Botrytis cinerea* R16, *Rhizoctonia solani* MUCL49235, *Aspergillus niger* MUCL 28698, *Pyricularia oryzae* MUCL9191 and *Colletotrichum* sp. available in the collection of the Microbial Processes and Interactions (MiPI) Laboratory, Gembloux Agro-Bio Tech, University of Liège, were used in the antagonism assays with the bacterial isolates from Burundi. In addition, fungi isolated from diseased leaves collected in Burundi, i.e. *A. solani*, *E.*

*turcicum* and *A. rabiei* were tested. *Bacillus velezensis* QST713 (ingredient of Serenade®), also from the MiPI collection, was used as a reference standard antagonist bacterium.

#### 6.3.2.2. Culture preparation

*Bacillus*-like isolates and plant pathogenic bacteria initially stored at -80˚C were plated on LBA and incubated at 30˚C for 24 h. Individual colonies were then pre-cultured the day before the experiment. On the day of the experiment, all bacterial cultures were centrifuged, and the pellet was washed twice with LB broth, resuspended in the same medium, and adjusted to $OD_{600}$ 0.1 and $OD_{600}$ 2 per mL for pathogenic and isolated bacteria, respectively. *B. velezensis* QST713 (hereafter referred to as QST713) was prepared under the same conditions. Five days' old fungal mycelia (cultured on PDA at 25°C) were used in dual confrontation assay with the most promising isolate based on antibacterial activity.

#### 6.3.2.3. Antagonistic activity assessment

A dual culture assay was performed to evaluate the antibacterial and antifungal activity of the bacterial isolates. Following the historical background of the sampling site where potatoes were previously grown, solidified Solanaceae root exudates mimicked medium (REM) (glucose 1 g/L, fructose 1.7 g/L, maltose 0.2 g/L, ribose 0.3 g/L, citrate 2 g/L, oxalate 2 g/L, succinate 1.5 g/L, malate 0.5 g/L, fumarate 0.5 g/L, casamino acids 0.5 g/L, $(NH_4)_2SO_4$ 1 g/L, $KH_2PO_4$ 0.3425 g/L, MOPS 11.5 g/L, $MgSO_4 \times 7H_2O$ 0.25 g/L, KCl 0.25 g/L, yeast extract 0.5 g/L, 50 µL of $Fe_2(SO_4)_3$ 1.2%, 50 µL of $MnSO_4$ 0.4%, 50 µL of $CuSO_4$ 1.6%, 50 µL of $Na_2MoO_4$ 4%, Agar 15 g/L) (Nihorimbere et al., 2012) was selected for antibacterial activity assessment. Suspension of pathogenic bacteria ($OD_{600nm}$ 0.1) was spread homogeneously on REM agar medium in square plates (12 cm×12 cm) and were incubated at room temperature for approximately one hour to dry. Bacterial isolates and QST713 suspensions ($OD_{600nm}$ 2) were spotted (5 µL) on the plate

coated with the pathogenic bacteria. Incubation was performed at 30˚C for 48 h. Each bacterial suspension was tested in triplicate against each plant pathogen, with two independent experiments (n = 6). Activity was expressed as mean values (± δƐ) of the inhibition diameter (mm) around the bacterial spot. The antibacterial potential of the cell-free REM culture supernatant was tested in a 96-well microtitre plate (200 µL per well including a mixture of LB culture of phytopathogenic bacteria adjusted at $OD_{600nm}$ 0.1 and 15% of filtered (0.22 µm pore size) cell-free REM culture supernatants of the tested bacteria grown for 48 h at 30°C, 160 rpm) and incubated at 30°C, 250 rpm in microplate incubator. Optical density readings (at 600 nm) were performed on a microplate reader (TECAN, SPARK) and the experiment was performed two times independently with three replicates per experiment (n = 6). For monitoring the kinetic growth of pathogens treated with BDI-IS1 cell-free extracts, incubation of microplates was performed however inside the TECAN machine with continuing shaking (30°C) and readings (at 600 nm) recorded automatically over time (each 30 min). The antibacterial activity of various constructed mutants (see section 6.3.5) of the most prominent isolate was evaluated using the dual culture assay protocol as described above.

For antifungal activity evaluation, a mycelial plug (5 mm of diameter) of each fungus grown on PDA was placed at the centre of a plate on PDA medium and 5 µL of the best bacterial isolate (based on antibacterial potential) suspension ($OD_{600}$ 2) was spotted 25 mm away from the centre. The inhibition radius was recorded when control growth reached a radius of 25 mm and the percentage of inhibition was calculated using the following formula: Inhibition (%) = ((A-B)/A) X 100), where A and B are the growth radius of the control and treated fungi, respectively. Each confrontation was tested in triplicate and two independent experiments were performed (n=6). The antifungal activity of different constructed mutants of BDI-IS1 (see section 6.3.5) was assessed following the same protocol as detailed above.

### 6.3.3. Bacterial isolates molecular characterization

Genomic DNA from bacterial isolate was extracted using the Thermo Fischer Scientific Gram-positive bacteria genomic DNA Extraction Kit according to the manufacturer's standard procedure and was quantified using a Nanodrop ND-1000 spectrophotometer. The 16S rRNA gene was amplified by PCR using the primers 8F (5' - AGAGTTTGATCHTGGCTCAG - 3') and 1492R (5' - GGHTACCTTGTTACGACTT - 3'), Q5® High-Fidelity DNA Polymerase (New England BioLabs). After electrophoresis (1% agarose gel), the amplicons were further purified using the Thermo Fischer Scientific GeneJET Genomic DNA Purification Kit according to the manufacturer's standard procedure. The 16S rRNA amplified fragments were sequenced by Eurofins Genomics (Anzinger, Germany) and were blasted for homology searching to the NCBI Genebank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Whole genome sequencing (wgs) was also performed for the most promising isolate. Libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina), and the sequencing was performed on the Illumina MiSeq v3 platform using 600 bp paired-end chemistries. Illumina read adapters and low-quality bases were trimmed with Trimmomatic0.39. Draft genomes were assembled using SPAdes v3.13.0.

Comparative genome mining for important biosynthetic gene clusters (BGCs) was carried out using the web tools AntiSMASH 7.0.1 (Blin et al., 2023) and BAGEL4 (van Heel et al., 2018) in the available public genome sequences of strains belonging to the same species as the one sequenced in this study.

### 6.3.4. Bioactive secondary metabolites identification of the isolate BDI-IS1

Metabolome characterization of the BDI-IS1 cell-free supernatant was performed by LC-ESI-qTOF-MS (Agilent 1290 Infinity II coupled with

mass detector (Jet Stream ESI-Q-TOF 6530)) in positive mode with the following source parameters: capillary voltage of 3.5 kV, nebulizer pressure of 35 psi , drying gas of 8 $L.min^{-1}$ , gas temperature of 300°C, sheath gas flow rate of 11 $L.min^{-1}$, sheath gas temperature of 350°C, fragmentor voltage of 175 V, skimmer voltage of 65 V, and octopole radiofrequency of 750 V. Accurate mass spectra were recorded in the m/z range of 100 to 1,700 (acquisition rate 2 spectra/s). For an optimal separation, a C18 Acquity UPLC BEH column (2.1 mm; 50 mm; 1.7 µm; Waters) was used at a flow rate of 0.6 mL $min^{-1}$ and a temperature of 40°C (injection volume: 10 µL). A gradient of acidified water (0.1 % formic acid) (solvent A) and acidified acetonitrile (0.1% formic acid) (solvent B) was chosen as the mobile phase, starting at 10 % B, and rising to 100 % B in 20 min. Solvent B was kept at 100 % for 4 min before returning to the initial ratio. Where possible, metabolites were identified based on retention time, by comparison with *in-hous*e database constructed with *B. velezensis* mutants impaired in the production of the different metabolites (Andrić et al., 2023), and accurate mass. In addition, all structures were confirmed by MS/MS fragmentation.

### 6.3.5. BDI-IS1 mutants' construction

Knockout mutant strains of the promising isolate BDI-IS1 depleted in the biosynthesis of each of the bioactive secondary metabolites and in all non-ribosomally produced peptides (sfp mutant) were constructed by gene replacement and homologous recombination. The primers used for this purpose are listed in the supplementary material (Table 6-A1). A cassette containing a chloramphenicol resistance gene flanked by about 1kb of the upstream and downstream regions of each target gene was constructed by three fragments joining PCR. BDI-IS1 transformation was carried out according to the protocol of Hoff et al. (2021) with minor modifications. Shortly, one colony of BDI-IS1 was grown in LB medium (37°C, 150 rpm) for 4h, and washed twice with MMG liquid medium (19 $g.L^{-1}$ $K_2HPO_4$ anhydrous, 6 $g.L^{-1}$ $KH_2PO_4$, 1 $g.L^{-1}$ $Na_3C_6H_5O_7$ anhydrous, 0.2 $g.L^{-1}$ $MgSO_4$ $7H_2O$, 2 $g.L^{-1}$ $Na_2SO_4$,

50 mM $FeCl_3$, 2 mM $MnSO_4$, 8 $g.L^{-1}$ glucose, and 2 $g.L^{-1}$ L-glutamic acid; pH 7.0) and the $OD_{600}$ was adjusted to 0.01. The recombinant cassette (0.5 – 1 µg) was added to 1 ml of the latter prepared suspension and incubated at 37°C for 24h. Transformed colonies that have integrated the recombinant cassette, were further selected on LBA medium supplemented with chloramphenicol (5 µg/mL).

### 6.3.6. Biocontrol assays against early blight and Northern leaf blight

The biocontrol potential of the best antimicrobial bacterial isolate BDI-IS1 against two important plant diseases prevalent in Burundi, tomato early blight and Northern corn leaf blight, was assessed under greenhouse conditions. The bacterium *B. velezensis* QST713 was tested under the same conditions for comparison purposes.

#### 6.3.6.1. Plant material and culture conditions

The varieties Ruganda for tomato and ZM605 for maize were selected for their relative susceptibility to early blight of tomato and Northern leaf blight of maize, respectively. Maize and tomato seeds were disinfected in sodium hypochlorite (0.05 %), washed 3 times in distilled water, pregerminated and then sown in pots (2 L) containing potting soil, then placed in greenhouse at a temperature of 22°C ± 2°C and 18 °C ± 2°C day and night respectively, a relative humidity of 80 % ± 5 % and a photoperiod of 16 h.

#### 6.3.6.2. Treatments and experimental design

The activity of the two tested bacteria (BDI-IS1 and QST713), after leaf or root application, against tomato early blight and Northern corn leaf blight was evaluated in separate bioassays (see point 6.3.6.3) on tomato or maize, including twelve treatments : $T_1$= plant + BDI-IS1 leaf + fungus, $T_2$= plant + BDI-IS1 root + fungus, $T_3$= plant + QST713 leaf + fungus, $T_4$= plant + QST713 root + fungus, $T_5$= plant + BDI-IS1 leaf + water tween , $T_6$= plant + BDI-IS1 root + water tween , $T_7$= plant +

QST713 leaf + water tween, $T_8$= plant + QST713 root + water tween , $T_9$= plant + $MgCl_2$ buffer on leaf + water tween , $T_{10}$= plant + $MgCl_2$ buffer on root + water tween , $T_{11}$= plant + $MgCl_2$ buffer on leaf + fungus ,$T_{12}$= plant + $MgCl_2$ buffer on leaf. The treatments were tested in a completely randomized block design (CRBD) in two independent experiments, with 16 (4 replicates of 4 plants: N=16) and 12 (3 replicates of 4 plants: N=12) plants per treatment in trial one and two, respectively.

#### 6.3.6.3. Culture preparation, biocontrol agents' application and pathogen inoculation

*A. solani* was cultured on V8 media (V8 20 % (v/v), $CaCO_3$ 1 g/L, Agar 15 g/L) under neon lamp light, alternating light, and darkness (12 h/12 h) for 10 days. *E. turcicum* was cultured on PDA (potato dextrose agar 39 g/L, agar 2 g/L) and incubated at 27°C for 21 days. To collect the conidia, 3 ml of sterilized water and Tween 20 (0.05%) were added to Petri dishes and the mycelia and conidia were repeatedly scraped with a sterilized blade and collected in Falcon tube. Conidia were obtained by filtration through a double layer of cheesecloth and the concentration of the conidial suspension was counted using Fuchs Rosenthal cell and light microscopy. Adjusted concentrations of $2 \times 10^4$ conidia/ml for *A. solani* and $4.5 \times 10^4$ conidia/ml for *E. turcicum* were used for plant inoculation. The bacterial isolate BDI-IS1 and QST713 were revived from cryotubes (-80°C) on LBA, 16 h at 30°C. An individual bacterial colony was used to carry out a pre-culture incubated for 24 h at 30°C, 120 rpm, then used to perform a culture (initial $OD_{600}$ = 0.001, 100 mL in 250 mL flasks) for 24 h, at 30°C, 120 rpm. The cultures were centrifuged, the pellets washed twice with $MgCl_2$ (2.5 g/L) and resuspended in 10 L of $MgCl_2$ (equivalent to $10^6$ CFU/mL) before application on plants.

Two application methods were tested, i.e. on roots (root treatment) or on leaves (leaf treatment). For the root treatment, 100 mL of bacterial suspension was poured onto the plant substrate (about 2 L potting

soil/plant), twice, at seven days and one day before pathogen (or control) inoculation, i.e. on 24 and 28 days-old tomato plantlets and 11 and 16 days-old maize plantlets, respectively, for trial one and two. For foliar treatment, the bacterial suspension was sprayed on all leaves of treated plants until run-off, the day before inoculation. The control plants were treated with $MgCl_2$ solution only.

The fungal inocula of *A. solani* or *E. turcicum*, as prepared above, were sprayed until run off on the leaf surface of plants of 25 and 29 days-old tomato plantlets and 12 and 17 days-old maize plantlets, respectively for trial one and two.

### 6.3.7. Data collection and analysis

Leaf necrotic area (LNA) was used to assess the severity of early blight and Northern corn leaf blight on three (low, middle and top) and two leaves (middle and top) of each plant for tomato and maize, respectively. The LNA of tomato early blight was estimated daily using a scale from 1 to 9, where 1 = 0-5 % of leaf area affected by the disease, 3 = 5-10 %, 5 = 10-20 %, 7 = 20-50 % and 9 = 50-100 %. Northern leaf blight severity was scored at two-day intervals on a scale from 1 to 9 (Figure 6-A1), where 1 = 0 % of leaf area affected by the disease, 2 = 10 %, 3 =20 %, 4 = 30 %, 5 = 40 %, 6 = 50 %, 7 = 60 %, 8 = 70 %, 9 = 80-100 %. The disease severity (DS) for NLB was obtained from the mean of the disease severity of the related scores recorded on two leaves, while DS for TEB represent the mean of disease scores recorded on three leaves obtained using the following formula as described by Willocquet et al. (2004):

$DS = \sum_{i=1}^{k}(SR_i \times NP_i)/TNP \times HS) \times 100$, where *i* is the disease score, *k* the number of classes in disease scale (k = 5), while $SR_i$ and $NP_i$ represent the score rating and number of plants with *i* score, respectively. TNP and HS stand for the total number of assessed plants and high score (HS = 9), respectively.

The area under disease progress curve (AUDPC) for tomato early blight and Northern corn leaf blight was calculated using the following formula:

$\text{AUDPC} = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1)}}{2}\right) X(t_{i+1} - t_i)$, Where $y_i$: disease severity of $i^{th}$ day,$y_{i+1}$: disease severity of $i^{th+1}$ day, $t_i$:$i^{th}$ day, $t_{i+1}$: $i^{th+1}$ day and n = total number of observations.

The Protection Index (PI) conferred by a treatment was calculated as described by Caulier et al. (2018):

$$\text{PI} = \left[\left(1 - \frac{\text{AUDPC treatment}}{\text{AUDPC control}}\right) * 100\right]$$

The data on disease severity collected in the greenhouse for tomato early blight and Northern corn leaf blight were analyzed using Generalized Linear Mixed Model (GLMM) with R 4.0.5 software, where the bacteria, treatment method and trial were fixed factors, and block was a random factor. Degrees of freedom were calculated using the Kenward-Roger method, while the Estimated Marginal Means (emmeans) package was used to compare the different means. The differences between PI were assessed using t-test within R 4.0.5.

For the *in vitro* antagonist assays, data analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test ($p$ value = 0.05). All the graphs were constructed using Prism GraphPad 9.4.1 software.

## 6.4. Results

### 6.4.1. *B. nakamurai* strain BDI-IS1 as strong antagonist of plant pathogens

This work was initiated by screening bacteria in rhizosphere soil samples collected from crop fields at two different locations in Burundi. Our objective was to isolate endemic bacilli and we selected a subset of sixteen isolates characterized by a rapid growth (≤ 20h) and typical white *Bacillus*-like colonies on solid LB medium. These isolates were first tested for their antagonistic potential against a range of phytopathogenic bacteria including species known as important plant pathogens with high economic impact notably in tropical and subtropical regions (Ndayihanzamaso et al., 2016; Nihorimbere et al., 2024). Antibacterial activity was chosen as prime criterion for screening because this trait is not only critical to the overall efficacy of broad-spectrum biocontrol agents (Wang et al., 2022) but also important for the ecological fitness and persistence in the competitive rhizosphere niche (Afridi et al., 2022; Andrić et al., 2023; Weiland-Bräuer, 2021). Moreover, the outcome of bacterial interactions in general and of antagonistic competition in particular may vary according to various conditions including the medium (Garrido-Sanz et al., 2023; Sun et al., 2022). It is why, instead of common laboratory media, we performed these confrontation assays on an agar-solidified Root Exudate-Mimicking medium (REM) designed to reflect the nutritional context of the rhizosphere in Solanaceae plants (Hoff et al., 2021).

Based on the size of the inhibition zone developed around the colony (Figure 6-1A&B), the isolate BDI-IS1 displayed the highest and most consistent antibacterial activity against all the tested bacterial pathogens and at a level similar to the one observed for the commercial strain *B. velezensis* QST713, a well-known biocontrol agent in agriculture (EFSA, 2021). A very high inhibition was observed against *C. michiganensis*, *R. fascians* and *X. campestris* for both BDI-IS1 and

QST713, while *Pectobacterium carotovorum*, *Pseudomonas cichorii* and *Pseudomonas fuscovaginae* were moderately inhibited (Figure 6-1A). We next used microplate-based assays to test the inhibitory effect of cell-free extracts obtained from BDI-IS1 liquid cultures in REM. Growth of *C. michiganensis* and *R. fascians* cells were fully repressed upon addition of BDI-IS1 extract (15 % v/v); while *X. campestris*, *P. carotovorum* and *P. cichorii* growth inhibitions were partial, ranging from 90% to 40% depending on the pathogen (Figure 6-1C & 1D). This correlates well with results obtained in plate confrontation assays and evidences the crucial role of secreted soluble BSMs in mediating antibacterial activities. The isolate BDI-IS1 was also evaluated for its antifungal activity against a range of phytopathogenic fungi reported as threats to agriculture in tropical regions including species that we isolated from diseased plants in Burundi (Figure 6-1E). Data revealed the broad-spectrum activity of BDI-IS1, at levels comparable to QST713, with very high antifungal potential against *P. oryzae* and *Colletotrichum* sp., good inhibitory potential against *Ascochyta rabiei*, *Alternaria solani* and *E. turcicum*, and moderate inhibition towards *Aspergillus niger*, *B. cinerea* and *F. oxysporum* (Figure 6-1E & F).

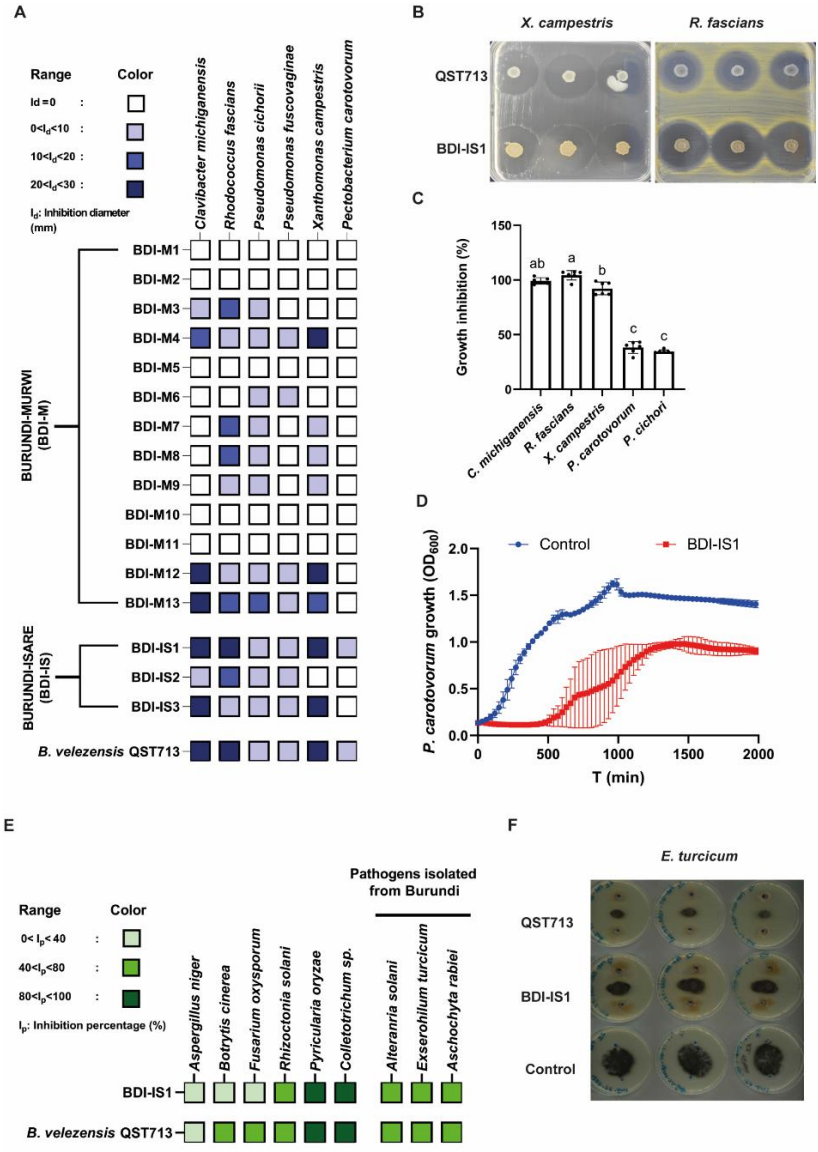


Figure 6-1: The Burundi isolated strain BDI-IS1 retains a great antagonistic potential against phytopathogens.

*A. Antibacterial activity of bacterial isolates (BDI-M1 to BDI-M13 and BDI-IS1 to BDI-IS3) against a set of bacterial phytopathogens in comparison to* Bacillus velezensis *QST 713 (Serenade®). This was assessed through a dual culture assay carried out on gelified REM (30 mL, 30°C, 48 h) and the inhibition potential is illustrated in blue boxes with a gradual increase of color font in line with the increasing range of inhibition diameter (Id); data are means of inhibition diameter (±*

*SE, mm) and evolve from two independent experiments conducted each in triplicate (n=6) (Table 6-A2). B. Illustration of inhibition growth of* X. campestris *and* R. fascians *exhibited by the isolate BDI-IS1 and QST713. C. Growth reduction of bacterial phytopathogens by cell-free REM culture supernatants (CFS) of BDI-IS1; overall growth reduction (%) after 48 h of bacterial (pathogenic) culture supplemented with 15 % (V/V) of CFS. Bar graphs (with error bars) represent mean (± SD) values calculated from three repetitions with two independent assays (n=6), letters a to c indicate significant statistical differences of means following the one-way analysis of variance (ANOVA) and Tukey's HSD test (Honestly significant different, α =0.05). D. Growth curve of* P. carotovorum *cultured with or without 15 % (v/v) BDI-IS1 CFS (red and blue curves, respectively); data points are means (± SD) values calculated from three repetitions with two independent assays (n=6). E. Antifungal activity of the isolate BDI-IS1 and QST713 against fungal phytopathogens. Dual confrontation assay was used and inhibition percentages (partitioned into different ranges) are represented in green boxes with color intensity increasing with inhibition percentage range, data are means (± SE) of triplicates repeated independently twice (n=6) (Table 6-A3). F. Illustration of antagonistic activity of BDI-IS1 and QST713 against* E. turcicum *isolated from diseased maize leaves from Burundi, in a dual culture assay set-up.*

Genomic DNA was further extracted and the 16S rRNA gene was sequenced (NCBI accession number OP546292) for identification purpose. The sequence alignment with NCBI database showed the highest homology to *Bacillus nakamurai* NRRL B-41091 with 99.7% of identity. This was supported by the black-pigment production by the strain when cultured on tryptic soy agar (TSA) (Figure 6-A2), a phenotypic feature typical of newly described species "*Bacillus nakamurai*" (Dunlap et al., 2016). The black, melanin-like pigment observed with *B. nakamurai* strains is also reported in other members of *Bacillus subtilis* group including among others *B. atrophaeus* (Nakamura, 1989), and *B. subtilis* 4NP-BL (Ghadge et al., 2020). This melanic pigment is associated to environmental stresses resistance such as UV rays oxidative stress (Ghadge et al., 2020; Saxena et al., 2002) and was found to have antibacterial activity against some plant pathogens including *X. campestris* (Ghadge et al., 2020). *B. nakamurai* is a less-studied species that has been first described in 2016 and only five strains including BDI-IS1, were sequenced up to date: *B. nakamurai* NRRL B-41091 (NZ_LSAZ00000000.1) and *B. nakamurai*

NRRL B-41092 (NZ_LSBA00000000.1), *B. nakamurai* MZ03-67 (NZ_JANBMN000000000.1) and *B. nakamurai* B-41093 (NZ_JARLZY000000000.1). Other isolates that have not been fully sequenced are also described as putative strains of *B. nakamurai* based on 16S rRNA gene sequencing (Chaouachi et al., 2021; Shaikh et al., 2023).

