THE UNIVERSITY OF YAOUNDE I
FACULTY OF SCIENCE

POST-GRADUATE AND TRAINING SCHOOL OF LIFE SCIENCE, HEALTH AND ENVIRONMENT

UNIVERSITE DE YAOUNDE I
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CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE SCIENCE DE LA VIE, SANTE ET ENVIRONNEMENT

DEPARTMENT OF BIOCHEMISTRY

DEPARTEMENT DE BIOCHIMIE

ANTIMICROBIAL AND BIOCONTROL AGENTS UNIT

LABORATORY FOR PHYTOBIOCHEMISTRY AND MEDICINAL PLANTS STUDIES

Biological potential of ten endophytic bacteria isolated from *Euphorbia antiquorum* against *Ralstonia solanacearum* in tomato (*Solanum lycopersicum L.*)

THESIS

Presented in partial fulfilment of the requirements for the award of a Doctorat/Ph.D in Biochemistry

BY

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SUPERVISED BY:

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Academic year: 2023-2024

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CERTIFICATE OF CORRECTION OF THE PH. D THESIS

ATTESTATION DE CORRECTION DE LA THESE DE DOCTORAT PH. D

The undersigned members of jury involved in the Ph.D thesis in Biochemistry entitled: **'Biological potential of ten endophytic bacteria isolated from *Euphorbia antiquorum* against *Ralstonia solanacearum* in tomato (*Solanum lycopersicum* L.)'**, defended on Wednesday, 13th of March 2024 at 9h.00 am in the Multimedia room of the Faculty of Sciences by **Mrs. YIMTA YOUMBI Diane (registration number 12R1000)**, are hereby certifying that the candidate has effected the corrections of the above mentioned thesis as requested by the examiners.

Therefore, they are satisfied with the corrections made, and are recommending the Doctorate/Ph.D degree to be awarded to the candidate.

Yaounde, the 13 JUIN 2024

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UPDATE : January 2024

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27	SIMO Elie	Associate Professor	On duty
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4	NGOS III Simon	Professor	On duty
5	NKOUMBOU Charles	Professor	On duty
6	NZENTI Jean-Paul	Professor	On duty
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14	GANNO Sylvestre	Associate Professor	En poste
15	GHOGOMU Richard TANWI	Associate Professor	**Head of division /Univ. Bertoua**
16	MBIDA YEM	Associate Professor	On duty
17	MOUNDI Amidou	Associate Professor	**TC /MINIMDT**
18	NGO BIDJECK Louise Marie	Associate Professor	On duty
19	NGUEUTCHOUA Gabriel	Associate Professor	**CEA/MINRESI**
20	NJILAH Isaac KONFOR	Associate Professor	On duty
21	NYECK Bruno	Associate Professor	On duty
22	TCHAKOUNTE Jacqueline épse NUMBEM	Associate Professor	**Chief of Cell /MINRESI**
23	TCHOUANKOUE Jean-Pierre	Associate Professor	On duty
24	TEMGA Jean Pierre	Associate Professor	On duty
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27	BEKOA Etienne	Senior Lecturer	On duty

28	ESSONO Jean	Senior Lecturer	On duty
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38	TEHNA Nathanaël	Senior Lecturer	On duty
39	FEUMBA Roger	Senior Lecturer	On duty
40	MBANGA NYOBE Jules	Senior Lecturer	On duty
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42	NGO'O ZE ARNAUD	Assistant lecturer	On duty
43	TENE DJOUKAM Joëlle Flore, spouse KOUANKAP NONO	Assistant lecturer	On duty