### 6.4.2. Characterization of the *B. nakamurai* secondary metabolome

The strong antagonistic potential of BDI-IS1 reflects the ability of the strain to efficiently secrete antimicrobial metabolites and therefore, we inspected its genomic content for biosynthetic gene clusters (BGCs) responsible for the synthesis of BSMs. The whole genome (3,811,863 bp of size) of BDI-IS1 was sequenced via Illumina MiSeq (NCBI acc. # NZ_JAJJBV000000000.1) and genome mining was performed using the softwares antiSMASH 7.0.1 (Blin et al., 2023) and BAGEL 4 (van Heel et al., 2018). It first revealed the presence of BGCs responsible for the biosynthesis of a range of non-ribosomal products such as the two families of cyclic lipopeptides surfactin and iturin A (Wang et al., 2023), the polyketide bacillaene (and its variant dihydrobacillaene) (Miao et al., 2023), the siderophore bacillibactin (Dertz et al., 2006), and the dipeptide bacilysin (Islam et al., 2022). Genes encoding several ribosomally produced and post-translationally modified peptides (RiPPs) including amylocyclicin (Scholz et al., 2014), plantazolicin (Molohon et al., 2016), bacinapeptin (Xue et al., 2022) and LCI (Gong et al., 2011) were also identified (Figure 6-2). Plantazolicin is a thiazole and oxazole heterocycle-containing molecule with a demethylated bioactive form (plantazolicin A) and its inactive non-methylated precursor peptide (plantazolicin B) (Kalyon et al., 2011).

Further chemical characterization of BSMs produced by BDI-IS1 in REM was performed via UPLC-q-TOF-$MS^2$ analysis of cell-free extracts. All the predicted non-ribosomal compounds and plantazolicin

were detected and identified based on their exact mass and fragmentation patterns (Figure 6-3A and Figure 6-A3). With this method we also elucidated the panoply of co-produced structural variants for the lipopeptides surfactin and iturin families, which differ in the length of the fatty acid chain ranging from $C_{12}$ to $C_{16}$ for surfactin and $C_{14}$ to $C_{17}$ for iturin A.

We next inspected the genomic content in BGCs responsible for BSMs synthesis in the other strains of *B. nakamurai* sequenced so far in order to evaluate to what extent this secondary metabolome is conserved at the species level. It reveals that synthesis of the non-ribosomal compounds surfactin, iturin A, bacillaene, bacilysin and bacillibactin is conserved across the strains. Together with plantazolicin predicted in four out of five strains, these molecules thus constitute the core metabolome typical of the *B. nakamurai* species. By contrast, the content in BGCs predicted to encode for RiPPs (bacinapeptin, thermoactinoamide, ericin_S, plantazolicin, amylocyclicin, LCI, pumilarin and mersacidin) is more variable and strain-specific (Figure 6-3B).

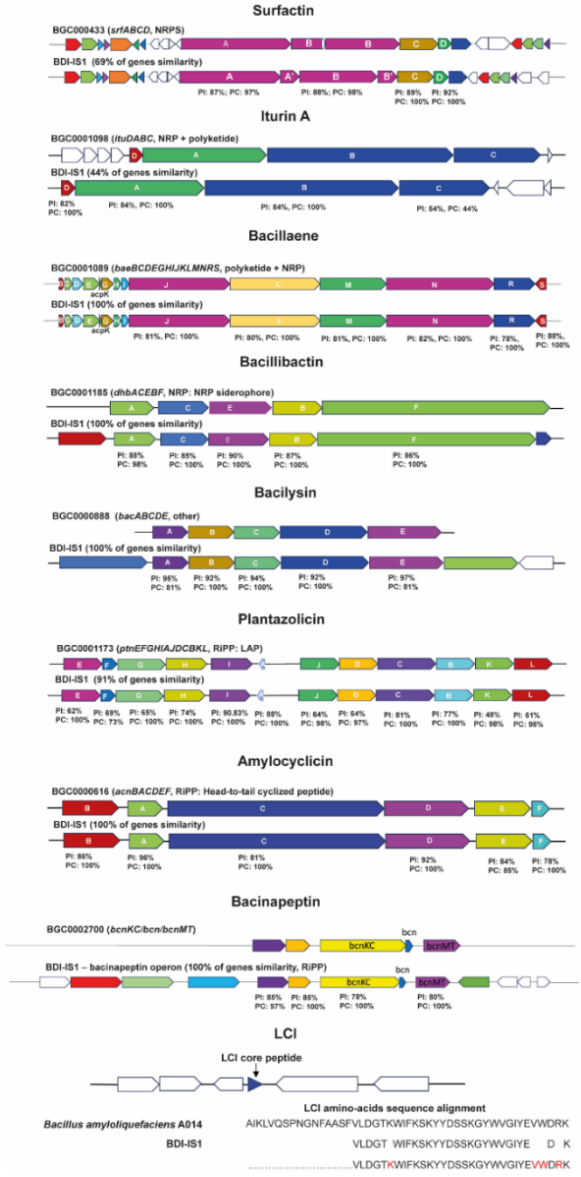


Figure 6-2: Predicted biosynthetic gene clusters (BGCs) in BDI-IS1 genome sequence.

*Prediction was performed using AntiSMASH 7.0 (relaxed detection strictness) and Bagel 4 softwares. The percentage of identity and coverage below each gene of BDI-IS1 were generated from alignment to corresponding gene sequence of the reference strain (reference strain above and query strain, BDI-IS1 below). The architecture of the genes was adapted in line with the prediction tools information.*

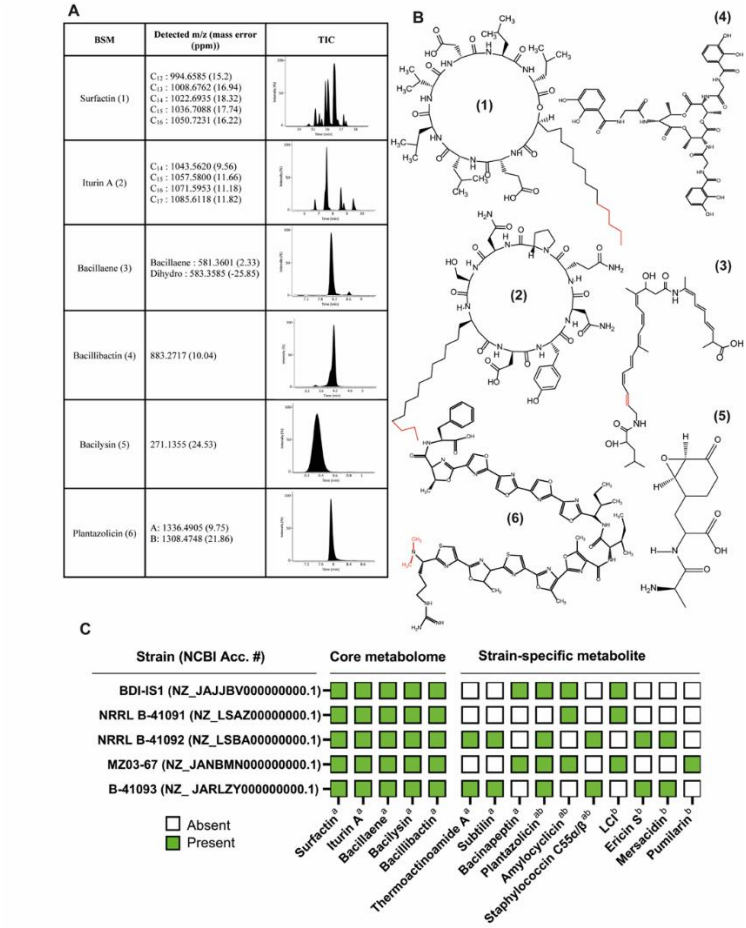


Figure 6-3: Secondary metabolome of BDI-IS1 and comparative genome mining profile of all the *B. nakamurai* strains.

***A.*** *Detected bioactive secondary metabolites with their m/z ratio (error in ppm) and extracted ion chromatogram (TIC); cell-free supernatant from a 48h old culture of BDI-IS1 (REM) was analysed with the hyphaneted method ultra-perfomance liquid choromatography (UPLC)- mass spectrometry (MS) with quadrupole time of flight (qtof) as method of detection in a positive mode.* ***B****. Chemical structures of different detected compounds were drawn with ChemDraw softaware*. ***C****. Comparative genome mining of BGCs in all available genome sequences of the different* B. nakamurai *strains. The letters a, b, ab, imply the typical compound was predicted either by AntiSMASH or by Bagel 4 or by the two webtools, respectively.*

### 6.4.3. Specific involvement of BSMs in the antimicrobial activity of BDI-IS1

We next wanted to determine the relative contribution of each BSM in the broad-spectrum antimicrobial activity displayed by *B. nakamurai*. For that purpose, we generated a library of mutants specifically repressed in the synthesis of one compound and a *Δsfp* mutant lacking the 4'-phosphopantetheinyl transferase (sfp) gene essential for proper NRPS functioning (Wu et al., 2023) and thus impaired in the synthesis of all non-ribosomal metabolites (Table 6-A1).

All these mutants were tested for their antagonistic activity against a set of bacterial and fungal pathogens described above and data revealed different situations (Figure 6-4). In some cases, the *Δsfp* mutant completely lost its inhibitory potential but the antimicrobial activity clearly relies on one single compound such as bacillaene (and/or its -2H form) against *P. carotovorum* and *R. fascians* or iturin A against *A. niger* and *R. solani*. In other cases, the antagonistic activity of *Δsfp* is drastically reduced and we could identify bacilysin (against *P. fuscovaginae*) and iturin A (against *E. turcicum*) as main active ingredients, but other unidentified compounds also contribute to the inhibition. As a third situation, some sfp-independent metabolites are mainly involved in the inhibition of pathogens like plantazolicin which plays some role in the antagonistic effect against *X. campestris* (partial loss of activity of *ΔpznA*). However, the main metabolite(s) interplaying in the antagonism of the latter pathogen together with *C. michiganensis*, *P. cichorii*, *B. cinerea* and *A. solani* still remain to be identified (Figure 6-4).

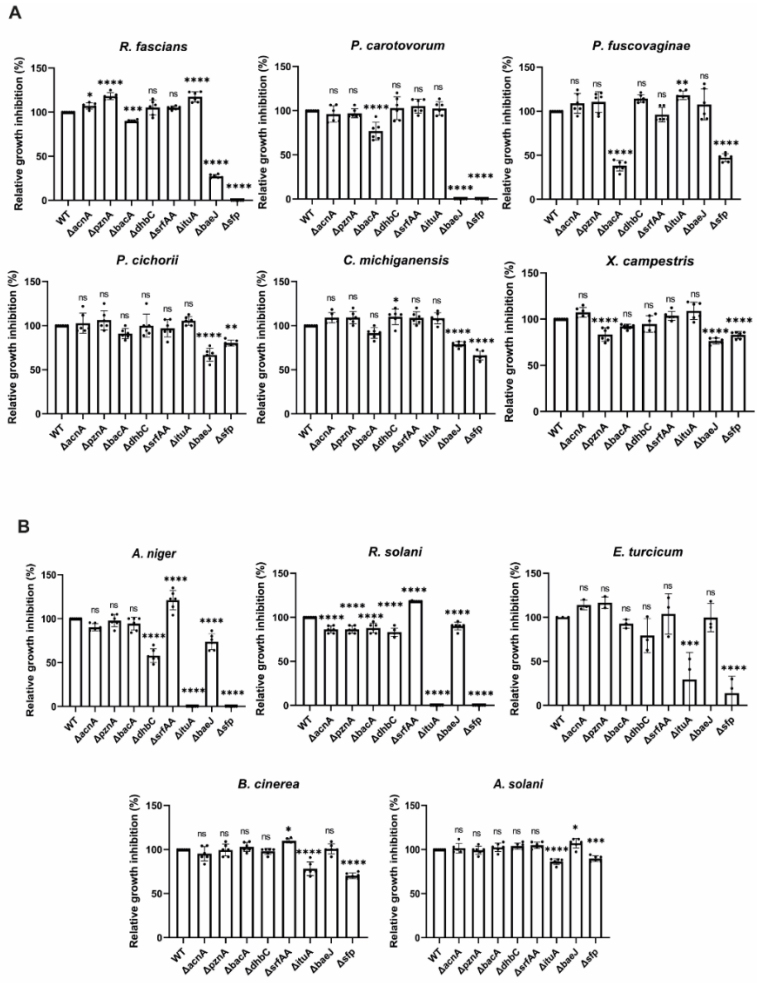


Figure 6-4: Antagonistic activities of BDI-IS1 mutants against selected bacterial and fungal phytopathogens.

*A. Antibacterial activity (relative growth inhibition) of different BDI-IS1 mutants against some bacterial phytopathogens, in comparison with the wild type. The confrontation assay was set on gelified REM (30ml, 30°C, 48h) and inhibition diameter (mm) was recorded and expressed in percentage (compared to the wild type). B. Antifungal activity of BDI-IS1 mutants against some important fungal phytopathogens. Relative growth inhibition of pathogens in confrontation with the different mutants was observed after seven days (20ml of PDA, 23-25°C), except for* A. solani *where incubation was for 21 days and grown on REM Agar. The plotted data (with error bars) for A&B are means (±SD) of three technical replicates, conducted twice in independent experiments (n=6). Statistical comparison of means was performed with one-way ANOVA coupled with Tukey's test ($\alpha = 0.05$) and ns represent non-significant difference, while asterisks *, **, ***, **** implies significant differences at $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.*

### 6.4.4. Biocontrol potential of BDI-IS1

Greenhouse experiments were next conducted to evaluate the potential of BDI-IS1 to control two major diseases prevalent in Burundian agro-ecological zones and caused by the foliar pathogens *A. solani* on tomato and *E. turcicum* on maize. Prior to infection, plants were treated with BDI-IS1 either by aerial spraying (as a foliar treatment) or by soil drenching (as a root treatment) to evaluate the biocontrol activity mediated via direct antagonism in the first case or via the induction of systemic resistance in the second case. The leaves of tomato or maize plants were then inoculated with *A. solani* or *E. turcicum*, respectively. Tomato early blight and Northern corn leaf blight diseases were assessed by determining the necrotic leaf area according to a specific scale of symptoms (Figure 6-A1). Comparison of the dynamics of disease severity revealed that symptoms occurrence was delayed by 1 to 3 days in plants pretreated with BDI-IS1, depending on the pathosystem and application method (Figure 6-5). We also observed a slower progression of disease severity compared to untreated plants, particularly on tomato plants upon leaf treatment with the bacteria, and on maize plants upon root and leaf treatments (Figure 6-5).

The reduction of disease severity was significant from the 13$^{th}$ day post inoculation until the time when symptoms no longer evolved on control plants (Tables 6-A4 & A5). BDI-IS1 delayed disease development by acting efficiently at early stage, as shown by the disease progression curves (Figure 6-5). Root-treated plants did not show symptoms until 4 days post inoculation with *A. solani*, while a 10% disease severity (DS) was observed on control plants and for those treated on leaves. NLB symptoms appeared on control and treated plants from the 7$^{th}$ and 10$^{th}$ day post inoculation, respectively. Overall protection indexes of approximately 25% and 35% for TEB and 65% and 50% for NLB (Figure 6-5) were obtained after root and leaf treatments in two independent greenhouse experiments under severe infection pressure, and no significant differences were observed between leaf and root treatments. These results highlight the biocontrol potential of BDI-IS1

acting locally as antagonist (leaf treatment) or systemically by eliciting plant resistance (root treatment). Furthermore, the levels of protection provided by BDI-IS1 against TEB or NLB are in the same range as the ones observed for *B. velezensis* QST713 tested under the same conditions, except for leaf treatment on tomato, where no disease reduction was observed with that strain (Figure 6-A4 & Table 6-A6). Despite the same global trend in protection levels for each treatment method adopted, the data collected from the two independent trials show a huge variation due to the different weather conditions in which the trials were conducted (Table 6-A7).

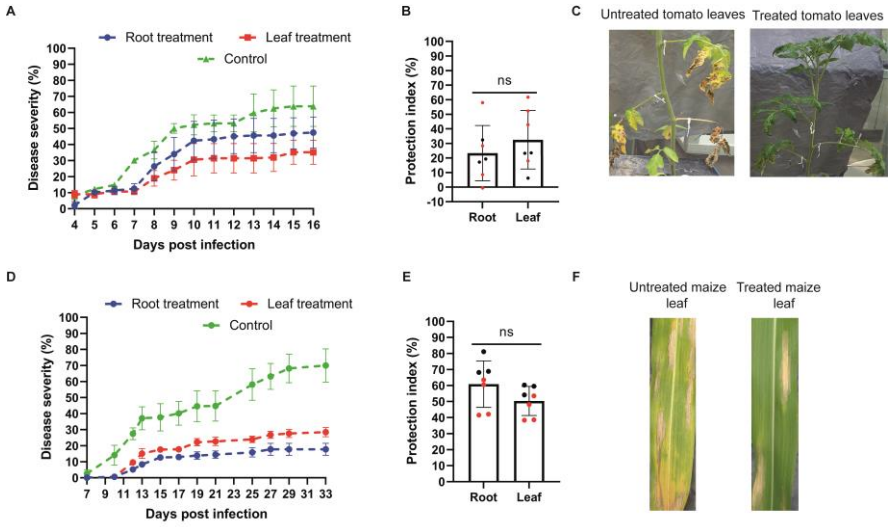


Figure 6-5: Biocontrol efficacy of BDI-IS1 against tomato early blight (TEB) and Northern corn leaf blight (NLB).

***A&D*** *represent the reduction in disease severity of TEB and NLB on plants treated on the roots (blue) or leaves (red) with BDI-IS1 followed by inoculation with* A. solani *or* E. turcicum, *respectively, and control plants (in green) inoculated with the respective fungus, under greenhouse conditions. Each point in graphs A and D represents the mean (±SE) of disease severity from four replicates (n=4) and three replicates (n=3) for experiments one and two, respectively.* ***B&E*** *represent the protection index (PI) of BDI-IS1 when applied to roots or leaves of tomato and maize plants inoculated with* A. solani *and* E. turcicum, *respectively. PIs represent means (± SE) of two independent experiments (n=7) with four (red) and three (black) replicates per treatment for experiment one (n = 4) and two (n = 3), respectively. Means of PI are calculated from area under progress curve (AUDPC) data, which in turn depend on disease severity scores and the time interval between successive recording points (see formula in Section 6.3.7). The differences between PI of BDI-IS1 obtained from root and leaf treatment were analyzed using t-test method and there was no significant difference (ns) at p-value ≤5 %.* ***C&F*** *show the TEB and NLB necrotic lesions on tomato and maize plants treated on leaves with BDI-IS1 compared to untreated but inoculated plants.*

## 6.5. Discussion

In this work, we provide a first comprehensive genomic and chemical characterization of the secondary metabolome produced by an isolate belonging to the rare species *B. nakamurai*. Comparative genome mining for cognate BGCs revealed that a subset of this metabolome with compounds produced via the non-ribosomal pathway is well conserved in all strains sequenced so far. Due to the absence of the lipopeptide fengycin and the polyketides difficidin and macrolactin, this core metabolome of *B. nakamurai* appears less diversified compared to other closely related species such as *B. velezensis* or *B. amyloliquefaciens* in the *B. subtilis* clade but still can be used as a chemical fingerprint typical for the species and can be exploited in taxonomic identification (Yin et al., 2023). By contrast with the well-conserved non-ribosomal products and plantazolicin, the type and number of RiPPs are more strain-specific. This diversification of RiPPs at the strain level may be explained by the acquisition of BGCs from close relatives or other bacterial genera sharing the same agro-ecological niche via horizontal gene transfer (Grubbs et al., 2017; Malit et al., 2021) which may be facilitated by the *ComX* gene, involved in natural competence and quorum-sensing (Rahman et al., 2015), and present in all *B. nakamurai* strains (Pedreira et al., 2022).

Furthermore, we show for the first time possible genetic modification in this undervalued species and data generated from testing the different mutants allow to further exemplify the key role played by some of these specialized metabolites in the broad-spectrum antimicrobial activity displayed by BDI-IS1(Ajuna et al., 2024; Saiyam et al., 2024). Some bacterial phytopathogens were antagonized by BDI-IS1 via the production of bacillaene and/or its 2H-form or bacilysin or a synergy between the two compounds. The polyketide bacillaene (and its 2H-form) is an antibacterial compound against various pathogenic bacteria by interfering with protein synthesis (Erega et al., 2021; Miao et al., 2023), and it is reported to sustain other ecological roles like protecting

the producing strain from predation by soil competitors such as *Mycoplasma xanthus* (Andrić et al., 2023). Although not evidenced in our study, this compound was also shown to exhibit antifungal activity (Miao et al., 2023). Bacilysin retains strong antibacterial activity against a range of plant and food-borne pathogenic bacteria, presumably by inhibiting the synthesis of glucosamine 6-phosphate and hence the formation of peptidoglycan (Chen et al., 2009; Islam et al., 2022). Plantazolicin, a thiazole/oxazole containing-molecule was found to be involved in the inhibition of the brassica's black rot-causing pathogen *X. campestris*. Beyond exhibiting nematicidal activity against *Caenorhabditis elegans* (Liu et al., 2013), this metabolite was already reported to have antibacterial activity, but only against Gram positive bacteria including the obligate pathogen *B. anthracis* (Molohon et al., 2016; Scholz et al., 2011).

In some cases, we could not identify the metabolites involved in the antimicrobial activity suggesting that other products may interplay solely or in synergy with known BSMs in these antagonisms. The predicted antimicrobial peptide LCI was shown to have a strong inhibitory potential against *X. campestris* pv *oryzae* and *Pseudomonas solanacearum* PE1(Gong et al., 2011), whereas the LCI-like $APC_2$ is reported to antagonize *Fusarium solani*, the causal agent of ginseng root rot (Wang et al., 2021). Other lanthipeptides predicted by AntiSMASH such as bacinapeptin (Xue et al., 2022) have been described for their antibiotic activity against various Gram-positive bacteria.

Beyond these already predicted and elucidated compounds, we suspect that other unknown metabolites derived from the T3PKS (type III polyketide synthase), terpene and RRE-containing protein BGCs could putatively be among the mediators of the antagonistic activity of BDI-IS1. Indeed, although not yet identified in *Bacillus*, some compounds synthesized via the T3PKS like diacetylphloroglucinol in *Pseudomonas* sp., are described to play important biological functions in biocontrol activity via inhibition of fungal phytopathogens (Katsuyama and Ohnishi, 2012). Most terpenes produced by strains of the *B. subtilis*

group are volatile compounds (Caulier et al., 2019; Iqbal et al., 2023; Shafi et al., 2017), except the C-35 terpenes reported in broth culture of *B. subtilis* KSM 6-10 (Takigawa et al., 2010) and the oxidative stress alleviator sporulenes, tetracyclic terpenoids found in *B. subtilis* spores (Bosak et al., 2008). The roles of these terpenes in the antimicrobial potential of bacilli against phytopathogens is not well described, but it was shown that isoprene and monoterpene α-terpineol from *Bacillus subtilis* exhibit antagonistic activity against cyanobacteria and nematodes (Caulier et al., 2019). The RiPP Recognition Element (RRE) domain is represented in almost half of RiPPs-producing prokaryotes (Ren et al., 2023) and this RRE-containing protein BGC in BDI-IS1 would code for a novel RiPP with putative antagonistic properties (Hudson and Mitchell, 2018). Other BSMs like surfactin and bacillibactin are not involved in direct antagonistic activity but still these compounds can play key functions for the ecological fitness of *B. nakamurai* considering their well-recognized role in space colonization through cell motility and biofilm formation for surfactin (Crouzet et al., 2020; Hoff et al., 2021) and iron acquisition for bacillibactin (Yu et al., 2011).

Globally, our data illustrate the huge chemical diversity of BSMs in *B. nakamurai* and this probably explains the broad-spectrum antimicrobial activity of strains like BDI-IS1, which extends much beyond the antagonistic activity of *B. nakamurai* occasionally reported against *Erwinia amylovora* (Leathers et al., 2020), *Fusarium poae* (Zanon et al., 2024) and *B. cinerea* (Chaouachi et al., 2021). As *B. nakamurai* targets bacterial and fungal pathogens affecting different crops of worldwide economic importance, this antagonistic potential is an important trait for any biocontrol candidate.

Basing our prospect on the new strain *B. nakamurai* BDI-IS1 isolated from arable soils collected from highlands of tropical region (1880m of altitude, Burundi), we unravel the great biocontrol potential of this endemic *Bacillus* strain against two important tropical plant diseases, tomato early blight and Northern corn leaf blight. The biocontrol

efficacy of BDI-IS1 against important fungal diseases such as Northern corn leaf blight and tomato early blight is interesting as it reaches a level similar or higher than the commercialized strain QST713 in greenhouse experiments. The bacteria were applied to the roots or leaves of the plants, followed by a fungal inoculation on the leaves. When applied preventively, BDI-IS1 delayed the severity of TEB and NLB by one to three days and thus reduced disease pressure on young plants, allowing faster growth and higher resistance to pathogen invasion or to abiotic stresses. This observation was also reported by (Galiano-Carneiro and Miedaner, 2017), where high yield losses were observed after early infection by *E. turcicum*, before silking.

Our results showed an average protection of 25% and 35% for TEB after root or leaf treatment with BDI-IS1. Previously, other *Bacillus* species such as *B. amyloliquefaciens* strain bact-03 have been reported to control TEB caused by *A. solani* strain As-9003, with a significant disease reduction (50%) after leaf treatment under greenhouse conditions (Imran et al., 2022). Although BDI-IS1 protects tomato plants by either root or leaf treatment, the fact that the leaf treatment guaranteed relative high protection for BDI-IS1 strongly suggest that the strain utilizes mostly the direct route in controlling TEB. This may involve to some extent the antifungal iturin A active against several pathogens such as *Phytophthora infestans* (Wang et al., 2020), *B. cinerea* (Ambrico and Trupo, 2017) and *Fusarium* spp.(Liu et al., 2020). Noteworthy, these pathogens are also prevalent in the tropics, threatening various economic crops and could be controlled by BDI-IS1 alone or in combination with other plant disease control strategies.

The efficacy of *Bacillus* spp. in controlling NLB was previously reported, with a reduction in disease severity ranging from 40% to 56% following leaf treatment in a greenhouse experiment (Sartori et al., 2017a). In this study, we demonstrate for the first time the high potential of *B. nakamurai* to protect maize against NLB upon the two modes of application, suggesting a dual mode of action relying on direct antagonism and/or induction of plant systemic resistance. Based on *in*

*vitro* assays, production of iturin A may be tightly involved in local restriction of *E. turcicum* development on leaves. Regarding ISR, the cyclic lipopeptide surfactin produced by all species of the *B. subtilis* clade, including *B. nakamurai*, has been widely reported as elicitor of plant defense in several pathosystems (Caulier et al., 2019; Crouzet et al., 2020; Khan et al., 2021; Pršić and Ongena, 2020). However, iturin A has also been reported as trigger of ISR (Han et al., 2015; Lam et al., 2021; Yamamoto et al., 2015) and further experiments are needed to identify the molecule(s) from *B. nakamurai* that is (are) active on tomato and maize in our study.

Although not equipped like *B. velezensis* QST713 in terms of secondary metabolites reported to be involved in the biocontrol of fungal diseases (i.e. absence of fengycin), BDI-IS1 demonstrated significant protection against TEB and NLB under controlled conditions at 22°C, either upon leaf or root treatment at levels similar to QST713; except in the tomato/*A. solani* pathosystem upon leaf treatment where QST713 did not reduce tomato early blight severity. This suggests a specific adaptation of BDI-IS1 to provide similar or better protection against local plant diseases, as it has been shown that abiotic factors affect the *in planta* efficacy of biocontrol agents (Ayaz et al., 2023).

## 6.6. Conclusions

Isolated from Burundian soil, *B. nakamurai* BDI-IS1 exhibited strong *in vitro* antimicrobial activities against a range of important bacterial and fungal phytopathogens. We characterized the core metabolome of the species and identified some non-ribosomal specialized metabolites for their involvement in pathogen inhibition. However, the weaponry of *B. nakamurai* is quite diverse and several RiPPs may also contribute to the global antimicrobial activity. A lot remains to be discovered in the diversity of bioactive natural products potentially formed by this understudied species. The biocontrol potential assessment against tomato early blight and Northern corn leaf blight revealed that BDI-IS1 provides its protective effect upon leaf or root treatment and to a level

similar or higher compared to *B. velezensis* QST713 (Serenade®) under greenhouse conditions, suggesting two main modes of action including direct and indirect antagonism. Further studies should explore the mechanisms underlying these biocontrol properties and investigate other potential benefits in terms of plant growth promotion or resistance to abiotic stresses (Enebe and Babalola, 2018; Etesami et al., 2023a).

The high potential of this locally isolated strain to control major plant diseases prevalent in the tropical region highlights the importance of seeking adapted solutions to local problems by exploiting indigenous microbial resources (Y. Liu et al., 2023; Novello et al., 2023).

## Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

## Data availability

All data supporting the findings of this work are available within the paper and in supplementary materials.

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Table 6-A1: Primers utilized in the BDI-IS1 mutant strains construction.

| **Targeted molecule (s)** | **Primer name** | **Oligonucleotide sequence** |
|---|---|---|
| Bacillaene | baeJ verif | TACATGGACTCGCTGAACGAC |
| | dwn baeJ F | GTAAAACGACGGCCAGTGAGGTTTAATGTGAAGAACTTC |
| | dwn baeJ R | CCTTCAAGAGATGCGGAGAC |
| | up baeJ F | TCAGACCGGGATAATCAAGC |
| | up baeJ R | CAGGAAACAGCTATGACTTTCATAGAGCTGCCTCCAT |
| Bacilysin | bacA verif | GCTTGCTTTAATTTTCGGCG |
| | dwn bacA F | GTAAAACGACGGCCAGTACTGAACAAGATTTGCAGG |
| | dwn bacA R | CGTATCAATCGCTGACTGGA |
| | up bacA F | ATGGACGTTTTGGATTTTCG |
| | up bacA R | CAGGAAACAGCTATGACAATCATGAGCACCAACCAAT |
| Iturin A | ituA verif | GGAGAATATTCAGCTCTTGTCT |
| | dwn ituA F | GTAAAACGACGGCCAGTAACTTTTAGGAAGCAAGGGGA |
| | dwn ituA R | AGTTCGGCTTCCAGGGTAAT |
| | up ituA F | TTGATGCAAAATGCAGATCC |
| | Up ituA R | CAGGAAACAGCTATGACAAACATAGGTTTCCTCCAAGG |
| Plantazolicin A | pznA verif | CCCGGAGTTAAATACCTATGT |
| | dwn pznA F | GTAAAACGACGGCCAGTACGTTCTAACCTCTAAGACAAA |
| | dwn pznA R | ACCGCTGTAGAATTGGGACA |
| | up pznA F | GCCATCAACAATAACGCAAA |
| | Up pznA R | CAGGAAACAGCTATGACAACCATATTGTGATTTATAT |
| Bacillibactin | dhbC verif | TAATCAGCCGTGCCCTGCGG |
| | dwn dhbC F | GTAAAACGACGGCCAGTATGTAATGGAGAAAGGGGAA |
| | dwn dhbC R | AGCACTTGGAGGCTGGATAA |
| | up dhbC F | TCGGACTAAAAACGGCTGAT |
| | Up dhbC R | CAGGAAACAGCTATGACTGACATGTCTGTACCTCCTT |

| Targeted molecule (s) | Primer name | Oligonucleotide sequence |
|---|---|---|
| Amylocyclicin | acnA verif | AGTGTTCCCGTAACAATTACTGA |
| | dwn acnA F | GTAAAACGACGGCCAGTATTTAGCCCGGGGGAGAGCA |
| | dwn acnA R | TGACTGCCCGATAAACACAG |
| | up acnA F | TTACGGAATGGGTTTTGAGC |
| | up acnA R | CAGGAAACAGCTATGACACCTATCATATTACCATCCT |
| Surfactin | srfAA verif | TGATCTCGGAGGACGGGGTT |
| | down srfAA F | GTAAAACGACGGCCAGTGCTTGGACCAGCTCCCTCTT |
| | down srfAA R | GGGATTTGCCTTTCCATGCG |
| | up srfAA F | CCATTCCGGAGCTTGTGTCC |
| | up srfAA R | CAGGAAACAGCTATGACCGCCTCCGGCTTTCAAAATC |
| NRPs | sfp verif | CGATTCAGCCTGAGCTGATG |
| | down sfp F | GTAAAACGACGGCCAGTGCACAGCCCGATTTTTGTGA |
| | down sfp R | CGATGCAGTCCCCGGATTAT |
| | up sfp F | GGCTGGACAATGGTTGTCGT |
| | up sfp R | CAGGAAACAGCTATGACGTCCAACATCCTCCGTCTGC |

Table 6-A2: Antibacterial activity (mm of inhibition diameter ± SE) of the isolated bacterial strains from Burundian soils against six plant pathogenic bacteria.

| Samples/ pathogen | *X. campestris* | *C. michiganensis* | *P. cichori* | *P. fuscovaginae* | *R. fascians* | *P. carotovorum* |
|---|---|---|---|---|---|---|
| BDI-M1 | 0 | 0 | 0 | 0 | 0 | 0 |
| BDI-M2 | 0 | 0 | 0 | 0 | 0 | 0 |
| BDI-M3 | 6 ± 0.6 | 3.2 ± 0.17 | 5.5 ± 1.04 | 0 | 12.5 ± 0.29 | 0 |
| BDI-M4 | 25.5 ± 0.29 | 19.7 ± 0.44 | 8.3 ± 0.31 | 8.6 ± 0.17 | 9.3 ± 0.17 | 0 |
| BDI-M5 | | 0 | | 0 | 0 | 0 |
| BDI-M6 | | 0 | 8.3 ± 0.44 | 8.7 ± 0.44 | 0 | 0 |
| BDI-M7 | 3.5 ± 0.44 | 0 | 7.7 ± 0.6 | 0 | 11 ± 0.29 | 0 |
| BDI-M8 | 5.5 ± 0.35 | 0 | 4.3 ± 0.17 | 0 | 12.5 ± 0.58 | 0 |
| BDI-M9 | 4.8 ± 0.44 | 0 | 5.5 ± 0.87 | 0 | 8.5 ± 0.29 | 0 |
| BDI-M10 | 0 | 0 | 0 | 0 | 0 | 0 |
| BDI-M11 | 0 | 0 | 0 | 0 | 0 | 0 |
| BDI-M12 | 24.8 ± 0.17 | 20 ± 0.28 | 6.6 ± 0.33 | 8.7 ± 0.44 | 5.8 ± 0.17 | 0 |
| BDI-M13 | 14 ± 0.5 | 17.3 ± 0.44 | 8.2 ± 0.73 | 8.7 ± 0.44 | 10.3 ± 0.17 | 0 |
| BDI-IS1 | 27.8 ± 0.5 | 26.7 ± 0.6 | 6.8 ± 0.22 | 8.8 ± 0.34 | 28.6 ± 0.26 | 7 ± 0.27 |
| BDI-IS2 | 5.8 ± 0.17 | 7 ± 0.29 | 5.3 ± 0.44 | 0 | 4.5 ± 0.29 | 0 |
| BDI-IS3 | 26.3 ± 0.82 | 24.3 ± 0.33 | 8 ± 0.5 | 8 ± 0.00 | 5.7 ± 0.17 | 0 |
| *B. velezensis* QST 713 | 26.2 ± 0.28 | 27.6 ± 1.17 | 7.6 ± 0.13 | 8.7 ± 0.39 | 19.2 ± 0.34 | 6.6 ± 0.15 |

Table 6-A3: Antifungal activity (mm of inhibition radius ± SE) of the isolated bacterial strain BDI-IS1 against nine phytopathogenic fungi.

| Samples/ pathogen | *A. niger* | *B. cinerea* | *A. solani* | *A. rabiei* | *E. turcicum* | *F. oxysporum* | *R. solani* | *P. oryzae* | *Colletotrichum sp.* |
|---|---|---|---|---|---|---|---|---|---|
| BDI-IS1 | 5.4±0.15 | 8.8±0.21 | 14±0.11 | 12±0.36 | 16±0.28 | 8.3±0.17 | 14±0.73 | 21±0.47 | 21±0.42 |
| *B.velezensis* QST 713 | 9.4±0.20 | 14±0.66 | 17±0.32 | 15±0.37 | 20±0.34 | 12±0.33 | 16±0.62 | 21±0.4 | 21±0.38 |

Table 6-A4: Analysis of the daily variation (DAI) in the severity of early blight in tomato using a general linear mixed model by trial and by treatment method (trial 1&2). R: Root treatment, L: Leaf treatment. Values with different letters are significantly different.

| Trial | Days post inoculation | Treatment method | BDI-IS1 | QST 713 | Control | Trial | Days post inoculation | Treatment method | BDI-IS1 | QST 713 | Control |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 4DAI | R | 2.08a | 4.17a | 8.10a | 2 | 3DAI | R | 18.52a | 16.67a | 23.15a |
| 1 | | L | 9.03a | 19.60a | 8.10a | 2 | | L | 20.37a | 25.31a | 23.15a |
| 1 | 5 DAI | R | 10.19a | 17.82a | 12.27a | 2 | 4 DAI | R | 22.84ab | 19.75a | 30.86b |
| 1 | | L | 9.03a | 19.60a | 12.27a | 2 | | L | 22.22a | 28.40a | 30.86a |
| 1 | 6DAI | R | 11.34a | 20.37a | 14.81a | 2 | 5DAI | R | 31.48a | 33.95a | 40.74a |
| 1 | | L | 10.88a | 23.84a | 14.81a | 2 | | L | 33.33a | 45.06a | 40.74a |
| 1 | 7DAI | R | 12.50a | 25.46a | 30.09a | 2 | 6DAI | R | 32.72a | 35.19ab | 43.83b |
| 1 | | L | 10.6a5 | 32.18a | 30.09a | 2 | | L | 33.95a | 46.30aa | 43.83 |
| 1 | 8DAI | R | 26.39a | 30.09a | 36.57a | 2 | 7DAI | R | 35.19a | 35.19a | 45.68a |
| 1 | | L | 18.98a | 43.98a | 36.57a | 2 | | L | 38.89a | 50.62a | 45.68a |

| Trial | Days post inoculation | Treatment method | BDI-IS1 | QST 713 | Control | Trial | Days post inoculation | Treatment method | BDI-IS1 | QST 713 | Control |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 9DAI | R | 34.03a | 35.19a | 50.00a | 2 | 8DAI | R | 37.04a | 41.36a | 52.47b |
| 1 | | L | 24.07a | 48.92ab | 50.00b | 2 | | L | 41.98a | 56.79a | 52.47a |
| 1 | 10DAI | R | 42.36a | 36.34a | 52.31a | 2 | 9DAI | R | 40.74a | 44.44a | 59.88b |
| 1 | | L | 30.56a | 51.70a | 52.31a | 2 | | L | 46.30a | 59.88a | 59.88a |
| 1 | 11DAI | R | 43.29a | 36.81a | 53.24a | 2 | 10DAI | R | 46.91a | 50.62a | 63.58b |
| 1 | | L | 31.48a | 51.70a | 53.24a | 2 | | L | 50.62a | 61.73a | 63.58a |
| 1 | 12DAI | R | 45.14a | 37.27a | 53.24a | 2 | 11DAI | R | 51.23a | 50.62a | 67.28b |
| 1 | | L | 31.48a | 52.16a | 53.24a | 2 | | L | 54.94a | 67.28a | 67.28a |
| 1 | 13DAI | R | 45.60a | 37.27a | 59.72a | 2 | 12DAI | R | 56.17a | 50.62a | 69.14b |
| 1 | | L | 31.48a | 52.16ab | 59.72b | 2 | | L | 59.26a | 67.90a | 69.14a |
| 1 | 14DAI | R | 45.60a | 39.58a | 62.50a | 2 | 13DAI | R | 56.79a | 51.23a | 69.75b |
| 1 | | L | 31.94a | 52.85ab | 62.50b | 2 | | L | 59.26a | 68.52a | 69.75a |
| 1 | 15DAI | R | 46.99a | 39.58a | 63.89b | 2 | 14DAI | R | 56.79a | 52.47a | 70.99b |
| 1 | | L | 35.19a | 52.85ab | 63.89b | 2 | | L | 60.49a | 69.14a | 70.99a |
| 1 | 16DAI | R | 46.99a | 39.58a | 63.89b | 2 | 15DAI | R | 58.02a | 53.09a | 71.60b |
| | | L | 35.19a | 52.85ab | 63.89b | 2 | | L | 61.73a | 69.75a | 71.60a |
| | | | | | | 2 | 16DAI | R | 58.02a | 53.09a | 71.60b |
| | | | | | | 2 | | L | 61.73a | 69.75a | 71.60a |

Table 6-A5: Analysis of the daily variation in the severity of the Northern corn leaf blight disease using a general linear mixed model by trial and by treatment method (trial 1 & 2).
*R: Root treatment, L: Leaf treatment. Values with different letters are significantly different at 0.05 threshold.*

| Trial | Days post inoculation | Treatment method | BDI-IS1 | QST 713 | Control |
|---|---|---|---|---|---|
| 1 | 23DAI | R | 23.44a | 26.70a | 56.71b |
| 1 | | L | 27.08a | 22.38a | 56.71b |
| 1 | 38DAI | R | 47.19a | 55.94a | 78.02b |
| 1 | | L | 54.06a | 53.44a | 78.02b |
| 2 | 7DAI | R | 0.10a | 0.00a | 2.58a |
| 2 | | L | 0.08a | 0.08a | 2.58a |
| 2 | 10 DAI | R | 0.58a | 5.92a | 14.08a |
| 2 | | L | 0.58a | 4.42a | 14.08a |
| 2 | 12DAI | R | 5.17a | 7.92a | 27.42a |
| 2 | | L | 9.63a | 13.67a | 27.42a |
| 2 | 13DAI | R | 8.38a | 10.08a | 37.1b |
| 2 | | L | 15.13a | 17.50a | 37.1a |
| 2 | 15DAI | R | 12.67a | 10.54a | 37.1b |
| 2 | | L | 17.63a | 18.83a | 37.1a |
| 2 | 17DAI | R | 12.96a | 12.79a | 40.21b |
| 2 | | L | 17.79a | 18.88b | 40.21b |
| 2 | 19DAI | R | 13.83a | 16.96a | 44.58b |
| 2 | | L | 22.22a | 21.58a | 44.58b |
| 2 | 21DAI | R | 14.46a | 16.96a | 44.75b |
| 2 | | L | 22.69b | 21.58a | 44.75b |
| 2 | 25DAI | R | 15.88a | 18.21a | 58.17b |
| 2 | | L | 23.99a | 23.17a | 58.17 b |
| 2 | 27DAI | R | 17.75a | 22.79a | 63.17 b |
| 2 | | L | 26.78a | 24.50a | 63.17b |
| 2 | 29DAI | R | 17.75a | 24.46a | 68.17b |
| 2 | | L | 27.64a | 27.00a | 68.17b |
| 2 | 33DAI | R | 17.75a | 24.46a | 70.04b |
| 2 | | L | 28.47a | 27.04a | 70.04b |

Table 6-A6: Area under the disease progression curve (AUDPC) and protection index (PI) of BDI-IS1 and QST713 against Northern corn leaf blight and tomato early blight.
*AUDPCR: Area Under Disease Progress Curve after root treatment. AUDPCL: Area Under Disease Progress Curve after leaf treatment. PIR: Protection Index after root treatment. PIL: Protection Index after foliar treatment.*

| Northern corn leaf blight | | | | | | Tomato early blight | | | |
|---|---|---|---|---|---|---|---|---|---|
| Trial | Treatment | AUDPCR | AUDPCL | PIR | PIL | AUDPCR | AUDPCL | PIR | PIL |
| 1 | BDI-IS1 | 799.31± 97.24 | 920.01± 62.09 | 51.93 ±5.85 | 44.67 ±3.73 | 388.19± 48.30 | 287.85± 34.93 | 23.59± 12.96 | 43.70± 9.39 |
| 1 | QST713 | 926.91± 146.90 | 825.94± 45.27 | 44.25 ±8.84 | 50.32 ±2.72 | 377.66± 100.11 | 518.40± 114.78 | 26.67± 19.80 | -0.15±23.96 |
| 2 | BDI-IS1 | 316.08± 48.75 | 487.44± 22.68 | 72.76 ±4.20 | 58.00 ±1.95 | 564.81± 35.02 | 604.01± 41.98 | 22.96± 4.78 | 17.62± 5.73 |
| 2 | QST713 | 395.75± 103.14 | 494.83± 107.49 | 65.90 ±8.89 | 57.36 ±9.26 | 553.40± 23.52 | 738.89± 27.61 | 24.52± 3.21 | -0.78±3.77 |

Table 6-A7: Temperature and relative humidity in greenhouses during the experimental period.

| Pathosystem | Trial | Period | Temperature (°C) | | | Relative humidity (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | | Minima | mean | maxima | minima | mean | maxima |
| Maize + *E. turcicum* | 1 | Day | 14.40 | 20. 8 | 26.00 | 59.00 | 72.00 | 85.00 |
| | | Night | 14. 40 | 16. 05 | 17. 60 | 73.00 | 80.00 | 86.00 |
| | 2 | Day | 15. 40 | 21. 95 | 26 | 55.00 | 70.00 | 85.00 |
| | | Night | 15. 80 | 17.06 | 18. 90 | 71.00 | 78.00 | 85.00 |
| Tomato + *A. solani* | 1 | Day | 13. 90 | 19. 91 | 24.00 | 62.00 | 81.00 | 96.00 |
| | | Night | 14,00 | 16.00 | 20.00 | 73.00 | 86.00 | 96.00 |
| | 2 | Day | 17. 30 | 20. 85 | 24.00 | 69.00 | 82.00 | 99.00 |
| | | Night | 17. 20 | 17. 5 | 18.00 | 83.00 | 88.00 | 95.00 |




Figure 6-A1: Scale developed for the evaluation of tomato early blight (A) and Northern corn leaf blight (B).
*The scale is based on necrotic leaf area, with tomato early blight scored on a scale of 1 to 9, where 1 = 0-5% of leaf area affected by the disease, 3 = 5-10%, 5 = 10-20%, 7 = 20-50% and 9 = 50-100%. Northern leaf blight was also scored on a scale from 1 to 9, where 1 = 0% of leaf area affected, 2 = 10%, 3 = 20%, 4 = 30%, 5 = 40%, 6 = 50%, 7 = 60%, 8 = 70%, 9 = 80-100%. Asymptomatic samples are not included in the Northern corn leaf blight scale but will obviously have 0% necrotic area.*

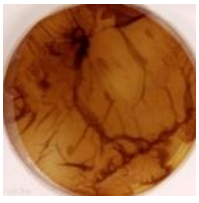

Figure 6-A2: Black-brown pigment developed by Burundi isolate BDI-IS1.
*This isolate BDI-IS1 (later identified as* B. nakamurai *BDI-IS1) was grown on tryptic soy agar (TSA) and incubated at 30°C for 72h.*