DISTRIBUTION OF PERMANENT LECTURERS IN THE FACULTY OF SCIENCE OF THE UNIVERSITY OF YAOUNDE I ACCORDING TO DEPARTMENTS

NUMBER OF LECTURERS					
DEPARTMENT	**Professor**	**Associate Professor**	**Senio lecturer**	**Assistant lecturer**	**Total**
BCH	8 (01)	15 (11)	13 (03)	7 (05)	**43 (20)**
ABP	14 (01)	16 (09)	18 (04)	4 (02)	**52 (16)**
PBP	6 (01)	12 (02)	13 (07)	3 (00)	**34 (10)**
IC	7 (01)	15 (04)	5 (01)	1 (00)	**28 (06)**
OC	6 (01)	18 (04)	11 (04)	2 (00)	**37 (09)**
CS	2 (00)	2 (00)	14 (01)	4 (00)	**22 (01)**
MAT	1 (00)	8 (00)	17 (01)	7 (02)	**33 (03)**
MIB	2 (01)	7 (03)	8 (04)	7 (02)	**24 (10)**
PHY	15 (01)	15 (04)	11 (01)	2 (00)	**43 (06)**
ES	8 (00)	17 (03)	15 (04)	3 (01)	**43 (08)**
Total	**69 (07)**	**125 (40)**	**125 (30)**	**40 (12)**	**359 (89)**

A total of **359 (89)** including

- Professors **70 (08)**
- Associate professors **125 (40)**
- Senior lecturers **125 (30)**
- Assistant lecturer **40 (12)**

( ) = Number of women **89**

Dedication

I dedicate this thesis to my parents

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I would like to first of all, thank the holly **GOD** for strength and protection he provided for the accomplishment of this work.

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All those whose names are not mentioned here, your individual contributions have been of great help to the success of this work.

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LIST OF ABBREVIATIONS

Symbol	Full name
Ab	Absorbance
AUDPC	Area Under Disease Progress Curve
AI	Attenuation index
AmBcAU	Antimicrobial & Biocontrol Agents Unit
ANOVA	Analysis of Variance
BCA	Biological Control Agent
CFU	Colony Forming Unit
CLSI	Clinical Laboratory Standard Institute
CSD	Completely Simple Design
DNA	Deoxyribose Nucleic Acid
EAI	Enzymatic Activity Index
EDTA	Ethylenediaminetetra-acetic acid
ET	Ethylene
FAO	Food and Agriculture Organization
FAOSTAT	Statistic of Food and Agricultural Organization
$FeCl_3$	Ferric-chloride
GI	Germination Index
GR	Germination Rate
GPX	Gaicol Peroxidase
HCl	Hydrogen Chloride
HCN	Hydrogen Cyanide
HPLC	High Performance Liquid Chromatography
IAA	Indol Acetic Acid
ISR	Induced Systemic Resistance
JA	Josmonis Acid
LB	Lurea Bertani
LBA	Lurea Bertani Agar
LC/MS	Liquid Chromatography/Mass Spectrometry
MAMPs	Microbs Associated Molecular Patern
MINADER	Ministry of Agriculture and Rural Development
MET	Mean Emergence Time

MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
NB	Nutrient Broth
NBT	Nitroblue Tetrazolium
OD	Optical Density
ONU	Organisation of United Nation
PAL	Phenyl Alanine Ammonia Lyase
PSI	Percentage Severity Index
PGP	Plant Growth Promoting
PGPB	Plant Growth Promoting Bacteria
PGPR	Plant Growth Promoting Rhizobacteria
PR-protein	Pathogenic Related Protein
PWP	Percentage of Wilt Plant
R. solanacearum	*Ralstonia solanacearum*
RCBD	Randomized Complete Block Design
ROS	Reactive Oxygenated Species
SA	Salicylic Acid
SAR	Systemic Aquired Resistance
SDW	Sterile Distilled Water
S. lycopersicum	*Solanum lycopersicum*
SOD	Superoxid Dismutase
SR	Systemic Resistance
VOCs	Volatil Organic Compound
WS	Wilt Severity