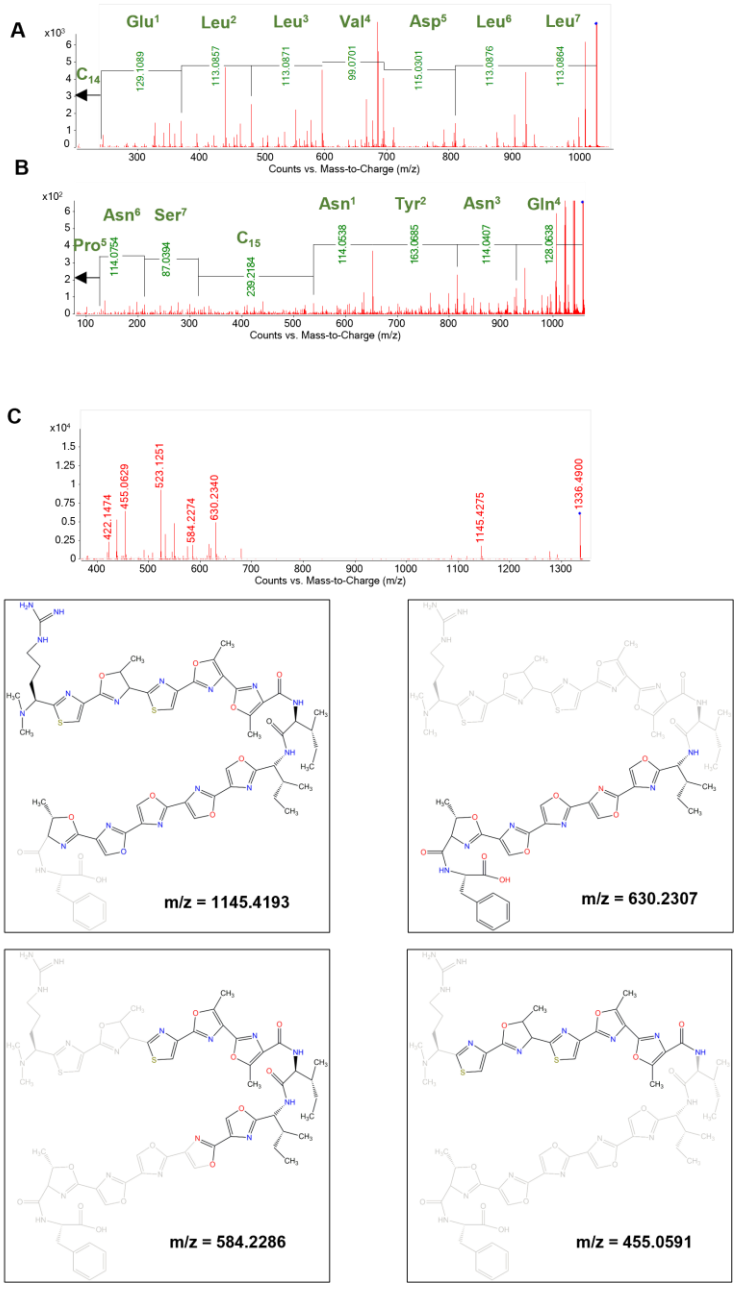


Figure 6-A3: MS2 patterns of surfactin (A), iturin A (B), and plantazolicin (C).
*Analysis was performed with the tandem mass spectrometry ($MS^2$) in a positive mode. Plantazolicin $MS^2$ profile (C) is provided together with chemical structures highlighting of the main fragmentation patterns of the compound.*

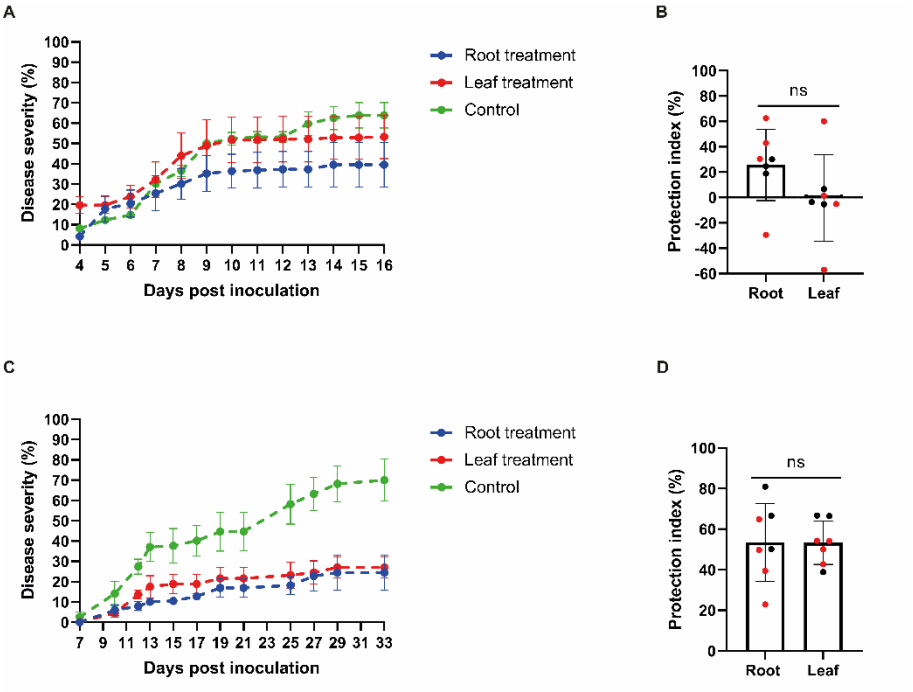


Figure 6-A4: Biocontrol efficacy of QST713 against tomato early blight (TEB) and Northern corn leaf blight (NLB).

A&*C. Reduction in disease severity of TEB and NLB on plants treated in the roots (blue) or leaves (red) with QST713 followed by inoculation with* Alternaria solani *or* Exserohilum turcicum, *respectively, and control plants (in green) inoculated with the respective fungus, under greenhouse conditions. Each point in graphs A and C represents the mean (±SE) of disease severity from four replicates (n=4) and three replicates (n=3) for experiments one and two, respectively. B&D. Protection index (PI) of QST713 when applied to roots or leaves of tomato (B) and maize (D) plants inoculated with* A. solani *and* E. turcicum, *respectively. PIs represent means (± SE) of two independent experiments with four (red) and three (black) replicates per treatment for experiment one (n = 4) and two (n = 3), respectively. Means of PI are calculated from area under progress curve (AUDPC) data, which in turn depend on disease severity scores and the time interval between successive recording points (see formula in methods). The differences between PI of QST713 obtained from root and leaf treatment were analyzed using t-test method and there was no significant difference (ns) at p-value ≤5%.*

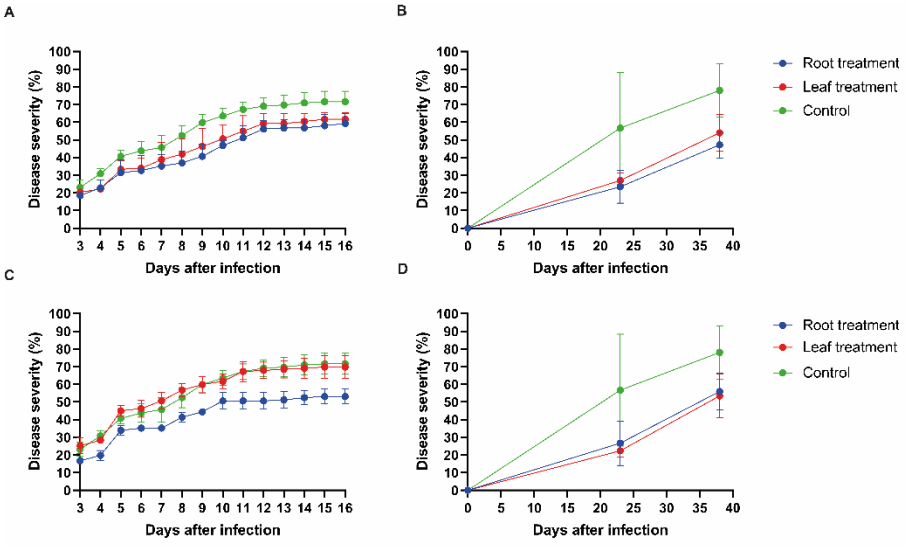


Figure 6-A5: Biocontrol efficacy of BDI-IS1 and QST713 against tomato early blight (TEB) and Northern corn leaf blight (NLB).
*Reduction in disease severity of TEB (A&C) and NLB (B&D) on plants treated in the roots (blue) or leaves (red) with BDI-IS1 (A & B) or QST 713 (C&D) followed by inoculation with* A. solani *or* E. turcicum, *respectively, and control plants (in green) inoculated with the respective fungus under greenhouse conditions. Each time point represents the mean (±SE) of disease severity from three replicates (n=3) and four replicates (n=4) for TEB and NLB in experiments two and one, respectively. The difference between DS was analyzed using general linear mixed models at p-value ≤5% (Table 6-A4 & A5).*

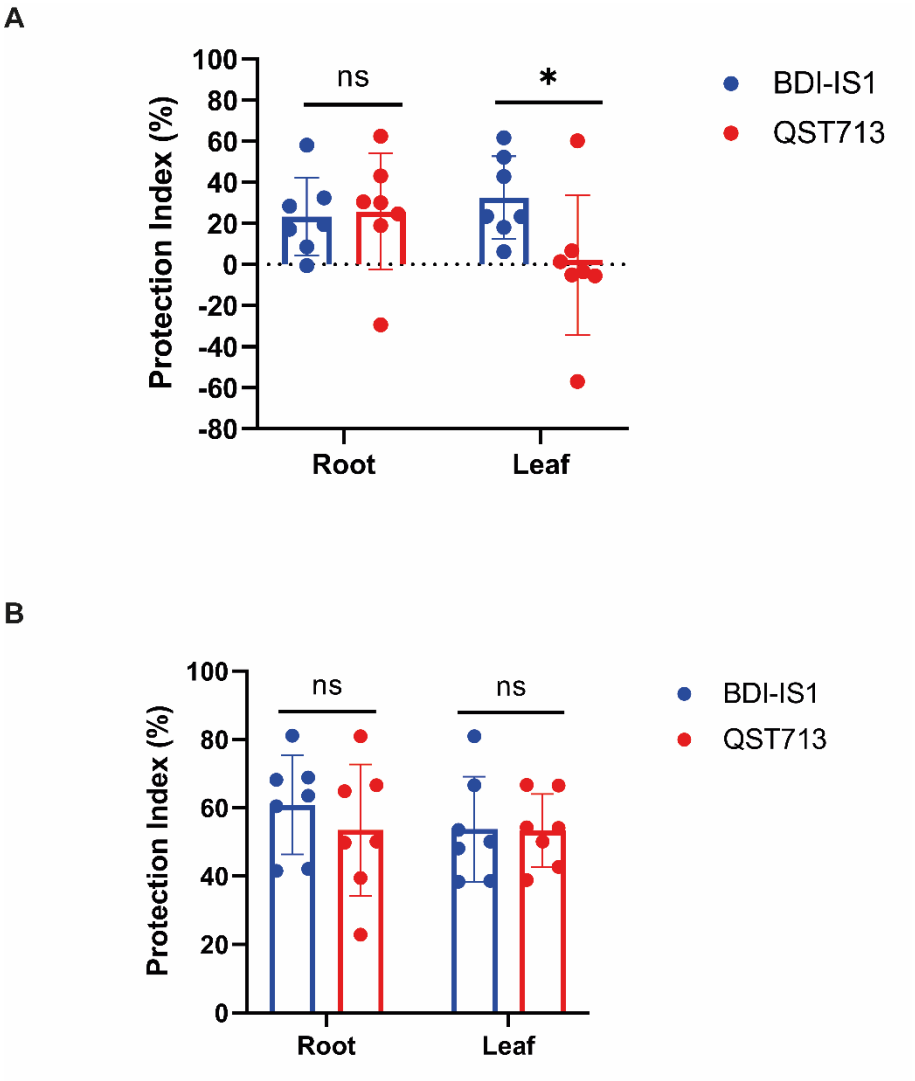


Figure 6-A6: Comparison of protection provided by BDI-IS1 and QST713 against TEB and NLB.

*Protection index (PI) of BDI-IS1 and QST713 when applied to roots or leaves of tomato (A) and maize (B) plants inoculated with* A. solani *and* E. turcicum*, respectively. PIs represent means (± SE) of two independent experiments (n = 7) with four and three replicates per treatment for experiment one and two, respectively. Means of PI are calculated from area under progress curve (AUDPC) data, which in turn depend on disease severity scores and the time interval between successive recording points (see formula in section 6.3.7). The differences between PI of BDI-IS1 and QST713 obtained from root and leaf treatment were analyzed using t-test method, ns implies that there was no significant difference, while * shows significant statistical difference at p-value ≤5%.*

# CHAPTER VII.

# BIOCONTROL OF NORTHERN CORN LEAF BLIGHT BY *BACILLUS NAKAMURAI* BDI-IS1 THROUGH DIVERSE MODES OF ACTION

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## 7.1. Abstract

The bacterium BDI-IS1 has been shown to control Northern corn leaf blight (NLB) under controlled conditions. The biocontrol efficacy of BDI-IS1 under field conditions and the mechanisms of action involved in the control of NLB are not known. The aim of this study was to evaluate the biocontrol efficacy of BDI-IS1 against NLB and to elucidate its modes of action under field conditions. Field trials were conducted in Burundi to evaluate the biocontrol efficacy of bacterial cell and bean residues-based BDI-IS1 bioproduct to control NLB. The BDI-IS1 knocked out in iturin A and surfactin production were used to identify the roles of these secondary metabolites in biological control. To elucidate the indirect activity of BDI-IS1 on maize plants against NLB, a transcriptomic approach was used to quantify the expression of key defense genes at different time points. BDI-IS1 reduced NLB upon leaf or root treatment by application of bacterial cell-free supernatant and seed coating with bioproduct, suggesting the multiple modes of action including direct and indirect mechanisms involved by BDI-IS1 to control NLB. Direct antagonism was mediated by iturin A, while surfactin was involved in the indirect activities of BDI-IS1 against NLB. BDI-IS1 induced maize defense by enhancing the expression of

phenylalanine ammonia lyase (PAL), allene oxide synthase (AOS) and MYC7 genes, which are involved in the salicylic acid (SA) and jasmonic acid (JA) plant defense signaling and defense pathways, respectively. This bacterium reduced the NLB prevalent in Burundi and could therefore be incorporated into crop protection strategies to control NLB.

Key words: Biocontrol, *Bacillus nakamurai* BDI-IS1, iturin, surfactin, *Exserohilum turcicum*, Induced Systemic Resistance, phenylalanine ammonia lyase, allene oxide synthase, MYC7

## 7.2. Introduction

Biological control is gaining recognition as a sustainable crop protection strategy and an eco-friendly alternative to chemical pesticides. Many microorganisms from the genera *Bacillus*, *Pseudomonas* and *Trichoderma* have demonstrated their efficacy in controlling various plant diseases in greenhouse trials and, to a lesser extent, under field conditions (Lahlali et al., 2022; Nihorimbere et al., 2024). However, the global market for these biological products within agrochemical phytoproducts represents only 10%, which is relatively low despite their advantages in sustainable disease and pest management, as well as in protection of human and environment health (Marrone, 2023). The limited growth of the biological control agent market is hindered by their relatively low efficacy compared to chemical products and their strong dependence on biotic and abiotic environmental conditions. The risk of disturbing the ecological balance by introducing a new micro-organism into a different environment limits the development and expansion of this sector. To address these challenges, various solutions are proposed, including the use of native microorganisms, the combination of microorganisms with different modes of action, and the integration of biological control with other plant protection strategies, such as plant resistance and chemical control, to enhance the efficacy of BCAs in managing plant diseases and pests.

The bacterium *Bacillus nakamurai* BDI-IS1, isolated in Burundi, exhibited *in vitro* antagonistic activity against a wide range of bacterial and fungal pathogens, including *Alternaria solani* and *Exserohilum turcicum*, the causal agents of tomato early blight (TEB) and Northern corn leaf blight (NLB), respectively-two major fungal diseases affecting tomatoes and maize in Burundi. The biocontrol efficacy of BDI-IS1 against NLB and TEB through root and leaf treatments was demonstrated in greenhouse trials (Nimbeshaho et al., 2024). The effectiveness of this bacterium in both leaf and root treatments suggests the potential for multiple modes of action, including direct and indirect antagonism, depending on the treatment method. The bacteria applied on maize leaves can interact directly with *E. turcicum*, while those treated on roots are distantly separated to the fungus and could indirectly acting on fungus by inducing plant defenses.

Additionally, BDI-IS1 has been characterized at the molecular and biochemical levels and found to produce several bioactive compounds, including non-ribosomal peptides such as iturin A, surfactin, bacillaene, bacilysin, and bacillibactin, and ribosomal peptides such as bacinapeptin, plantazolicin, amylocyclicin, and LCI (Nimbeshaho et al., 2024). These metabolites play key roles in bacterial survival, competition, and the establishment of symbiotic relationships with plants.

Among these secondary metabolites, the cyclic lipopeptides, particularly iturin A and surfactin, are well-characterized for their biocontrol efficacy against various diseases and pests affecting major crops globally (Caulier et al., 2019; Iqbal et al., 2023; Kaspar et al., 2019; Ongena and Jacques, 2008; Salazar et al., 2023). In recent studies, a direct effect of BDI-IS1 was shown to be mediated by iturin A, as bacteria impaired in iturin production lost their ability to inhibit the mycelial growth of *E. turcicum* (Nimbeshaho et al., 2024).

The efficacy of biological control agents is highly dependent on biotic and abiotic environmental conditions. Therefore, it is crucial to test their effectiveness in the various soil and climate conditions where they

will be used, particularly against prevailing plant diseases under field conditions. To the best of our knowledge, the efficacy of BDI-IS1 in controlling NLB under field conditions, as well as its associated modes of action, remains unknown, despite their importance for bioformulation and field application of this bacterial-based bioproduct.

The aim of this study is to evaluate the biocontrol efficacy of BDI-IS1 against NLB under field conditions and to elucidate its modes of action. Additionally, we aim to assess its potential application methods adapted to the tropical agro-ecological conditions of Burundi. This research will also provide valuable insights to guide the future use and application of this bacterium in field environments.

## 7.3. Materials and methods

### 7.3.1. Plant material

Maize seeds of ZM621, a variety widely grown in the mid-altitude zone of Burundi and susceptible to NLB, were used in our experiments. The certified seeds were provided by ISABU from prebasic seeds production.

### 7.3.2. Origins and inoculum production of bacterial and fungal strains

The strain BDI-IS1 of *B. nakamurai* and derived-mutant strains, depleted in the biosynthesis of iturin (ΔituA) or surfactin (ΔsrfAA) (Nimbeshaho, 2024) or transformed with green fluorescent protein (GFP)-transformed BDI-IS1, available in the collection of the Microbial Processes and Interactions (MiPI) Laboratory, Gembloux Agro-Bio Tech, University of Liège, were used in this study. *Bacillus velezensis* QST713, also from the MiPI collection, was used as a reference standard antagonist bacterium. The strains were preserved in glycerol (30%) at -80°C. They were cultured on Luria Bertani Agar (LBA) medium (tryptone: 10g/L, yeast extract: 5g/L, NaCl: 10g/L, agar: 15g/L) at 30°C for 24 hours. A single bacterial colony was

transferred to LB (tryptone: 10 g/l, yeast extract: 5 g/l, NaCl: 10 g/l) medium (50 ml in 250 ml flasks) as a preculture and incubated for 24 hours at 30°C, 120 rpm, then 1 ml of the preculture with an $OD_{600}$ of 0.1 was used to prepare a culture (initial $OD_{600}$ = 0.001, 100 ml in 250 ml flasks) for 24 hours at 30°C, 120 rpm. The cultures were centrifuged, and the pellets were washed twice with $MgCl_2$ (2.5 g/L) before being diluted in $MgCl_2$ to adjust its concentration to $10^6$ CFU/ml.

The strain MBC10105 of *E. turcicum* originated from Burundi was used in this study (Nimbeshaho et al., 2024). A conidial suspension preserved in glycerol (10%) at -80°C was inoculated on PDA (39g/L of potato dextrose agar + 2g/L of agar) and incubated at 27°C. After 15 days of incubation, the conidia were collected in sterilized water + Tween 20 (0.05%), filtered through a sieve (50µ) and the concentration of the suspension was adjusted after counting using a Fuch Rosenthal cell.

### 7.3.3. Biocontrol in field using bacterial inocula produced *in vitro*

Two independent trials were conducted to evaluate the efficacy of BDI-IS1 compared to QST713 to control NLB under field conditions. The experimental trials took place at the Mparambo Research Centre of the Institut des Sciences Agronomiques du Burundi (ISABU), located in a lowland region (Cibitoke, western Burundi, altitude: 750-1000 m, temperature >23°C), where maize is naturally infected with *E. turcicum*.

Maize seeds were sown directly into prepared holes without the addition of fertilizer. The first trial (season A) was carried out from November 11, 2021, to February 3, 2022, and the second trial (season B) took place from February 21 to May 12, 2022.

The experimental design consisted of 12 plots (four blocks * three treatments), each of 22.5 m² (6m x 3.75m) and each containing 66 maize plants. Three treatments including (i) plants treated with BDI-IS1, (ii) plants treated with QST713, (iii) plants treated with buffer ($MgCl_2$)

were tested in a fully randomized design with four replications. BDI-IS1 and QST713 were cultured and prepared as described above. The bacterial suspension, at a concentration of $10^6$ CFU/ml, was applied to the leaves weekly, starting from 20 and 7 days after sowing, resulting in a total of 3 and 7 applications in seasons A and B, respectively.

### 7.3.4. Biocontrol field assays using bacterial inocula produced on local substrates

Two independent bioassays were conducted in Burundi to evaluate the efficacy of the BDI-IS1-based bioproducts produced locally, ensuring their accessibility for further potential industrial-scale bacterial production. In these assays, three lignocellulosic substrates were tested for bacterial growth. In the first trial conducted in a tunnel under semi-controlled conditions at ISABU, lignocellulosic substrates, including bean, maize and rice residues, were ground in fine powders with a particle size of 1-5 mm, and then over-dried before sterilization. Ten grams of each substrate supplemented with rice husk (7:3) were placed in a 500 ml flask, humidified with distilled water, and adjusted to 1:5 v/v for aeration. The humidified substrates were sterilized at 121°C for 20 minutes. After cooling, 1 ml of a BDI-IS1 bacterial suspension ($OD_{600}$ of 0.1, prepared as described above) was added, and the cultures were incubated at 30°C for 5 days before being over-dried at 80°C for 15 minutes to produce spores.

These bioproducts were tested against *E. turcicum* in a trial conducted in a tunnel or insect-proof greenhouse at ISABU. The experimental layout included nine treatments: (1) BDI-IS1 + bean residues, (2) BDI-IS1 + rice residues, (3) BDI-IS1 + maize residues, (4) QST713 + bean residues, (5) QST713 + rice residues, (6) QST713 + maize residues, (7) bean residues, (8) rice residues, and (9) maize residues. These treatments were arranged in a randomized complete block design, with 12 plants per treatment (4 plants per experimental unit, replicated 3 times).

Pregerminated maize seeds were grown in 2L pots (1 grain per pot) containing soil collected from a field at the same site and mixed with 5 g of each bioproduct, with a concentration of either 0 or $10^{14}$ CFU/g, depending on the absence or presence of bacteria in bioproduct. After 14 days of growth, the plants were sprayed with a suspension of *E. turcicum* (prepared as described above) and then covered with a plastic bag. The disease severity was scored periodically as outlined previously.

A second field trial was conducted at the same ISABU center to test the efficacy of the BDI-IS1-based bioproduct in controlling NLB. The bean-based bioproduct was prepared as described above and 5g was poured into the seed bed, with a spacing of 75 cm x 50 cm. The trial was established following a randomized complete block design comprising two treatments: BDI-IS1-based bioproduct and bean residues. Each treatment consisted of 66 plants and was replicated three times, leading to a total of 396 plants (66 plants*3 replicas*2 treatments) for the entire trial.

### 7.3.5. Bioassays conducted in greenhouse to understand the modes of action of BDI-IS1

Two independent bioassays were carried out under greenhouse conditions to compare the efficacy of BDI-IS1 and its mutants in controlling NLB on maize. The plants were prepared as described previously by Nimbeshaho et al. (2024). The seeds of the maize variety ZM621 were disinfected in sodium hypochlorite (1%) and rinsed three times in sterile water, then pregerminated for three days. Each grain was then sown in a plastic pot (2L) filled with potting soil and incubated in a greenhouse (temperature: 18-23°C; relative humidity: 80 ± 5%; photoperiod: 16 h). The plantlets were watered three times a week.