ABSTRACT

Tomato wilt caused by *Ralstonia solanacearum* hampers tomato production worldwide, including Cameroon. Endospore-forming *Bacilli* could provide biological alternatives to curb the burden. Herein, we screened the suppressive traits of selected Bacilli from the desert spurge *Euphorbia antiquorum* against *R. solanacearum.* Out of the ten endophytic strains screened *in vitro* for their antagonist activities, six including *Bacillus velezensis* CBv_BE1, *Bacillus amyloliquefaciens* CBa_BFL2 and *Bacillus amyloliquefaciens* CBa_RA37, *Bacillus megaterium* CBm_RR10, *Bacillus amyloliquefaciens* CBa_BFL1 and *Lysinibacillus* CBa_LPR19 exhibited direct and indirect antibacterial potentials. The inhibition diameter varied from 15 to 43 mm, with MICs ranging from 1000 to 31.25 µg/ml and 62.5 to 7.812 µg/ml, respectively for their culture filtrates and ethyl acetate-based extracts, that completely suppressed the growth of *R. solanacearum.* Depending on the strains, they produced cell wall-degrading enzymes (amylase, protease and cellulase) and plant growth-promoting factors (ammonium, siderophores, indole acetic acid and salicylic acid) at different concentrations. The generated spontaneous antibiotic-resistant mutants showed the ability to form biofilms and colonize tomato seedling tissues, with an optimum at log10 CFU = 4.29/g fresh weight for an inoculum load of 0.5 x 10^8 CFU/ml. The combine application of *B. amyloliquefaciens* CBa_BFL2 and *B. amyloliquefaciens* CBa_RA37 (CBa_BFL2/CBa_RA37) significantly promoted shoot and root growth. Additionally, the CBa_BFL2/CBa_RA37 consortium suppressed the wilt incidence and severity by 90% and 89%, respectively. Pearson's model revealed significant and negative correlations between specific activities of guiacool peroxidases (GPX), phenylalanine ammonia lyase (PAL) and superoxide dismutase (SOD) and wilt severity, indicating an induced response upon bacterial infection compared to the negative controlThe famer field trials showed 100% severity after three months, compaed to the *Ralstonia solanacearum* treatment alone. The consortium BFL2/RA37 treatment significantly reduced the disease severity by 87.5% compared to the negative control. The positive control (streptomycin) proved to be ineffective with a disease severity of approximately 87.5%. Whereas treatment with biocontrol agents gave the best yield in terms of flowers and fruits (30.74Kg/ha) whencompared to the negative (3.6Kg/ha) and positive (7.3Kg/ha) controls. This investigation has demonstrated that the endophytic bacteria (CBa_BFL2 and CBa_RA37) from *E. antiquorum* L. possess the ability to both improving growth and protecting tomato plants, and thus are endowed with suitable potential for further development as biopesticides to help mitigate the impact of tomato bacterial wilt in the field. Investigations are currently ongoing for the formulation of a CBa_BFL2 and CBa_RA37 based biopesticide for validation in field trials.

Keywords: Tomato; *Ralstonia solanacearum*; Tomato wilt disease; Biocontrol; *Bacillus*;