Ten liters of each bacterial suspension were prepared independently as described above. A control (Blanco) was also prepared without bacteria. These suspensions were applied to the established maize plants in two modes: either to the roots (substrate) or to the leaves. For root

application, one-week-old plants were treated by pouring 100 ml of the bacterial suspension resuspended in $MgCl_2$ (2.5g/L) to be adjusted at $10^6$ CFU/ml or the control onto the plant substrate, followed by an additional treatment one week later. For leaf treatment, the bacterial suspension or control was sprayed until runoff on all the leaves of two-week-old plants.

One day after these applications, the plants were inoculated with *E. turcicum* by spraying a conidial suspension at 4.5 x $10^4$ conidia/ml until runoff on maize leaves (Nimbeshaho et al., 2024). Control plants were treated with $MgCl_2$ and inoculated with sterilized water + Tween 20 (0.05%). The inoculated plants were then incubated for three days in a humidity chamber at 90% humidity and a temperature of 20 ± 2°C.

In these assays, a total of 16 treatments were compared, including the treatments of plants with (1) ΔituA, (2) ΔsrfAA, (3) BDI-IS1, or (4) $MgCl_2$ on leaves, and (5) ΔituA, (6) ΔsrfAA, (7) BDI-IS1, or (8) $MgCl_2$ on roots, before inoculation with *E. turcicum* or a control solution with Tween 20 (treatments 9 to 16). Each treatment was applied with 4 replications (4 plants) distributed in a randomized block design in the greenhouse. Disease and growth parameters were assessed as described below.

Another bioassay was performed under greenhouse conditions to decifer the effect of BDI-IS1 on plant defense. This bioassay was conducted as described above. Four treatments were compared: (1) BDI-IS1 + *E. turcicum*, (2) $MgCl_2$ + *E. turcicum*, (3) BDI-IS1, and (4) $MgCl_2$ + Tween 20 (control). Each treatment was applied to 72 plants arranged in a completely randomized block design with four replications. The bacterial suspension was poured onto the root substrate, while the fungal inoculum was sprayed on the leaves, as previously described.

The plants were grown under the same conditions as in above bioassays. At five time points (0 h, 9 h, 3 days, 7 days, and 13 days post-inoculation), the leaves of 12 plants (4 replicates of 3 plants) were

harvested to quantify the expression of certain defense genes (see below in RNA extraction, and RT-qPCR). The progression of disease severity was assessed on 12 plants per treatment, up to 22 days post-inoculation, as described hereafter.

### 7.3.6. Assessment of NLB

The disease intensity on plants grown under greenhouse conditions was scored based on the number of chlorotic and necrotic lesions and the leaf necrotic area, assessed at 2-day intervals using a scale of 1 to 9 (Figure 6-A1, described in chapter VI), where 1 = 0% of leaf area affected by disease, 2 = 10%, 3 = 20%, 4 = 30%, 5 = 40%, 6 = 50%, 7 = 60%, 8 = 70%, and 9 = 80-100%. This scale was previously developed for evaluating NLB in the greenhouse (Nimbeshaho et al., 2024).

In contrast, the disease intensity on plants grown in the field was scored on 10 plants per treatment, based on the number of chlorotic and necrotic lesions observed at several time points, using a 1-5 scale developed by CIMMYT (1985). On this scale, 1 = traces or 1 to 2 lesions on lower leaves; 2 = lower leaves moderately affected, with some lesions merged; 3 = many lesions on lower leaves, with few on middle leaves; 4 = many lesions on both lower and middle leaves, extending to upper leaves; and 5 = many lesions on all leaves, with the plant potentially dying prematurely due to wilting.

Disease intensity in the greenhouse was calculated as the mean percentage of the disease necrotic area, while disease severity (DS) in the field was calculated using the formula described by Willocquet et al.(2004):

$DS = \sum_{i=1}^{k}(SR_i \times NP_i)/TNP \times HS) \times 100$, where i is the disease score, k the number of classes in disease scale (k = 5), while $SR_i$ and $NP_i$ represent the score rating and number of plants with i score, respectively. TNP and HS are the total number of assessed plants and highest score (HS = 9), respectively. The area under the disease

progression curve (AUDPC) was calculated using the following formula:

$AUDPC = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2}\right) X\ (t_{i+1} - t_i)$, where $y_i$: disease severity of $i^{th}$ day,$y_{i+1}$: disease severity of $i^{th+1}$ day, $t_i$:$i^{th}$ day, $t_{i+1}$: $i^{th+1}$ day.

The Protection index (PI) conferred by a treatment was calculated from the disease severity (DS) or AUDPC, using the following formula:

$$PI = \left[\left(1 - \frac{\text{AUDPC treatment}}{\text{AUDPC control}}\right) * 100\right]$$

### 7.3.7. RNA extraction and quantification of gene expression by quantitative RT-qPCR

Total RNA was extracted from the collected leaf samples stored at -80°C using the Trizol method, as previously described by the manufacturer (Invitrogen, Waltham, MA, USA). RNA quality was checked by spectrophotometry (NanoDrop ND 1000, Thermo Fisher Scientific), and its integrity was assessed by electrophoresis on a denaturing RNA gel (0.65% formaldehyde, 1.2% agarose), as previously described by Decroës et al. (2022). Nucleic acid concentration and 260/280 and 260/230 ratios were assessed, and samples with low concentrations (less than 50 ng/µl) and ratios (below 1.8) were discarded. Another sample was then taken from the stored powder and re-extracted using the same protocol. The extracted total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, U.S.A.) to remove DNA and was purified with phenol: chloroform alcohol: isoamyl alcohol (125:24:1), then eluted in 30 µl of RNase-free water. Two aliquots of each RNA were stored at -80°C.

cDNA was synthesized from 1 µg of RNA using an oligo (dT) anchored 25 (Eurogentec®) and M-MLV reverse transcriptase enzyme, as described by the manufacturer (Promega). Three reference genes (housekeeping genes) reported by Lin et al. (2014) to be stable in maize

metabolism, along with seven defense genes related to salicylic acid and jasmonic acid biosynthesis and signaling pathways, were quantified by RT-qPCR using the primers and conditions listed in Table 7-A1.

The specificity of the primers used to amplify these genes, the size of their amplification products, and the conditions were first checked and validated in silico using the NCBI platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the primer-blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The concentration of GC and primer self-pairing at the 3' and 5' ends were checked using PCR Primer STAT (https://www.bioinformatics.org/sms2/pcr_primer_stats.html). The sequences of the primers, the amplification products, and the annealing temperatures are described in the supplementary file (Table 7-A1).

For each primer pair, PCR assays were performed using cDNA from stored RNA of a set of samples to optimize the amplification conditions and obtain PCR products that would serve to establish a standard curve in the RT-qPCR. This was achieved by performing a tenfold serial dilution of the PCR product from a previously amplified sample selected based on its unique and distinct fragment observed on the electrophoresis gel.

To quantify the corresponding transcript for each gene, RT-qPCR was conducted using SYBR Green technology. For each reaction, a mixture containing 10 µl of Takyon No ROX SYBR 2X MasterMix blue dTTP-750 rXn (7.5 µl) (Eurogentec), 0.5 µl of each primer (20 µM), and 7 µl of RNase-free water was first loaded into the plate, followed by the addition of 2 µl of standard samples or the relevant cDNA to each specific well. Two technical replicates were performed for each sample.

The RT-qPCR reactions were performed on the CFX 96 real-time system (Bio-Rad) using the following amplification conditions: first denaturation at 95°C for 3 minutes, followed by 40 cycles of second denaturation at 95°C for 10 seconds, annealing at the specific temperature (see Table 7-A1) for 20 seconds, extension at 72°C for 20

seconds, and a melting curve from 55°C to 95°C with a heating rate of 0.5°C/sec.

### 7.3.8. Localization of GFP-transformed BDI-IS1 in maize plant

Two bioassays were performed to assess the colonization of BDI-IS1 inside or on the surface of maize plants following root treatment or seed coating. Thirty maize plants of ZM621 variety were grown in sterilized quartz in 1L pots under greenhouse conditions. The GFP-transformed BDI-IS1, stored at -80°C, was successively cultured in LBA and LB supplemented with 5 µg/ml of chloramphenicol and poured to the substrate of one-week-old fifteen maize plants (as described above), while $MgCl_2$ was poured onto the roots of the remaining fifteen control plants. In another bioassay fifteen maize seeds were immerged in a GFP-transformed BDI-IS1 suspension or in $MgCl_2$ (control seeds) for 2 hours prior to sowing. After five days in trial one and twelve days in bioassay two, the roots, stems, and leaf tissues of both treated and control three plants were collected to assess the colonization of this transformed bacterium in these organs. For each plant, three small sections for each organs, were collected gently, placed on slides, and visualized using confocal microscopy (Leica Stellaris Confocal 8 Flacon Laser Scanning Microscope) with HC PL APO CS2 63X/1.20 water objectives at the IMABIOL platform of UCLouvain, Belgium. The samples were analyzed both externally and internally, and the corresponding photographs were taken.

### 7.3.9. Data analysis

Data from the set of plants used to assess disease intensity/severity, obtained from all trials, were analyzed by using Generalized Linear Mixed Model (GLMM) with R 4.0.5 software, where the bacteria, treatment method and trial were fixed factors, and blocks were taken as random factor. The robust and suitable method of Kenward-Roger was used to calculate the degrees of freedom, while the Estimated Marginal Means (emmeans) package was used to compare the different means of

disease severity per day. The variation of disease severity at specific date was estimated by using one-way ANOVA, and in case of absence of normal distribution, the non-parametric test, Kruskall-Wallis was used to assess the variation between treatment, and the means were separated by using Tukey or Dunn test, respectively, at the threshold of 5%.

Data from RT-qPCR assays were analyzed using CFX Manager software. The annotated plates containing samples with amplification efficiencies between 90 and 110 were imported into CFX Manager software (Bio-Rad) for graph and gene expression analysis. The stability of the reference genes was assessed using an evolution curve constructed from the Cq data, where the genes with the same expression profile were retained for further analysis. The stability of the reference genes was also checked using CFX manager software, where the coefficient of variation (CV) and stability parameters (M) of each gene were obtained (Table 7-A2). The reference genes with CV<0.5 and M<1 was selected to normalize the expression of the tested defense genes. The $2^{-\Delta\Delta cq}$ method described by Livak and Schmittgen (2001) and integrated in the CFX manager software was used to obtain the relative normalized expression of each defense gene tested, while including the control samples at each time point and the three reference genes in the analysis.

## 7.4. Results

### 7.4.1. Biocontrol efficacy of the spraying of bacterial suspension on plants against *E. turcicum* in field conditions

The efficacy of BDI-IS1 in controlling NLB was evaluated under natural conditions at Mparambo research centre, in two independent trials carried out in season A (from November to February) and B (from February to May). The bacterial suspension was sprayed on leaves and the disease severity was assessed using a previously developed scale (Nimbeshaho et al., 2024). In both trials, a significant reduction in

disease severity was observed on plants treated with BDI-IS1 from 74 to 81 days after sowing compared to the control (see Table 7-A3). The number of chlorotic and necrotic lesions was significantly higher in control plants than in those treated with BDI-IS1. At the end of the season (81 days after sowing), the mean disease severity observed on plants treated with BDI-IS1 was significantly lower than that of the control in both seasons A and B, and even lower than that observed in QST713-treated plants in season A (Figure 7-1). Additionally, we noted a generally higher disease severity in the second trial conducted in season B compared to the first trial conducted in season A. For example, 74 days after sowing, the average NLB disease severity on plants treated with BDI-IS1 in trials 1 and 2 was 45% and 58.5%, respectively, while the disease severity on control plants was 56% and 66% (see Table 7-A3). Overall, disease reduction at the end of trials 1 and 2 was 34% and 11%, respectively.

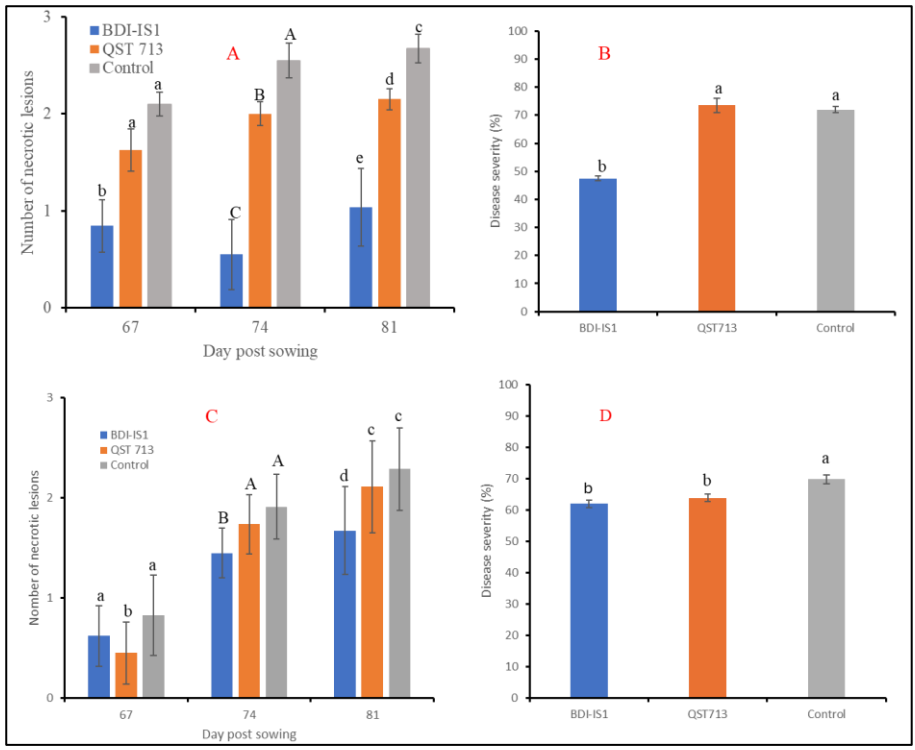


Figure 7-1: Number of necrotic lesions and disease severity recorded in two field trials performed in season A (A&B) and in season B (C&D).

*Maize plants were treated with BDI-IS1, QST713 or buffer ($MgCl_2$) (control). The number of necrotic lesions was counted on the ear and upper ear leaves and is the average of 80 leaves for each treatment and trial. Disease severity data presented are the average of 40 plants assessed for each treatment and for each trial. Variations in necrotic lesions and in disease intensity were analyzed using the general mixed linear model and means were separated using the emmeans package in R. At the end of experiment, the variation in disease severity was analyzed by using Kruskall-wallis test and Dunn tests were used to separate the means at 5% of threshold, respectively. Treatments with the same letters are not significantly different.*

### 7.4.2. Efficacy of BDI-IS1-based bioproducts added to the maize substrate against NLB

BDI-IS1 was cultured on bean, rice, and maize residues, demonstrating high sporulation levels, exceeding $10^{14}$ CFU/g for all three substrates (Figure 7-A1). The efficacy of BDI-IS1 spores produced from these lignocellulosic substrates was tested on maize plants grown in soil-filled pots within an insect-proof tunnel at ISABU, in season B. The

BDI-IS1 based bioproducts were applied in pots at the same time as the maize seed. When applied into maize substrate, the progression of NLB was slower on plants treated with BDI-IS1-based bioproducts. Both inoculated substrates reduced significantly NLB severity from 4 dpi up to 30 dpi (Figure 7-2 & Table 7-A4). Thirty days after *E. turcicum* inoculation, disease severity in BDI-IS1-treated plants ranged from 15% to 20%, compared to 70% to 80% in control plants treated with extracts from plant residues without bacteria (Figure 7-2). Overall, the protection index of BDI-IS1 grown on these substrates exceeded 60%, with the highest protection index (67%) observed for BDI-IS1 grown on bean residues, followed by rice residues (66%) and maize residues (61%). However, the differences in protection indexes among the three substrates were not statistically significant.

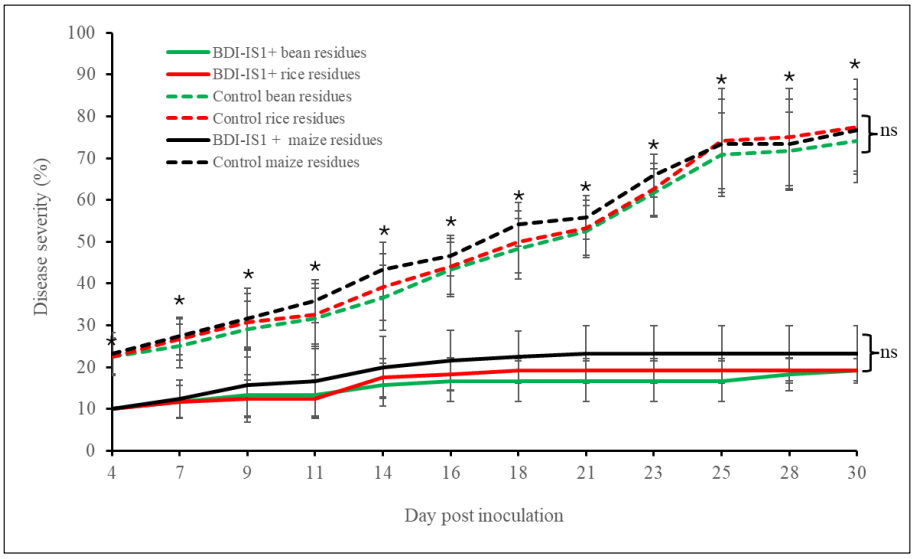


Figure 7-2: Progression of NLB on maize plants treated with bioproducts from bean, rice and maize residues supplemented with rice husk, inoculated or not with *B. nakamurai* BDI-IS1.

*Each point in graph represents an average of disease severity recorded on 12 plants grouped in 3 blocks (4 plants per treatment), individually scored on two leaves, giving a total of 24 leaves scored per treatment, from 4 dpi to 30 dpi. The bar represents the standard deviation of disease severity per treatment and per day. The analysis of variance was performed by General Mixed Linear Model (GML) and the means were separated by the emmeans method at 5% of the threshold. The ns: indicate the non-significant difference between the treatment at different date. *: indicate the difference between the treatment at specific date are significant.*

Based on the results obtained in the tunnel experiment, the bean substrate yielded high bacterial sporulation (Figure 7-A1) and provided strong protection against NLB. Therefore, this substrate was selected to produce the inoculant for field trials conducted at the Mparambo Research Centre in season B, where NLB occurs naturally every season. The BDI-IS1-based bioproduct was prepared as described in the materials and methods section, with more than $10^{10}$ CFU/g obtained for use in the field trial (Tasiaux, 2024). The bacterial inoculant was incorporated into the seed-bed, followed by maize seed sowing as previously described. A slower increase of disease severity on BDI-IS1-treated plants compared to untreated plants, with significant differences observed between 31 and 37 days post-sowing (see figure 7-3B). The number of necrotic lesions counted at 31 days post-sowing was significantly lower on BDI-IS1-treated plants (Figure 7-3A). Furthermore, disease reduction increased over time, with a protection rate of 13% at 27 days post-sowing, which rose to 26% by the end of the evaluation period (37 days post-sowing).

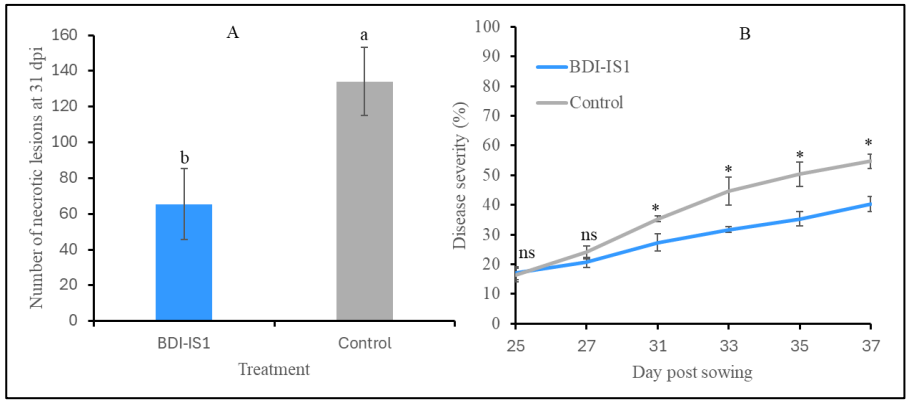


Figure 7-3: Biocontrol efficacy of a BDI-IS1-based bioproduct obtained on bean residues against NLB.

*Number of necrotic lesions at 31 days post sowing (A) and severity of NLB (B) on plants treated or not with BDI-IS1-based bioproduct. The trials were conducted at the Mparambo Research Centre (lowland) under natural inoculation with* E. turcicum, *the causal agent of NLB. The data presented are the means of necrotic lesions (Figure 7-3A) recorded at 31 post sowing and the disease severity (Figure 7-3B) recorded on two leaves per 30 plants (60 points). The bar indicates the standard deviation within the four replications of each treatment and the * indicates the level of significance at the 5% threshold. Treatments with the same letter are not significantly different.*

### 7.4.3. Role of secondary metabolites produced by BDI-IS1 to control NLB

The efficacy of BDI-IS1 in reducing NLB disease, previously observed in greenhouse experiments (Nimbeshaho et al., 2024), was confirmed in this study under field conditions using both foliar spray and seed coating applications. The role of secondary metabolites produced by BDI-IS1 in its biocontrol efficacy against NLB was further explored by comparing the protection index achieved with BDI-IS1 and the mutants ΔituA and ΔsrfAA, which are deficient in the production of iturin A and surfactin, respectively. These secondary metabolites were selected based on the reported direct activities of iturin A in *in vitro* assays (Nimbeshaho et al., 2024), and the widely reported induction of plant defense of surfactin, produced by various *Bacillus* spp., against many pathogens. Chlorotic lesions began appearing on inoculated plants four days after inoculation, while no symptoms were observed on uninoculated plants. By 22 days post-inoculation, disease severity differed significantly among treatments (Figure 7-4B).

Application of the wild-type BDI-IS1 to roots or leaves significantly reduced disease severity by 37% and 35%, respectively. Plants treated with ΔituA or ΔsrfAA on the leaves exhibited higher disease severity compared to those treated with the wild-type bacterium, but lower than in untreated plants. However, these treatments did not result in significantly different disease severity compared to either wild-type or untreated plants. In contrast, when applied to roots, ΔituA reduced disease severity as effectively as the wild-type, while ΔsrfAA resulted in an intermediate disease index.

Furthermore, disease severity in plants treated on the roots with the surfactin-deficient mutant (ΔsrfAA) and inoculated with *E. turcicum* was not significantly different from that of the control plants (Figure 7-4A). The protection level provided by the surfactin mutant against NLB was reduced by up to 26% compared to the wild type.

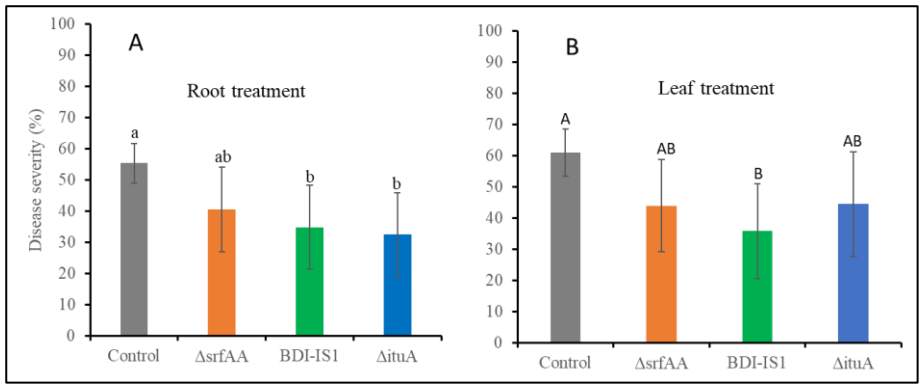


Figure 7-4: Biocontrol efficacy of BDI-IS1 and defective mutants in the production of secondary metabolites (ΔituA: iturin A, ΔsrfAA: surfactin) against maize Northern corn leaf blight.
*A: Disease severity recorded on plants treated or not on roots with mutants or BDI-IS1 and inoculated with* E. turcicum, *B: Disease severity recorded on plants treated or not on leaves with mutants or BDI-IS1 and inoculated with* E. turcicum. *The data presented are the means of disease severity at the end of the experimental process, recorded on 32 plants (2 x 16 plants per treatment (spread into 4 replications) and per experiment) and 64 leaves per treatment, in two independent experiments. The entire recorded data are represented in a supplementary file (Figure 7-A3). The bar represents the standard deviation calculated on 32 plants per treatment. The analysis was carried out using the Kruskal-Wallis method, while the means were separated using the Dunn test. Treatments within each graph with the same letter are not significantly different at the 5% threshold.*

### 7.4.4. Induction of systemic resistance by BDI-IS1

To assess the potential induction of systemic resistance in maize by BDI-IS1, a greenhouse bioassay was conducted in which the bacterium was applied to the roots, followed by a spray of *E. turcicum* conidial suspension on the leaves of maize plants. The dynamics of expression of specific defense genes selected to be involved in SA or JA biosynthesis and defense pathways were monitored at several time points alongside assessments of disease severity, in both inoculated and uninoculated maize plants with *E. turcicum*. Small chlorotic lesions, corresponding to individual infection points, were observed as early as 3 days post-inoculation (dpi) on plants inoculated with *E. turcicum*, while no symptoms were observed on uninoculated plants. The number

of chlorotic lesions was more than halved on BDI-IS1-treated plants (15.5) compared to untreated plants (36.5) (Figure 7-5A). By 14 dpi, the differences between treated and control plants were very clear (Figure 7-5D&E), while the number of lesions that had become necrotic was also reduced on treated plants (2.5) compared to untreated plants (6.6) (Figure 7-5B). Subsequently, disease severity continued to increase, reaching 69% on untreated plants and 26% on BDI-IS1-treated plants by the end of the observation period (Figure 7-5C).

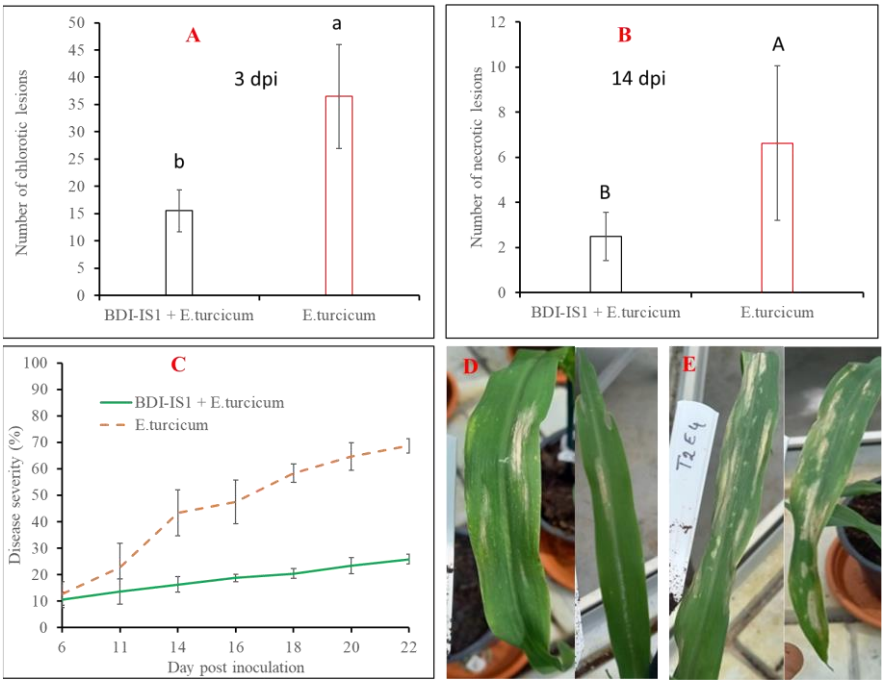


Figure 7-5: Evaluation of NLB on maize treated or not treated with BDI-IS1.
*A: number of chlorotic lesions 3 days post inoculation (dpi), B: number of necrotic lesions 14 days post inoculation, C: leaf necrotic area/disease severity on treated (green) or not treated plants (orange), D: leaves from plants treated with BDI-IS1 and inoculated with* E. turcicum *(photo taken 14 days post inoculation), E: leaves from plants not treated with BDI-IS1 and inoculated with* E. turcicum *(photo taken 14 days post inoculation, from where the differences were very distinct with the characteristic cigar shape symptoms of NLB). The data presented are the means of chlorotic lesions/necrotic lesions/disease severity recorded on 12 plants and 24 leaves per treatment. The bars represent the standard deviation of 12 means values from 24 data per treatment. One-way ANOVA was performed, and the means were separated by Tukey at 5% of the threshold. Treatments within each graph with the same letter are not significantly different.*

Furthermore, we evaluated the expression dynamics of a selected set of defense related marker genes at five timepoints in maize plants treated or not with BDI-IS1 and inoculated or not with *E. turcicum*. The expression levels of most tested genes did not differ significantly between treatments across time points (Figure 7-A4), except for *PAL*, *AOS1*, and, to a lesser extent, *MYC7* (Figure 7-6).

At 3 days post-inoculation (dpi), the *PAL* gene was significantly up-regulated in BDI-IS1-treated and inoculated plants, with a relative normalized expression increase of more than 18-fold compared to untreated and inoculated plants (Figure 7-6A). The transcription factor *MYC7* was also significantly up-regulated at this timepoint, in treated and inoculated plants, with a relative normalized expression change of 4-fold compared to control plants (Figure7-6B). By 7 dpi, the *AOS1* gene was significantly up-regulated in treated and inoculated plants, showing a relative normalized expression change of over 32-fold compared to untreated and inoculated plants (Figure 7-6C). Overall, the bacterium BDI-IS1 induced strong expression of *PAL* and *AOS1*, and a moderate expression of *MYC7*. *PAL* is involved in biosynthesis of phenolic compounds and phytoalexins (Klessig et al., 2018), with potential antagonistic activity against many pathogens, while *AOS1* and *MYC7* are involved in biosynthesis and transcription of defense genes mediated by JA, respectively (H. Cao et al., 2023; Fu et al., 2020).

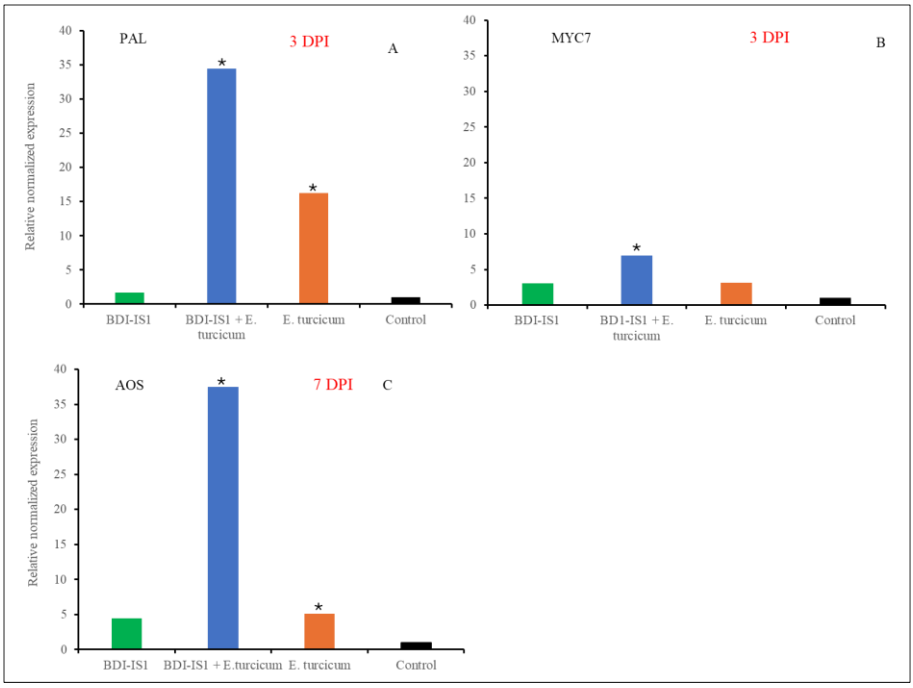


Figure 7-6: Differential expression of defense genes in maize plants treated or not with BDI-IS1 and inoculated or not with *E. turcicum.*

*PAL (A) and MYC7 (B) were highly to moderately expressed, respectively, in maize plants treated on roots with BDI-IS1 followed by inoculation with* E. turcicum *at 4.5 X* $10^4$ *conidia/ml at 3 dpi (A). AOS1 was highly expressed in plants treated on roots with BDI-IS1 followed by inoculation with* E. turcicum *at 4.5 X* $10^4$ *conidia/ml at 7 dpi (C). Data presented are the means of four biological samples replicated in duplicate. Each biological sample consists of six leaves collected from three plants (2 leaves per plant) and pooled to form one sample. Gene expression analysis was performed using CFX manager software and the expression of these genes was normalized using the control samples as well as the three housekeeping genes: actin, EF and EIF (Figure 7-A2 & Table A2). *Significance of the normalized expression of the target gene in the sample compared to its expression in the control sample.*

### 7.4.5. Localization of the BDI-IS1 in maize plant

To decipher how BDI-IS1 interacts with maize plants, a bioassay was conducted to determine the preferred niche occupied by BDI-IS1 after root treatment. GFP-transformed BDI-IS1 was either applied to maize roots or used to coat maize seeds, and its subsequent localization on or within the roots, stems, and leaves was determined using confocal microscopy five- and twelve-days post-treatment in bioassay 1 and bioassay 2, respectively. For both treatment methods, the bacteria were detected in the rhizosphere surrounding the roots, but no bacteria were observed on or within the stems or leaves of maize plants. Additionally, the spread of bacterial suspension on roots led to a high number of bacteria, while seed coating resulted in a localized bacterial niche closely associated with the absorbent root hairs. A few bacterial cells were detected within the cortical cells, although no bacteria were visible in the root vessels (Figure 7-7B).

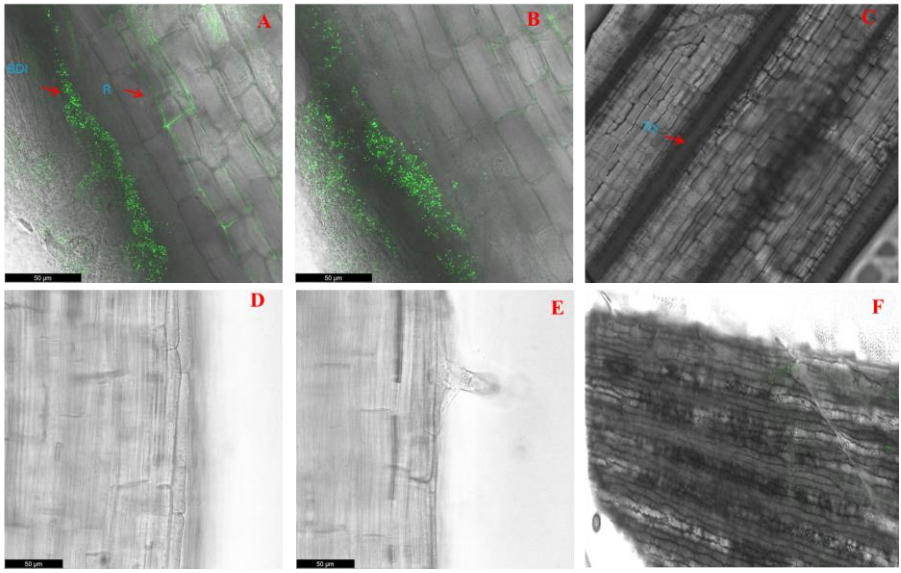


Figure 7-7: Localization of BDI-IS1 on the maize plant using confocal microscopy. A*&B: Root taken from plant treated with GFP-transformed BDI-IS1 at the root, D&E: Root taken from untreated plant, C&F: Stem and leaf taken from plant treated with GFP-transformed BDI-IS1 at the root. Microscope objectives were set at 63X. BDI: GFP-transformed BDI-IS1, R: root, xy: xylem.*

## 7.5. Discussion

The first part of this study investigates the biocontrol efficacy of BDI-IS1 in controlling Northern corn leaf blight (NLB) under field conditions. The biocontrol efficacy of BDI-IS1 against NLB was first assessed during the two main growing seasons A and B of Burundi. Several applications of BDI-IS1 on the leaves of maize resulted in a significant reduction in NLB severity under field conditions. The disease reduction varied between trials, with a higher protection (34%) observed in the first trial, carried out in season A compared to season B (11%). This better protection observed in season A could be linked to disease pressure, which was higher in season B. These results demonstrated that BDI-IS1 is capable of limiting the disease under field conditions in Burundi. The biocontrol efficacy of *Bacillus* spp. upon leaf treatment against NLB had already been reported in a previous

assay in Argentina by Sartori et al. (2017). This author reported a disease reduction slightly higher than the protection obtained in this study. The difference in NLB reduction after leaf treatment with BDI-IS1 and another *Bacillus* spp. could be explained by several reasons, linked to the genomes and the metabolites produced by the biocontrol agents, the environments as well as to the disease pressure. The virulence of the strains of *E. turcicum* prevalent in Burundi and Argentina can also differ. Furthermore, the difference in biocontrol efficacy of the two BCAs could also be explained by the treatment method used. Sartori et al. (2017) sprayed a surfactant-based vegetable oil before applying the bacterial cell-free supernatant to maize leaves. The use of this surfactant was not included in our trial, although it could enhance bacterial attachment and colonization, thereby improving the protective efficacy of BDI-IS1 against NLB.

A higher reduction in the severity of NLB was observed in season A, from September to January, compared to season B, from February to May. It could be explained by contrasting disease pressure. In season A, the population of *E. turcicum* is still low and gradually increased after moderate temperature and high relative humidity, which occurred in December towards the end of season A. The positive correlation between NLB severity or incidence and relative humidity has been reported by several authors in different pathosystems (Jackson-Ziems, 2016; Kapange et al., 2019; Sharma, 2023). The higher reduction in NLB severity observed during season A could also be explained by the late infection of *E. turcicum* observed at the flowering stage and by a potential lower inoculum leading to lower disease pressure. This gave the bacteria a chance to colonize the maize plants and produce the compounds needed to fight the pathogens. The effect of pathogen pressure on the biocontrol efficacy of BCA has also been reported by several authors (Collinge et al., 2022; Desmedt et al., 2021; Flors et al., 2024), where BCA, which acts by inducing plant resistance, is often ineffective under high pathogen pressure. The high NLB severity observed in season B may be explained by the overlapping of fields cropped with young and mature plants, resulting in the continuous

presence of maize plants or residues, which are source of *E. turcicum* inoculum (Carpane et al., 2020; De Rossi et al., 2022; Jakhar et al., 2017).

The protection obtained with BDI-IS1 against NLB in the field following leaf treatment was two times lower than the previously reported disease reduction in greenhouse (Nimbeshaho et al., 2024). This slighter reduction of biocontrol efficacy under field conditions was also reported by several authors in various pathosystems (Bardin et al., 2015; Bonaterra et al., 2012; Nicot, 2011) and could be explained by the environmental conditions prevailing in the field. The bacterial cell suspension sprayed on maize leaves in the field are exposed to high temperatures (>30°C) and to ultraviolet radiation, which can be harmful to bacterial cells, especially as BDI-IS1 was isolated from the rhizosphere (Nimbeshaho et al., 2024). High rainfall occurring in Mparambo probably could lead to run-off of the BDI-IS1 bacterial cells applied to the maize leaves. An enhanced formulation to promote bacterial adherence and longevity should be taken into account for foliar treatments.

The encouraging results of the first assays performed in fields and the fact that BDI-IS1 was more effective against NLB when applied to the roots under controlled conditions (Nimbeshaho et al., 2024) led us to try to multiply BDI-IS1 on lignocellulosic substrates and produce spore formulation of BDI-IS1, designed for a soil application. The three tested substrates, including bean, maize or rice, resulted in the production of more than $10^{14}$ CFU/g of bacterial spores. This concentration was reported to be sufficient to induce protection against pathogens (Khan Amir et al., 2023). The application of these bioproducts on soil at the sowing in a tunnel experiment resulted in a significant reduction in NLB severity, showing that the three tested lignocellulosic substrates were suitable for the production of high quantities of bacterial endospores and assumed their potential bioactivities and production of secondary metabolites active against the disease. The efficacy of these residues as substrates for bacterial growth is explained by their high content of

carbon, sulfur, calcium and iron nutrients used by this bacterium for growth and reproduction (Ndabacekure, 2022). Several authors have also reported the importance of carbon content in lignocellulosic-based bioproducts for bacterial growth and reproduction (Gopalakrishnan et al., 2016; Khan et al., 2023; Llamas et al., 2023). Carbon is an energy source used by bacteria and is mainly found in cellulosic or hemicellulosic compounds of plant tissues, including bean, which is produced in all growing seasons (A, B and C) in Burundi and is therefore widely available. Our work shows that BDI-IS1 degrades cellulose, the main component of the lignocellulosic substrates used in this study, as source of carbon (Mugisho, personal communication). This feature of BDI-IS1 is further supported by Nimbeshaho et al. (in preparation), who characterized the genome of BDI-IS1 and identified a set of genes associated with polysaccharide degradation. These include α-amylases (e.g., *amyE*) for starch degradation (Rajesh and Gummadi, 2022), cellulases (e.g., *licH, bglA, licABC,* and *yckE*) involved in the breakdown of cellulose, hemicellulose, and related products (Ejaz et al., 2021; G. Liu et al., 2023), pectate lyases (e.g., *pelBC*) involved in the degradation of plant pectin (Boubsi, 2019; Pavlović et al., 2024), and enzymes such as laccase, heme-containing peroxidase, and manganese catalase, which are implicated in the biodegradation of lignin compounds present in crop residues (Dhankhar et al., 2020; Pfanzagl et al., 2018; Rashid and Bugg, 2021).

The biocontrol efficacy of a bean-based bioproduct against NLB was then assessed in the field at the Mparambo Research Centre. The bioproduct applied in seed-bed at sowing period, provided significant protection against NLB compared to the control plants. However, the protection was lower than observed in the tunnel experiment. This difference could also be explained by the same reasons explained above linked to the environmental conditions prevailing in the field, as well as to the fitness of the bacteria or the competition with other microorganisms from the soil. The current bioformulation of BDI-IS1 on lignocellulosic substrates gave promising results. It opens windows for further improvement by mixing with other compatible

microorganisms or working with other potential carriers such as peat, which is readily available in Burundi. The use of multiple strains of biocontrol agents has been reported to limit the inconsistency of biocontrol efficacy observed under field conditions (Price-Christenson and Yannarell, 2023). In addition, the biocontrol efficacy of bacteria could be enhanced by combining them with beneficial fungi. This approach has been demonstrated by Anckaert et al. (2024), who reported that the combination of *Bacillus velezensis* GA1 and *Rhizophagus irregularis* improved the general fitness of both partners and increased plant protection. Assays combining BDI-IS1 with *Rhizophagus irregularis* could be conducted, as well as those combining BDI-IS1 with *Trichoderma harzianum* KUFA0710, which has been shown to be active against NLB (Limdolthamand et al., 2023).

The second part of this work aims at give insights into the modes of action of BDI-IS1. BDI-IS1 was shown to express direct antagonism against several species *in vitro* and *in vivo*. This bacterium produces several secondary metabolites, including iturin A, surfactin and bacillibactin, that can be involved in direct antagonistic activity or in the induction of plant defense (Nimbeshaho et al.,2024). The lower efficacy of BDI-IS1 impaired in iturin, surfactin and bacillibactin production in controlling NLB compared to the wild type of the strain, when applied on leaves, suggested that both secondary metabolites could be involved in the direct antagonism. In a recent study, we reported that the iturin deficient-BDI-IS1 mutant loss its inhibition against *E. turcicum* mycelial growth, showing that production of iturin A may be tightly involved in local restriction of *E. turcicum* development on leaves (Nimbeshaho et al., 2024). The direct antagonistic activity of iturin produced by *Bacillus* spp. is reported in several pathosystems. Application of iturin-rich extract from *Bacillus subtilis* strain ET1 significantly reduced fruit rot diseases caused by *Penicillium digitatum* and *Botrytis cinerea*, with a 74% and 68% reduction in disease incidence, respectively (Ambrico and Trupo, 2017). Iturin A has also been identified as responsible for the direct activity of *Bacillus velezensis* GA1 against *Sclerotium rolfsii* infecting

groundnut in the Democratic Republic of Congo (DRC) (Korangi Alleluya et al., 2023). The mechanism of action of iturin A is reported to involve the formation of ion-conducting pores and disruption of pathogen cell membranes (Wang et al., 2013). In contrast, the role of surfactin in direct antagonism against fungal pathogens is rarely reported (Salazar et al., 2023). In our experiment, we also observed a high level of disease severity on plants treated on leaves with bacterium impaired in bacillibactin (ΔdhbC) production, suggesting a potentially high involvement of this secondary metabolite in the direct activity of BDI-IS1 against *E. turcicum*. In our previous *in vitro* assay using the ΔdhbC, we also suggested a relatively moderate antagonistic activity of bacillibactin in inhibiting the mycelial growth of *E. turcicum* (Nimbeshaho et al., 2024). The efficacy of bacillibactin in direct antagonism against *Fusarium oxysporum* affecting tomato plants was also reported by Dimopoulou et al.(2021). These authors hypothesized that the direct antagonism of bacillibactin against this pathogen is due to the co-localization of gene clusters involved in both bacillibactin and antimicrobial synthesis. The same hypothesis would explain why BDI-IS1 impaired in bacillibactin production does no longer produces the antibiotics or other enzymes involved in direct antagonism against pathogens. Furthermore, bacillibactin was also reported to play a key role in bacterial colonization (Dimopoulou et al., 2021). This could be further validated for BDI-IS1 by assessing the leaf colonization ability of BDI-IS1 impaired in bacillibactin production, compared to the wild type.

Based on our results, iturin, surfactin and bacillibactin produced by BDI-IS1 may act synergistically to antagonize *E. turcicum*. However, other potential secondary metabolites non tested *in planta* such as bacilysin could also contribute to the direct antagonism of BDI-IS1 against NLB, since the inhibition efficacy of these mutants against mycelial growth of *E. turcicum* was slightly reduced *in vitro* assay (Nimbeshaho et al., 2024).

The biocontrol efficacy of BDI-IS1 against NLB through indirect action (action not directly targeting the fungus) was assessed by evaluating the performance of this bacterium impaired in iturin and surfactin production when applied to the roots (soil), compared to the wild-type and untreated plants. The reduction in activity of the mutant suggests a potential role of surfactin in ISR in maize against NLB. This indirect effect of BDI-IS1 against NLB could probably be linked to the stimulation of the plant defenses against pathogens, as reported in several studies (Adeeb et al., 2022; Korangi Alleluya et al., 2023; Ongena et al., 2007). Indeed, monitoring the location of bacteria after root treatment using confocal microscopy shows that the GFP mutant BDI-IS1 remains and develops consistently in the rhizosphere but did not migrate to the leaves or the vessels. Since the bacteria is not translocated to aerial parts of the plants, a signal is probably sent from the roots to the aerial part of the plant. The induction of systemic resistance by surfactin has been reported by several authors for various maize pathosystems. Adeeb et al. (2022) demonstrated that *B. velezensis* M20 induced systemic resistance in maize infected with *Rhizoctonia solani*, leading to a high expression of defense-related enzymes, including *PAL*, superoxide dismutase, ascorbate peroxidase, catalase (CAT), and chitinase.

Similarly, root treatment with *B. velezensis* GA1 or pure surfactin significantly reduced stem rot severity and incidence in groundnut by inducing systemic resistance (Korangi Alleluya et al., 2023). A similar role of surfactin in inducing systemic resistance has also been demonstrated in bean and tomato plants affected by *Botrytis cinerea* (Ongena et al., 2007).