RÉSUMÉ

Le flétrissement bactérien de la tomate causé par *Ralstonia solanacearum* entrave la production de la tomate dans le monde entier et surtout au Cameroun. Les bactéries endophytes du genre bacillus formant des endospores leur permettant de mieux résister aux conditions de stress, pourraient être une solution biologique pour réduire les pertes dues à ce phytopathogène. Le présent travail a consisté à évaluer les effets suppressifs des bactéries endophytes isolées d'*Euphorbia antiquorum* contre *Ralstonia solanacearum in vitro*, en pot et en champ. Des dix (10) souches endophytes criblées *in vitro* pour leur activité antagoniste, six (6) souches à savoir *Bacillus velezensis* CBv_BE1, *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37, *Bacillus megaterium* CBm_RR10, *Bacillus amyloliquefaciens* CBa_BFL1 and *Lysinibacillus* CBa_LPR19 ont présenté des propriétés antibactériennes en confrontations directe et indirecte avec des diamètres d'inhibition variant de 15 à 43 mm et des concentrations minimales inhibitrices (CMI) variant de 1000 µg/ml à 31, 25 µg/ml et de 62, 5 µg/mL à 7,812 µg/ml respectivement pour les filtrats de culture et les extraits à l'acétate d'éthyle ayant complètement inhibé la croissance de *Ralstonia solanacearum*. Ces souches ont montré également la capacité à produire les enzymes dégradant la paroi cellulaire (amylase, protéase, et cellulase), les facteurs favorisant la croissance des plantes (ammoniac, sidérophores, acide indole acétique et acide salysilique) avec des concentrations variantes selon la souche bactérienne. Les mutants spontanés générés, résistants aux antibiotiques ont montré la capacité à former un biofilm et à coloniser l'intérieur des tissus des plants de tomate avec un maximum de colonisation observée à log10 UFC (4,29/g de poids frais) à une charge d'inoculum de 0.5. 10^8 UFC/mL. L'application combinée de *B. amyloliquefaciens* CBa_BFL2 et *B. amyloliquefaciens* CBa_RA37 (CBa_BFL2/CBa_RA37) a favorisé la croissance aérienne et racinaire. De plus, le consortium CBa_BFL2/CBa_RA37 s'est démarqué en supprimant l'incidence et la sévérité de la maladie de 90 et 89% respectivement. Le modèle de Pearson a révélé une corrélation négative significative entre les activités spécifiques des gaiacol peroxydases (GPX), de la phénylalanine ammoniac lyase (PAL), et des superoxydes dismutases (SOD) et la gravité du flétrissement indiquant une réponse de défense innée renforcée de la tomate lors de la bactérisation par les agents de lutte biologique. Une étude en champ a été effectuée pour révéler son potentiel en tant qu'alternative aux produits agrochimiques pour le contrôle du flétrissement bactérien et l'amélioration de la productivité. Les résultats en champ ont révélé que le consortium CBa_BFL2/CBa_RA37 gardait son activité avec une protection de 87.5% contre le flétrissement bactérien. Par ailleurs le traitement en présence de *Ralstonia solanacearum* seule a entrainé une sévérité de 100% de la maladie au bout des trois mois d'expérimentation. Le control positif (streptomycine) s'est avéré inefficace en champ avec une sévérité de 87.5% de la maladie. De même le traitement en présence des agents de lutte a montré un meilleur rendement en termes de fleur et fruit comparé aux contrôles négatif (traitement en présence de *R. solanacearum* seul. Cette étude a démontré que les bactéries endophytes (CBa_BFL2 et CBa_RA37) isolées d'*Euphorbia antiquorum*, sont capables d'améliorer la croissance des plantes, mais surtout de

protéger les plants de tomate contre le flétrissement bactérien. Elles ont par conséquent le potentiel d'être développées comme biopesticide. A cet effet, des recherches sont actuellement en cours pour la formulation d'un biopesticide à base du consortium CBa_BFL2/CBa_RA37 qui sera utilisé en champ pour des études complémentaires.

Mots clés : Tomate; *Ralstonia solanacearum*; Flétrissement bactérien; Biocontrole; *Bacillus*;

INTRODUCTION

INTRODUCTION

Tomato with the scientific name *Solanum lycopersicum* L. is a species of plant belonging to Solanacea family and native to the Northwest of South America. Tomato is the first most cultivated vegetable and the second largest food resource in the world and is the subject of several scientific researches. The latter presents numerous advantages on differents cases such as nutrition, medecinen and economy. However, tomato production faces biotic contraints broken by organisms such as nematodes (Vinod *et al*., 2020; Mahfouz *et al*., 2021), viruses (Chen *et al*., 2019; Rao and Gurivi, 2019), fungi (Toghueo et al., 2016; Eke et al., 2016; Shuping and Eloff, 2017; Yan *et al*., 2021), and bacteria (Merga *et al*., 2019; Chen *et al*., 2019).