The upregulation of *AOS1*, *PAL* and *MYC7* after the root treatment with BDI-IS1 are supporting this hypothesis. *AOS1* is a key enzyme involved in JA biosynthesis (Farmer and Goossens, 2019) and the JA signaling plays a central role in plant defenses. JA is synthesized in response to mechanical injury or in case of biotic or abiotic stress and is derived from unsaturated fatty acids, requiring enzymes such as lipoxygenase

(*LOX*), *AOS*, and allene oxide cyclase (*AOC*), which sequentially produce 13-hydroperoxide, OPDA, and JA (Borrego and Kolomiets, 2016; Sugimoto et al., 2022). High expression of *AOS1* has also been reported in the protection of maize treated with *Trichoderma gamsii* IM05 against *Fusarium verticillioides* (Galletti et al., 2020). It has been identified in the sugarcane genome as contributing to resistance against *Sporisorium scitamineum* (Sun et al., 2023). Our results revealed a significant upregulation of *MYC7* in treated and inoculated plants compared to inoculated plants alone. Similarly, *MYC7* has been reported to be upregulated in the roots and stems of maize treated with *Bacillus velezensis* SQR9 in response to *Fusarium graminearum* (Cao et al., 2023). *MYC7* is a transcription factor known as a master regulator of JA-dependent signaling responses. Upon activation by JA, *MYC7* is released from the JAZ complex and initiates the transcription of defense genes involved in downstream JA biosynthesis pathways (H. Cao et al., 2023; Fu et al., 2020). The high expression of *MYC7* could trigger defense responses mediated by the JA signaling pathway, including MAPK cascades, calcium channels, and other physiological and biochemical processes associated with plant growth regulators such as ethylene, salicylic acid, abscisic acid, and others (Maithani et al., 2021).

In turn, *PAL* is involved in the phenylpropanoid pathway, leading to the production of bioactive compounds such as SA phytohormone and other metabolic compounds including phenolics, phytoalexins, flavonoids, and lignin, which are toxic to many pathogens (Klessig et al., 2018). Previous studies reported that *PAL* was significantly upregulated in maize treated with *Klebsiella jilinsis* 2N3 after inoculation with *E. turcicum* (Zhang et al., 2021) and maize roots and stems treated with *Bacillus velezensis* SQR9 to control *Gibberella* stem rot (Y. Cao et al., 2023). These findings suggest that BDI-IS1 may also induce the SA signaling pathway to protect maize against NLB.

Thus, BDI-IS1 could trigger maize defense against *E. turcicum* by activating both JA and SA signaling pathways. To the best of our knowledge, this is the first report of BDI-IS1, a member of the *Bacillus*

genus, demonstrating the activation of both JA and SA pathways to confer protection to maize against NLB. Previously, *B. velezensis* SQR9 was reported to induce systemic resistance via both pathways in maize affected by *Fusarium graminearum* (Y. Cao et al., 2023). Co-activation of these two pathways may provide strong protection against a broad range of pathogens, including both biotrophic and necrotrophic pathogens (Maithani et al., 2021). In a previous experiment, we demonstrated the potential of BDI-IS1 to control early blight in tomato and NLB in maize, caused by *A. solani* and *E. turcicum*, respectively, representing necrotrophic and hemibiotrophic pathogens (Nimbeshaho et al., 2024). The broad-spectrum activity of BDI-IS1 against multiple pathogens is particularly important for disease control in the Great Lakes region, where the climate is conducive to the development of multiple plant pathogens (Nihorimbere et al., 2024).

## 7.6. Conclusion

BDI-ISI is active in planta and in the field against NLB after foliar or root application. Bacterial spores of BDI-IS1 produced on lignocellulosic residues constitute active bioproducts that can be easily produced locally in tropical areas. The bacterium produces several bioactive metabolites that act directly against the pathogen or by stimulating the plant's defenses. Its foliar activity is attributed in part to the production of iturin, which has fungicidal activity. Its activity on plant defense after root application could be linked to surfactin.

Appendix

Table 7-A1: Primers used to quantify defense genes by RT-qPCR.

| Genes | Forward primer Sequence | Reverse primer sequence | Amp.size (bp) | An.tem (°c) | Reference |
|---|---|---|---|---|---|
| *Actin* 2 | 5' CTG-AGG-TTC-TAT-TCC-AGC-CAT-CC 3' | 5' CCA-CCA-CTG-AGG-ACA-ACA-TTA-CC 3' | 133 | 60 | Li et al. (2014) |
| *EF1α* | 5' TGG-GCC-TAC-TGG-TCT-TAC-TAC-TGA 3' | 5' ACA-TAC-CCA-CGC-TTC-AGA-TCC-T 3' | 135 | 60 | Li et al. (2014) |
| EIF4A | 5' CGT-CCA-GAG-GTT-CTA-CAA 3' | 5' CAT-CCT-TCG-CCA-CAA-TAC 3' | 183 | 53 | Li et al. (2014) |
| *PR5* | 5' TTG-TGT-GCG-TGC-TTC-GTA 3' | 5' CTT-GGT-GCT-CTT-AAG-CGA-AAT-G 3' | 127 | 57 | This study |
| *PR4* | 5' ATC-TCA-TGC-ACG-CCG-ATC-ACA 3' | 5' ATG-GCC-AGA-GCT-AGG-AGG-CA 3' | 94 | 60 | This study |
| *PAL* | 5' GGG-AGA-TGA-AGT-GAA-GTG-AAG-A 3' | 5' ACA-GTG-AGC-ACC-TTG-AAG-AC 3' | 100 | 55 | This study |
| *OPR2* | 5' GCG-TAT-GGG-AAG-CTG-TTC-TT 3' | 5' GTG-GAG-CGG-TCG-TAT-TTG-TT 3' | 102 | 60 | This study |
| *LOX10* | 5' CAT-CGA-CGA-GTG-CAA-CAA-CA 3' | 5' TGG-ACT-AAT-GCA-TGC-TGA-GGA 3' | 158 | 55 | Guche et al. (2022) |
| MYC7 | 5' TAT-ATG-GAT-CAG-CCG-TCG-TCC-T 3' | 5' TCC-ATT-GCA-AAG-CGA-AAT-CCT-G3' | 88 | 60 | Fu et al. (2020) |
| *AOS1* | 5' CCC-AGC-GTC-GAC-AAC-AA 3' | 5' TGT-CGT-AGC-GGA-GGA-AGA 3' | 91 | 57 | Shumilak, 2021 |

Table 7-A2: Coefficient of variation (CV) and stability (M)of housekeeping genes at different tested time points.

| **Genes** | **Time point (hour)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **0** | | **9** | | **72** | | **168** | | **312** | |
| | CV | M | CV | M | CV | M | CV | M | CV | M |
| Actin | 0.1062 | 0.239 | 0.1028 | 0.464 | 0.2192 | 0.5064 | 0.1956 | 0.4669 | 0.2366 | 0.5004 |
| EF | 0.0489 | 0.1947 | 0.2791 | 0.6275 | 0.1251 | 0.43 | 0.1805 | 0.4669 | 0.0518 | 0.3542 |
| EIF | 0.1521 | 0.3233 | 0.2887 | 0.655 | 0.2532 | 0.6082 | 0.3565 | 0.785 | 0.2261 | 0.5258 |
| **Mean** | **0.1024** | **0.2523** | **0.2239** | **0.5822** | **0.1992** | **0.5149** | **0.2442** | **0.5729** | **0.1715** | **0.4601** |

Table 7-A3: Daily analysis of NLB severity obtained in trial 1&2 carried out under field at Mparamba research center. *DAS: day after sowing; a and b letters indicate the significance level with same or different letters for absence and presence of significance, respectively.*

| **Trial1** | | | | **Trial2** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | 67DAS | 74DAS | 81DAS | 32DAS | 39DAS | 46DAS | 53DAS | 60DAS | 67DAS | 74DAS | 85DAS |
| BDI-IS1 | 42.5±2.52a | 45±2.58a | 48.5±1.91a | 35.5±1.91a | 41.5±2.31a | 42±2.31a | 45.5±1.91a | 51.9±1.73a | 57.4±1.90a | 58.5±1.91a | 61.9±2.69a |
| QST713 | 47±2.58a | 51.5±4.43b | 73.5±5.97b | 38±1.63a | 41.5±1a | 41.5±1a | 49.5±4.73ab | 56±4.90ab | 61.6±1.08ab | 61.6±1.08ab | 63.8±2.78a |
| Control | 47±2.58a | 56±2.83b | 72±2.31b | 38.5±1.91a | 46.5±3.79b | 47.5±3.42b | 52±3.65b | 57.5±4.12b | 64.6±0.950b | 66.1±2.73b | 69.7±3.31b |

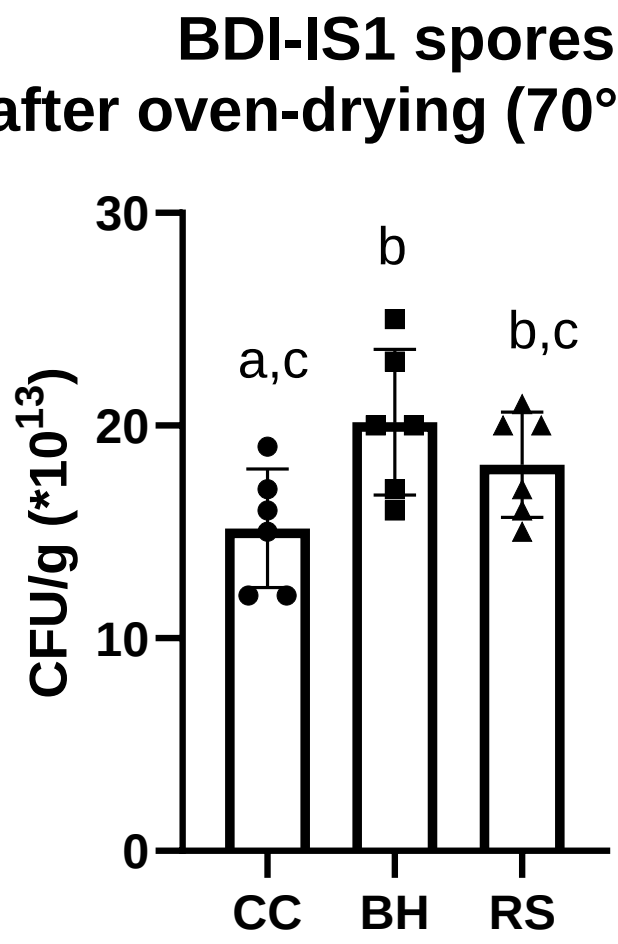


Figure 7-A1: Number of colonies from BDI-IS1 grown on lignocellulosic substrates. *CC: corn cob (maize residues), BH: bean residues, RS: Rice residues. The different letters indicate the level of significance, while the treatment with the same letters indicate that differences are not significant at 5% threshold*.

Table 7-A4: Variation of disease severity per day between inoculated substrates.
*The analysis was performed by using GLM where substrates, treatment and date post inoculation (dpi) were fixed variables and blocks are random variables. Letters indicate the significance of variance, with the same or different letters corresponding to the absence or existence of difference significance between treatments, respectively.*

| Substrates | 4 dpi | 7dpi | 9dpi | 11dpi | 14dpi | 16dpi | 18dpi | 21dpi | 23dpi | 25dpi | 28dpi | 30dpi |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BDI-IS1+ bean residues | 10 a | 11.67 a | 13.33 a | 13.33 a | 15.83 a | 16.67 a | 16.67 a | 16.67 a | 16.67 a | 16.67 a | 18.33 a | 19.17 a |
| BDI-IS1+ rice residues | 10 a | 11.67 a | 12.50 a | 12.50 a | 17.50 a | 18.33 a | 19.17 a | 19.17 a | 19.17 a | 19.17 a | 19.17 a | 19.17 a |
| BDI-IS1 + maize residues | 10 a | 12.50 a | 15.83 a | 16.67 a | 20.00 a | 21.67 a | 22.50 a | 23.33 a | 23.33 a | 23.33 a | 23.33 a | 23.33 a |
| Control bean residues | 22.5 b | 25.00 b | 29.17 b | 31.67 b | 36.67 b | 43.33 b | 48.33 b | 52.50 b | 61.67 b | 70.83 b | 71.67 b | 74.17 b |
| Control rice residues | 22.5 b | 26.67 b | 30.83 b | 32.50 b | 39.17 b | 44.17 b | 50.00 b | 53.33 b | 62.50 b | 74.17 b | 75.00 b | 77.50 b |
| Control maize residues | 23.33 b | 27.50 b | 31.67 b | 35.83 b | 43.33 b | 46.67 b | 54.17 b | 55.83 b | 65.83 b | 73.33 b | 73.33 b | 76.67 b |

**Selection of housekeeping or reference genes**

The reference genes reported in literature were tested to normalize the transcripts of the tested maize defense genes. Five genes including *Actin*, *EF*, *EIF*, *Tubulin* and *FGP* reported to be expressed in maize were amplified and quantified by RT-qPCR on a set of representative samples. The profile expression of these reference genes was performed and four of them (*Actin*, *EF*, *EIF*, *Tubulin*) were shown to be stable with an average cq varying from 25 to 35 (Figure 7-A2). However, the stability analysis of these reference genes was carried out using CFX manager software and three of them including *Actin*, *EF*, *EIF* with coefficient of variation (CV) less than 0.5 and the mean stability value (M) below 1, were selected and was used to normalize the expression of gene of interest (Hellemans et al. 2007). The average CV was between 0.1 and 0.2 while M stability varied between 0.2 and 0.5, corresponding to the homogenous expression of the tested reference genes.

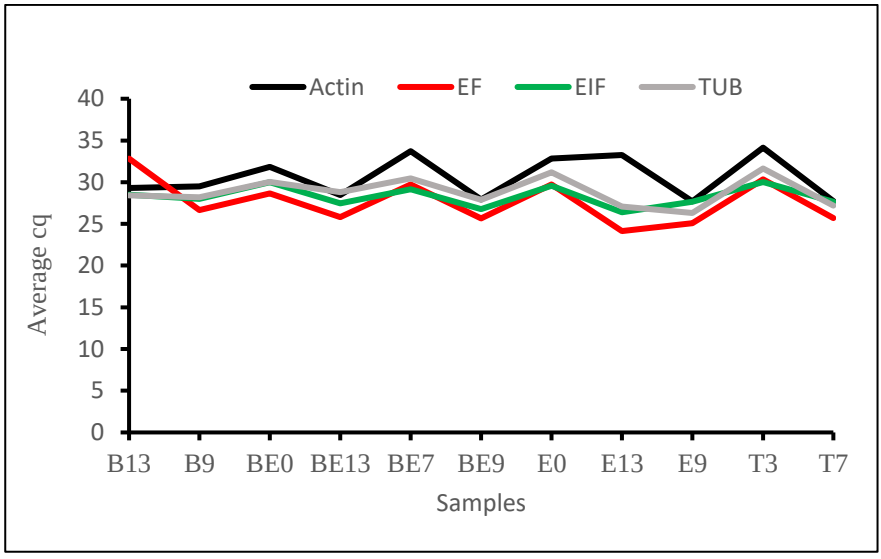


Figure 7-A2: Expression profile of housekeeping genes based on cq value obtained from qPCR assay.
*B: BDI-IS1, BE: BDI-IS1* + E. turcicum, *E.* E. turcicum, *T: Control. The numbers represent the time points, Oh, 9hpi, 3 dpi, 7 dpi, 13 dpi. Hpi: hour post inoculation, dpi: day post inoculation. Cq: quantification cycle.*

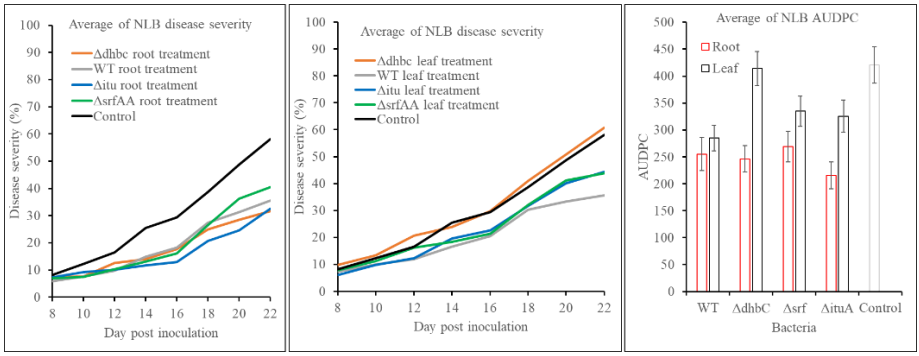


Figure 7-A3: Assessment of biocontrol efficacy of *Bacillus nakamurai* BDI-IS1 impeded in production of secondary metabolites (iturin A, surfactin and bacillibactin) against Northern corn leaf blight.
*Disease severity obtained from plants treated or not on roots with mutants or BDI-IS1 and inoculated or not with* Exserohilum turcicum (left), *disease severity obtained from plants treated or not on leaves with mutants or BDI-IS1 and inoculated or not with* Exserohilum turcicum (middle). *AUDPC calculated from disease severity recorded on plants treated or not on roots or leaves with mutants or BDI-IS1 and inoculated with* E. turcicum *(right). Each point of disease severity is an average of 16 plants per treatment, while the represented AUDPC is an average of 4 replicates per treatment. The standard deviation was also calculated from the 4 values of replicates*.

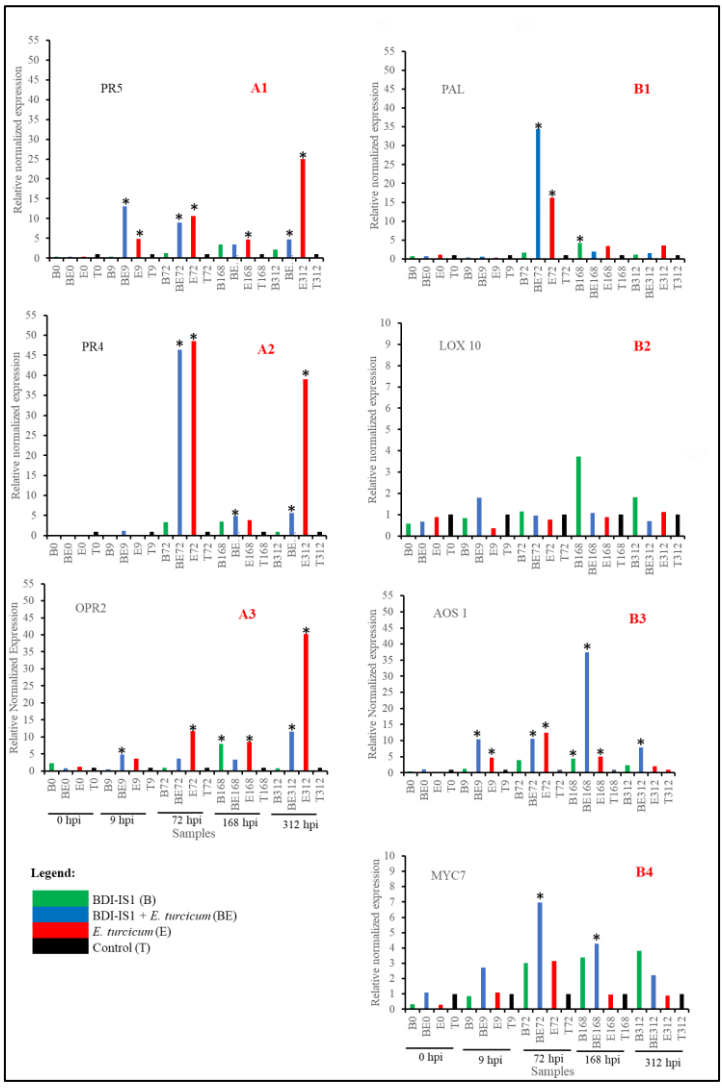


Figure 7-A4: Differential expression of defense genes in maize plants treated or not with BDI-IS1 and inoculated or not with *E. turcicum.*

*Samples were taken at 0 h, 9 hpi, 72 hpi (3 dpi), 168 hpi (7 dpi) and 312 hpi (13 dpi) after inoculation. A1: PR5; A2: PR4, A3: OPR2, B1: PAL, B2: LOX 10, B3: AOS 1, B4: MYC7. Samples are grouped according to the time of collection, B: bacteria only, BE: bacteria +* E. turcicum, *E:* E. turcicum, *T: control (untreated and uninoculated). Analysis was performed using CFX manager software. *Significance of expression compared to control.*

# GENERAL DISCUSSION AND CONCLUSION

# CHAPTER VIII.

# GENERAL DISCUSSION

## 8.1. Changing agricultural practices: embracing agroecology to reduce chemical pesticides in agriculture

Plant diseases and pests are increasingly affecting crop production and are commonly controlled by chemical pesticides. In Burundi, various diseases and pests impact crops both in the field and during storage, causing significant damage to crop production and food security (Nihorimbere et al., 2024; Ochilo et al., 2022; Okonya et al., 2019a). Although the use of chemical pesticides often provides rapid and effective solutions to plant diseases and pests, it has also led to adverse effects on human, animal, and environmental health (Ahmad et al., 2024; Zhou et al., 2024). In the Great Lakes countries of Africa, including Burundi, Rwanda, and the Democratic Republic of the Congo (DRC), the negative effects of chemical products are exacerbated by the use of highly toxic compounds, over 30% of which are not authorized in European countries (Nihorimbere et al., 2024). In Burundi, this situation is worsened by the failure to apply proper pesticide management practices. Misuse of chemical pesticides has been reported, including overdosing and the inappropriate use of fungicides in place of insecticides, and *vice versa* (Nihorimbere et al., 2024; Okonya et al., 2019b). This could lead to the development of pathogen resistance, the increase in pest or pathogen populations, and consequently to the absence of treatment efficacy and significant losses in crop production.

Therefore, there is an urgent need to rethink current crop protection practices to ensure sustainable diseases and pests control while securing food production for both present and future generations. Agroecological crop protection, derived from integrated pest and

disease management (IPM) is focusing on the factors that make agroecosystems more resilient to diseases and pests, are widely recognized as a holistic approach that integrates ecological principles into agriculture (Altieri et al., 2024). In practice, agroecology contributes to improving biodiversity, nutrient cycling, and soil health, as well as promoting better integrated pest and disease management with minimal external inputs to the ecosystems (Dushyant et al., 2024). It comprises good agricultural practices and biological control to substitute chemical pesticides and has proven effective in managing plant diseases and pests that affect crops (Deguine et al., 2023; Deguine and Penvern, 2014; Finger et al., 2024). Biological control involves the use of beneficial microorganisms to control plant diseases and pests (Jeanneret et al., 2021; Wezel et al., 2014). Various microorganisms, including bacteria and fungi, have been used, with bacteria being the most significant biocontrol agents, accounting for 75% of the bioproducts available on the market (Dunlap, 2019). These bacteria consist mainly of the *Pseudomonas* and *Bacillus* genera, which have demonstrated high biocontrol efficacy against numerous pathogens (Ali et al., 2024; Balthazar et al., 2022; Etesami et al., 2023b; Lee et al., 2023).

*Bacillu*s spp. have significant potential for agricultural applications due to their broad adaptability, spore-forming ability, and high production of secondary metabolites (Etesami et al., 2023b; Fira et al., 2018; Ongena and Jacques, 2008). Some species of this genus allocate more than 5-10% of their genome to the production of secondary metabolites including non-ribosomal lipopeptides, ribosomal lipopeptides, polyketides, and volatile organic compounds (Andrić et al., 2023; Caulier et al., 2019; Kaspar et al., 2019; Shahid et al., 2021).

The genus B*acillus* is diverse and contains many species clustered in *Bacillus subtilis* and *B. cereus* groups. The *B. subtilis* group comprises the species mostly used in agriculture including *Bacillus amyloliquefaciens*, *B. velezensis*, *B. siamensis*, and the recently discovered *B. nakamurai* (Caulier et al., 2019; Mageshwaran, 2024;

Wang et al., 2013). Most of these species are already available on the market, and their antifungal activities have been reported in various pathosystems, except for *B. nakamurai*, which is poorly documented. Five strains of *B. nakamurai* have been reported, namely NRRLB-41091, NRRLB-41092, MZ03-67, B-41093, and BDI-IS1 (Nimbeshaho et al., 2024). Their biocontrol activities are poorly documented, except the strain NRRLB-4091, which inhibits *Pectobacterium carotovorum* (Leathers et al., 2020). The biocontrol efficacy of the other strains has not yet been tested, although these bacteria have demonstrated broad adaptability to different environments, with pH levels ranging from 5.5 to 10 and temperatures from 17 to 50°C (Dunlap et al., 2016). Both NRRLB-41091 and NRRLB-41092 were isolated from tropical regions of Argentina, while BDI-IS1 was isolated in Burundi, indicating a wide adaptation potential for controlling prevalent plant diseases from this area. In the following paragraphs, we will discuss the efficacy of BDI-IS1 in controlling plant diseases occurring in Burundi, the mechanisms by which this bacterium antagonizes pathogens, its implication for crop protection in Burundi, and potential research areas for developing BDI-IS1-based bioproducts.

## 8.2. BDI-IS1 controls tomato early blight and Northern corn leaf blight

Bacterium *B. nakamurai* BDI-IS1 was isolated from soil previously used for potato cultivation at Isare (OAP, a private agricultural research and extension center) in Burundi. Our results showed that BDI-IS1 exhibits high activity against several phytopathogens, including bacteria and fungi. BDI-IS1 has antagonistic activity against major phytopathogenic bacteria, including *Clavibacter michiganensis*, *Rhodococcus fuscans*, *Pseudomonas syringae*, *Xanthomonas campestris*, and *Pectobacterium carotovorum*. Additionally, BDI-IS1 acts against a wide range of economically significant phytopathogenic fungi, including *Aspergillus niger*, *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Pyricularia oryzae*, *Colletotrichum* sp., *Ascochyta rabiei*, *Pseudocercospora griseola*, *Alternaria solani*, and

*Exserohilum turcicum.* This observation is an important feature for the development of a biological control agent, which can demonstrate broad activity against numerous pathogens to ensure competitiveness and successful colonization. This characteristic is also beneficial for a potential biocontrol agent to be suitable for tropical regions, where multiple pathogens can occur simultaneously under field conditions (García-Guzmán and Heil, 2014; Ghini et al., 2011; Nazarov et al., 2020). The *in vitro* antagonistic efficacy of BDI-IS1 against several bacteria and fungi was comparable to that of the commercial strain *B. velezensis* QST713 (Nimbeshaho et al., 2024). This further supports the selection of BDI-IS1 as a promising candidate.

We then tested the biocontrol efficacy of BDI-IS1 *in planta* under greenhouse and field conditions. The efficacy of BDI-IS1 was also compared with that of the commercial biocontrol agent QST713, with both treatments applied to the roots or leaves to assess their indirect and direct biocontrol efficacy against TEB and NLB, respectively. Our assays in greenhouse demonstrated the biocontrol efficacy of BDI-IS1 through root and leaf treatments against TEB and NLB. This shows the spectrum of activities of this bacterium against at least two important diseases caused by necrotrophic pathogen *A. solani* and hemibiotrophic pathogen *E. turcicum*. It also suggests that BDI-IS1 has multiple modes of action, including direct antagonism against pathogens (tested *in vitro* and in the greenhouse *via* leaf treatment) and indirect antagonism by interacting with plants or by translocating from roots to leaves within the plants (tested in the greenhouse *via* root treatment). This versatility is a significant advantage of BDI-IS1, as its multiple modes of action can be activated to control various plant diseases. This presents an important opportunity for crop protection in tropical regions like Burundi, where the climate is conducive to the development of major diseases and farmers have limited or no access to chemical pesticides. In our trials, a higher disease reduction was observed with NLB compared to TEB, which may be attributed to the strong inhibitory activity of BDI-IS1 against *E. turcicum* observed *in vitro*. Furthermore, the disease reduction in plants treated with BDI-IS1 was even greater

than those treated with QST713. This advantage can stem from the high adaptability of BDI-IS1 to tropical climates, in contrast to QST713, which was isolated from a temperate region (EFSA, 2021). The experiments were conducted at UCLouvain in greenhouse, with an average temperature of 22°C, which could provide the preferred native climatic conditions for BDI-IS1, facilitating its development and production of a wide range of compounds that enhance plant colonization, growth, and protection.

After the experiments under controlled conditions, the biocontrol efficacy of BDI-IS1 against NLB was tested under field conditions in Burundi. Our assays resulted in a significant reduction in disease following foliar treatment with BDI-IS1, and this bacterium led to a higher disease reduction compared to QST713. This superior performance could also be attributed to the environmental conditions occurring in Burundi and suitable to BDI-IS1, as a native strain, which consistently outperformed QST713 in all experiments. The high adaptability of BDI-IS1 under field conditions could potentially be explained by the dark pigment of its endospores, which contains melanin produced through the oxidation of phenolic compounds. This melanin provides tolerance to various biotic and abiotic stresses, including ultraviolet radiation, drought, and high temperatures (Singh et al., 2021). Melanin synthesis involves enzymatic families such as tyrosine, laccases, and polyketides (Ghadge et al., 2023; Singh et al., 2021). Among these, laccases and polyketides were predicted to be produced by BDI-IS1, and could thus contribute to the dark pigment of BDI-IS1 (Nimbeshaho, in preparation) and enhance its resistance to the harsh environmental conditions typical of tropical regions (Dunlap et al., 2016; Jacobs et al., 2004; Lønborg et al., 2021; Moeller et al., 2005).

The consistent biocontrol efficacy of BDI-IS1 was demonstrated in this work in all tests conducted *in vitro,* in the greenhouse, and in the field. The multiple modes of action of BDI-IS1 could account for its consistent biocontrol efficacy through direct or indirect antagonism. These mechanisms may also act synergistically, providing greater

protection. Several biocontrol agents demonstrate efficacy *in vitro* and in the greenhouse but fail under field conditions (Jones and Samac, 1996; Schisler et al., 2002; Spadaro and Gullino, 2005). The activity of a biocontrol agent is dependent to the plant host and pathogen, suggesting that the observed disease reduction results from the interaction among the three components: the plant, the pathogen, and the biocontrol agent (Bonaterra et al., 2022). Plants or pathogens may produce toxic compounds that inhibit biocontrol agents from colonizing and growing in the targeted plant niche, thereby limiting disease reduction. Additionally, the pathogen may be virulent to susceptible plants, leading to a high population that can evade the effects of biocontrol agents.

## 8.3. BDI-IS1 acts by direct antagonism and by stimulating the plant defense

The biocontrol efficacy of BDI-IS1 is probably linked to its capacity to produce secondary metabolites. Nimbeshaho et al. (2024) reported that the genome of BDI-IS1 contains genes encoding non-ribosomal lipopeptides, including iturin A, surfactin, bacilysin, and bacillibactin. These have been reported in other pathosystems to play a key role in direct or indirect antagonism against pathogens. Therefore, we tested the direct antagonistic activity of bacterial mutants impaired in the production of iturin, surfactin, bacillibactin, bacilysin, bacillaene, plantazolycin, and sfp against the mycelial growth of *E. turcicum*. Our results showed a significant loss of antagonistic activity in the mutants deficient in sfp and iturin A, while treatment with other mutants reduced the mycelial growth of *E. turcicum* similarly to the wild type. The lack of activity in the mutants impaired in sfp and iturin A production highlights the key role of these metabolites in the direct antagonism of BDI-IS1 against *E. turcicum*. Since sfp includes iturin A and other non-ribosomal lipopeptides, the direct antagonistic activity of BDI-IS1 against *E. turcicum* is probably primarily mediated by iturin A. The role

of iturin A in direct antagonism has been reported by several authors (Ambrico and Trupo, 2017; Lam et al., 2021; Wang et al., 2013).

Based on the literature and previous results obtained *in vitro*, we tested the biocontrol efficacy of BDI-IS1 impaired in iturin A (ΔituA), surfactin (ΔsrfAA) and bacillibactin (ΔdhbC) production against NLB under greenhouse conditions. Our results showed a significant increase in disease severity on plants treated with ΔdhbC, a moderate increase in disease severity on those treated with ΔituA and ΔsrfAA, compared to the wild type (BDI-IS1). These findings suggest a potential synergy between bacillibactin, iturin A and surfactin in the direct activity of BDI-IS1 against NLB. Such a synergistic activity has already been demonstrated for *Bacillus velezensis* Jt84 in protecting rice against *Ustilaginoidea virens* (Zhang et al., 2024). This study also demonstrated that surfactin and iturin A enhance biofilm formation, bacterial motility, and colonization on rice leaves, key features that ensure bacterial fitness and contribute to protection against pathogens.

The indirect effect of BDI-IS1 was tested by treating roots of maize plants with the wild type of BDI-IS1 or with the bacterium impaired in surfactin (ΔsrfAA), iturin A (ΔituA) and bacillibactin (ΔdhbC) production. Plants treated on the roots with ΔsrfAA were more diseased than those treated with BDI-IS1. This observation suggests a potential role of surfactin in the induction of ISR to NLB in maize. The role of this lipopeptidic compound in the induction of plant resistance has been reported by several authors in various pathosystems (Adeeb et al., 2022; Ongena et al., 2007; Rahman et al., 2015; Rodríguez et al., 2018; Zeriouh et al., 2014). ISR has been reported to be induced by several *Bacillus* species and proceeds through three main sequential steps: elicitation, signal transduction via the jasmonic acid or ethylene pathways into plant tissues (leading to a primed state), and the establishment of defense mechanisms that limit pathogen damage (Rodríguez et al., 2018). In this context, surfactin may act as an elicitor of ISR by interacting with lipid bilayers, such as sphingolipid microdomains located in the plasma membrane of maize cells (Henry

et al., 2011; Kour et al., 2024). Treatment with BDI-IS1 could induce a primed state in maize plants, characterized by rapid and strong defense reactions, including the production of phytoalexins, lignification of cell walls, oxidative bursts, chromatin modifications, hormone signaling, and the synthesis of antimicrobial compounds during infection with pathogens such as *E. turcicum* (Kour et al., 2024). The induction of systemic resistance in plants is an important feature, as it provides broad-spectrum protection against various pathogens, including bacteria, viruses, fungi, nematodes, and insects (Desmedt et al., 2021; Mariutto and Ongena, 2015). Therefore, BDI-IS1 could be a valuable biocontrol agent suitable for tropical regions, including Burundi, where several diseases occur simultaneously during the cropping season. To further validate BDI-IS1's ability to induce ISR, we investigated transcriptomic changes in specific defense-related genes previously reported to be induced by beneficial bacteria in maize. Our results demonstrated the high expression of defense genes, including *PAL* and *AOS1*, and to a lesser extent *MYC7*, in maize treated on roots with BDI-IS1 and inoculated on leaves with *E. turcicum* compared to control inoculated plants. The high expression of these defense genes after root treatment with BDI-IS1 supports its potential to induce resistance to NLB in maize. Indeed, the *PAL* and *AOS1* genes are involved in SA and JA plant defense signaling pathways, respectively, while *MYC7* is a transcription factor of defense genes mediated by JA (H. Cao et al., 2023; Fu et al., 2020; Klessig et al., 2018). *PAL* is also involved in the production of defense compounds, such as phenolics and phytoalexins, which have toxic properties against pathogens (Klessig et al., 2018; Zhang et al., 2021). *AOS1* was reported to be involved in biosynthesis of JA from oxylipins chain synthesis (Farmer and Goossens, 2019; Sugimoto et al., 2022). The high expression of *AOS1* in plants treated with BDI-IS1 and inoculated with *E. turcicum* could lead to the synthesis of JA and the activation of *MYC7*, which was also upregulated in our experiment. This, in turn, could potentially trigger the transcription of downstream defense genes in the JA defense pathway.

Considering all the elements, our study demonstrated that BDI-IS1 employs multiple modes of action, including both direct and indirect mechanisms, to antagonize pathogens. This bacterium is effective against necrotrophic and hemibiotrophic pathogens, *A. solani* and *E. turicum*, respectively. The diverse modes of action employed by BDI-IS1 to protect plants from disease are significant advantages of this biocontrol candidate, as they help prevent the development of pathogen resistance and could sustain biocontrol activity in the field. Furthermore, the multiple modes of action of BDI-IS1 enable both leaf and root applications of this bacterium. However, the foliar application of BDI-IS1 under field conditions, such as in Burundi, may be limited by various abiotic stresses, including high variability in environmental conditions, heavy rainfall and temperature often experienced in tropical regions. Additionally, foliar treatment may require several treatments and specific sprayers and personal protective equipment, which are often lacking in less developed countries. On the other hand, the efficacy of BDI-IS1 through root application confers some advantages to this BCA. Root application of this bacterium appears to be simple and potentially well-suited to the local agricultural conditions in Burundi. Practically, BDI-IS1 could be formulated for application simultaneously with seed sowing, thereby facilitating its use and enhancing its biocontrol efficacy.

## 8.4. Bioformulation of BDI-IS1 on bioresources substrates

Biocontrol agents must be formulated for use under field conditions prior to registration. The formulation of bacteria is essential to facilitate handling, increase biocontrol efficacy, and minimize the variability in efficacy that often occurs when moving from controlled conditions to field applications (Gopalakrishnan et al., 2016; Khan et al., 2023; Llamas et al., 2023). Bacteria are typically formulated on solid or liquid carriers. A good carrier should be rich in carbon sources and nutrients for microorganisms. In this study, we formulated and multiplied BDI-

IS1 on lignocellulosic substrates based on bean, maize, and rice residues. These substrates would contain sufficient carbon to support the potential growth and spore production of BDI-IS1. This bacterium has been reported to produce cellulase, pectase and other enzymes that degrade lignocellulosic substrates into smaller glucose molecules, serving as nutrient sources for BDI-IS1 (Nimbeshaho, in preparation). The formulated products were tested against NLB in a tunnel experiment using pots filled with field soil. We observed a significant reduction in NLB severity on treated plants compared to untreated ones. The disease reduction observed in treated plants was comparable to that previously reported in the greenhouse (Nimbeshaho et al., 2024). Although both bioproducts reduced the severity of NLB, a greater reduction was noted with the BDI-IS1 bean-based bioproduct, which was subsequently used to evaluate its efficacy under field conditions.

The assay conducted in field confirmed the interest of the BDI-IS1 bean-based bioproduct, which induced significant reduction in NLB disease following root treatment. The reduction was comparable to that obtained using bacterial cell-free supernatant. This observation also underscores the consistency of the biocontrol efficacy of BDI-IS1, whether used as a formulated bioproduct or not. Formulating BDI-IS1 on lignocellulosic substrates provides an economically viable alternative to liquid bacterial production and offers important advantages in the context of agriculture in Burundi. These substrates are derived from crop residues and are considered as agricultural waste. The valorization of crop residues as substrate for biocontrol agents is an important strategy that promotes environmental protection and creates new economic opportunities. Beans are cultivated by farmers throughout Burundi across the three seasons A, B, and C. A company focused on producing BDI-IS1 using bean residues could easily source raw materials through systematic collection from farmers' households. The extensive production of these staple crops also provides a market for both bean grains and residues. Additionally, these residues are underutilized in livestock production, as bean leaves are generally considered unpalatable to animals (Bennett Tiffany, 2015).

## 8.5. Perspectives

### 8.5.1. Sustainable utilization of BDI-IS1 for the control of plant diseases

The spores of BDI-IS1 produced from bean substrate has demonstrated its biocontrol efficacy. We have also shown that this product can be applied to roots as a seed coating. The product was stored in plastic bags for a short time before use, but we do not know how long the bacteria remain viable in the product. The shelf life of the biological product is highly correlated with the storage time and, to a lesser extent, with the frequency of application under field conditions (Teixidó et al., 2022, 2020). Extending the shelf life of bioproducts could increase demand and improve the economic and environmental viability of agroecosystems. The use of bio-products with a long shelf life is the most suitable crop protection tool for farmers with small plots of land and limited economic resources, such as those in Burundi. The product can be utilized in the A, B, and C seasons, as agriculture is practiced throughout the year. The long shelf life of the product also benefits the company involved in developing biological products, providing them with the opportunity to optimize production during a given period and allocate the remaining time to marketing and distribution. Further studies are needed to determine the shelf life of the formulated BDI-IS1 bean-based bioproduct and could be compared to QST713, already on the market. If the shelf life of this product is less than one year, improvements in production, formulation, packaging, and storage along the entire product value chain could be explored (Gotor-Vila et al., 2019).

In addition, we do not know the shelf life of this bioproduct in soil, on seed coating or how this bacterium might affect the existing microbial communities in the soil and plants. To improve the efficacy of the BDI-IS1 product for potential leaf application, bean residues could be mixed with oil to enhance adherence and provide protection against abiotic stresses. The addition of oil as a surfactant has been shown to improve

the efficacy of bioproducts formulated with *Bacillus* spp. in Argentina (Sartori et al., 2017). Our experiments conducted in pots demonstrated significant colonization of BDI-IS1 transformed with green fluorescent protein (GFP) in the rhizosphere of maize plants, while this bacterium was not detected in the stems or leaves, following root treatment. This study could be replicated under field conditions and complemented by further metagenomic studies to decipher the potential change in microbial communities prevailing on or in seeds, soil, and plants before and after the application of BDI-IS1. To increase its efficacy, a potential bioproduct combining BDI-IS1 and other microorganisms, such as *Pseudomonas* spp, *Trichoderma* spp. and mycorrhizal fungi, which are ubiquitously found in soil, could be tested for their disease control capabilities and persistence in soil. This assay would help develop suppressive soils that are effective in controlling several prevalent diseases in the fields of Burundi.

The efficacy of this bacterium has been tested under field conditions for only two pathosystems. These experiments could be extended to other pathosystems, including *Phytophthora infestans*, *Pythium* spp., and *Fusarium* spp., which are the main soil-borne pathogens affecting crops in Burundi. *P. infestans* causes late blight in potatoes and tomatoes and is the most damaging disease in highland areas. Currently, potato late blight is controlled by fungicides applied at high rates from emergence to harvest. *Pythium* spp. is an oomycete fungus that causes damping-off disease and represents a major constraint to taro (*Colocasia esculenta*) production in Burundi. *Fusarium* spp. is a harmful fungus that causes *Fusarium* wilt in many crops, including tomato, potato, and banana. Currently, there are no effective control measures for diseases caused by *Pythium* spp. and *Fusarium* spp. in Burundi. The potential biocontrol activity of BDI-IS1 against these diseases could reduce disease pressure and improve the production and sustainability of agriculture in the region.

### 8.5.2. Integration of BDI-IS1 into plant protection strategies in Burundi

BDI-IS1 has demonstrated its ability to reduce the severity of diseases caused by various pathogens, including *A. solani* (necrotrophic) and *E. turcicum* (hemibiotrophic), under both greenhouse and field conditions, for NLB. Root application (as discussed above) appears to be well-suited to the agricultural context in Burundi due to its ease of application and effectiveness. However, we observed a significant discrepancy in biocontrol effectiveness from greenhouse to field conditions, although the protection in both environments was notably significant compared to the control. This observation indicates that BDI-IS1 is not a definitive solution, but rather a promising opportunity for enhancing plant protection and sustaining agriculture in Burundi. The biocontrol of plant diseases using BDI-IS1 could be further improved by integrating it into a comprehensive pest and disease management framework, which has been shown to maintain pathogen pressure at low levels while allowing continuous production despite the presence of pathogens.

Several studies could evaluate the biocontrol efficacy of this bacterium when used in combination with good agricultural practices, resistant cultivars, and intercropping systems. Good agricultural practices enhance environmental conditions, reduce pathogens pressure, and promote the development of beneficial microorganisms by limiting competition with other pathogens. Additionally, improved fertilization using fertilizers and amendments provides nutrient sources for these beneficial microorganisms. Combining biological control with host resistance is a critical approach in plant protection. Several authors have reported a close association between microbial communities and resistant plants, which can confer protection and other benefits, such as enhanced plant growth, improved nutrition, and nitrogen fixation (Lengrand et al., 2024; Maciag et al., 2023; Pérez-García et al., 2011).

Crop association is an important practice that aims to reduce inoculum pressure by diluting the host, leading to decreased pathogen populations

and increased competitiveness of beneficial microorganisms. Associated plants can secrete various compounds that enhance the fitness and antagonistic activities of these microorganisms against pathogens. In more intensive agriculture, such as cash crops in Burundi (e.g. tomatoes), this bacterium could be used in combination with or as an alternative to chemical pesticides. This approach would not only ensure sufficient production but also reduce the quantity of pesticides applied. However, further studies are needed to assess the compatibility of BDI-IS1 with existing chemical pesticides.

Based on the reproducible results demonstrating the biocontrol efficacy of BDI-IS1 against diseases, the approval and registration processes into phytopharmaceutical products to be used in Burundi could be initiated. In Burundi, biological control agents or substances are categorized as pesticides, and their regulation follows the same legislation (Decree Law n°1/04 of 11/02/2021 on the control and management of pesticides). The registration of pesticides is overseen by an advisory council that collaborates closely with the Food, Veterinary Products, and Pesticides Regulation Board (ABRVPA). This council consists of scientific experts from the Ministry of Public Health and Environment, along with other stakeholders in pesticides management, who are appointed by decree. The ABRVPA evaluates the efficacy of pesticides, collects information from applicants, and presents the data to the advisory council for approval and registration. Once registered, the production and distribution of the BDI-IS1 bioproduct could be organized under the regulation of government agencies. Burundi has experience in the production and commercialization of *Rhizobium* inoculants, successfully transitioning from laboratory to farmers. In a short time, BDI-IS1 products could be produced and distributed through the existing *Rhizobium* supply chain. In fact, the production unit could be established at an agricultural research institute or the Faculty of Agriculture and Bioengineering at the University of Burundi, benefiting the scientific expertise of researchers. These researchers would receive the necessary support to refine the formulation based on the promising results obtained in this

work. Initially, production could be carried out on a small scale, supplying products for use in farmer field demonstration plots. This approach could also serve as an effective channel for marketing and organizing groups of farmers or cooperatives to adopt bioproducts.

The storage of the bioproducts could be managed through shops distributed across the country, strategically located near research centers or campuses to facilitate stakeholder access. At the outset, the entire process could be subsidized by the government to ensure the product is both affordable and accessible to farmers. To ensure use of bioproducts, farmers could pay a small amount in advance, following a model similar to their prepayment for other agricultural inputs such as seeds and fertilizers.

In the long term, the entire process, including production, storage, and distribution, could be handed over to a well-organized company acting under the supervision of government agencies to ensure proper regulation.

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# CHAPTER IX.

# GENERAL CONCLUSION

Plant diseases are increasingly constraining crop production and contributing to food insecurity in Burundi. Control measures are limited, with intensive use of chemical pesticides on cash crops including tomato and fruits, while diseases affecting staple crops such as maize and beans remain largely uncontrolled despite their high prevalence.

This thesis investigated the biocontrol efficacy of *B. nakamurai* BDI-IS1, a strain isolated from an agro-ecosystem in Burundi with biocontrol potential, focusing on its efficacy against major pathosystems and the mechanisms involved in controlling plant diseases. Multiple approaches, including *in vitro* and *in planta* trials, were used to assess the biocontrol potential of BDI-IS1. Based on *in vitro* assays, we demonstrated that BDI-IS1 exhibits strong antagonistic activity against a wide range of bacterial and fungal plant pathogens, including those responsible for some of the most prevalent diseases in Burundi, such as tomato early blight caused by *A. solani*, and Northern corn leaf blight caused by *E. turcicum*.

The biocontrol efficacy of BDI-IS1 against these fungal pathogens affecting leaves was tested in greenhouse trials through both root and leaf treatments, which resulted in significant reduction of the diseases. A particularly high reduction in disease severity was observed against NLB on maize. Therefore, we further tested the bacterial activity against this disease under field conditions. Root and leaf treatments of maize plants with BDI-IS1 resulted in significant disease reduction.

Based on the characterization of its genome (Nimbeshaho et al., 2024) and biocontrol assays, we postulated that the BDI-IS1 acts through multiple modes of action, including direct and indirect antagonism,

primarily mediated by the production of secondary metabolites. We demonstrated that the direct antagonism against *E. turcicum*, tested *in vitro* and *in planta via* leaf treatment, is mediated by the production of iturin A. The indirect activity after a treatment on soil, tested in both greenhouse and field trials, is mediated by surfactin.The overexpression of defense genes such as *PAL, AOS* and to lesser extent *MYC7* on maize plants treated with BDI-IS1 led to the hypothesis that the bacterium acts both in the SA and JA signaling and defense pathways. These plant defense phytohormones are known to play a key role in the production of defense enzymes and compounds toxic to pathogens, thus would contribute to the reduction of NLB.

This thesis also investigated the methods for mass production of the bacterium for field trials. We were able to multiply the BDI-IS1 on bean residues and obtain an active bioproduct consisting of bacterial spores on organic residues. This formulation showed interesting biocontrol properties and can be further improved.

Furthermore, this thesis argues for the interest of indigenous microbes in the development of locally adapted biological control agents. It also highlights the importance of combining multiple approaches, incorporating both phenotypic and molecular aspects, in the selection and evaluation of these agents. The intensification of *in vitro*, greenhouse, and field trials, by increasing the number of trials and plants, is a major strength of this study. This robust methodology provides a strong and consistent database that is valuable for the further development of biological control agents.

Further research is needed to assess the effects of this bacterium on existing microbial communities in soil and plants, which is essential for generating sufficient knowledge for its approval and registration. Key research activities include exploring the bacterium's potential to control other prevalent field diseases, enhancing its formulation and storability, and optimizing its production and distribution value chain. Additionally, the registration process must be thoroughly addressed before its official introduction and use.

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