Although 80% of plant diseases are caused by fungi (Li *et al*., 2017), reports indicate that plant pathogenic bacteria cause approximately one billion dollars of annual economic losses worldwide (Kannan *et al*., 2015; Paula *et al*., 2018). The strict soil-borne bacterium *R. solanacearum* is the second most damaging and economically impactful phytobacteriosis after *P. syringae* (Mansfield *et al*., 2012). The latter disseminates in agricultural fields through infested soil particles from previous seasons, surface and irrigation water, infested farm equipment, and latently infected planting materials. *R. solanacearum* is distributed worldwide, infecting approximately 200 distinct host plant species belonging to 50 different families, including important crops such as potato, eggplant, pepper, tobacco, banana, and tomato (Strange and Scott, 2005; Fanhong, 2013; Zhu *et al*., 2019). Yield losses were 20–30% in ginger (Liu *et al*., 2005) and 20-50% in chili (Tan *et al*., 2014). 80 - 100% in potato (Lemaga *et al*., 2001; Chen *et al*., 2005), 15-75% and up to 100% during extreme outbreaks in tobacco, 50–100% in peanut (Yu *et al*., 2011) and 10% to 80% in tomato (Singh *et al*., 2015; Wei *et al*., 2017). In tomatoes, the phytopathogen infects plants via wounded roots or emerging secondary roots, colonizes xylem vessels, and spreads rapidly to aerial parts of the plant through the vascular system (Vasse *et al*., 1995). Biofilm structures formed by the aggregation of bacterial cells prevent the free circulation of water and nutrients into the plant by clogging plant vessels via the production of exopolysaccharides, the major virulence factors (Kazusa *et al*., 2019). Symptoms in infected plants include browning of the xylem, chlorosis, stunting, and wilting. Infected plants usually die at an accelerated rate, and yield losses of up to 88-100% have been ascribed to this pathogen (Eyob and Desalegn, 2022).

Currently, taken individually, no control measure is effective against bacterial wilt (Jiang *et al*., 2017). However, chemicals such as fumigants, copper compounds and various antibiotics are being desperately used by farmers without much success (Fahime and Gholam, 2018).

Moreover, the hazardous effects of these chemically synthetized agricultural inputs on beneficial soil microflora, the environment and human health have been extensively proven. However, various recent reports indicate that suppression of bacterial wilt disease could be achieved by using endophytic bacteria (Yang *et al*., 2012; Tonial *et al*., 2020). The most frequently applied biocontrol bacteria are *Pseudomonas* spp. (Hu *et al*., 2016; Etminani and Harighi, 2018), *Streptomyces* spp. (Lu *et al*., 2013; Xiong *et al*., 2014), avirulent *Ralstonia* spp. mutants (Chen *et al*., 2004; Yang *et al*., 2008) and *Bacillus* spp. (Wei *et al*., 2011; Wang *et al*., 2015; Ayomide and Olubukola, 2020).

The production of broad-spectrum antibiotics (Lugtenberg *et al*., 2016; Ayomide and Olubukola, 2020), volatile organic antimicrobial compounds (Ekta, 2018) and cell wall hydrolytic enzymes (Imran *et al*., 2019) has been described as a direct mode of action employed by these bioagents to suppress pathogens. Indirect mechanisms such as the induction of host innate immune machinery through a regulated synthesis of salicylic and jasmonic acid (Kloepper and Ryu, 2006; Khare *et al*., 2016), the improvement of plant fitness by synthesizing and releasing phytohormones such as cytokinins, gibberellins, indol-3-acetic acid (IAA) and the facilitation of the acquisition of essential nutrients have equally been extensively reported (Zuniga *et al*., 2013; Shahsad *et al*., 2016).

As a biocontrol agent, the ubiquitous spore-forming and extremotolerant *Bacillus* spp. are particular (Won-II and Myong, 2020). A straight relationship has been drawn between the biotope and the efficiency of bioagents. It is well established that biological control agents living in extreme conditions are thought to be endowed with superior efficiency compared to those adapted to stress-free environments (Qiuwei and James, 2021). Hence, we anticipated that investigating endophytic Bacillus from extremotolerant plants could be advantageous over other sources to help agricultural plants cope with diseases and thrive in fluctuating environmental circumstances.

To date, no attempts have been made to investigate the biocontrol properties of the edophytic bacteria derived from the desert spurge *Euphorbia antiquorum*. Hence, this study aimed to test the antagonistic properties of some endospheric Bacillus species recovered from *Euphorbia antiquorum* naturally grown in Cameroon's Sahel region against the wilt-causing bacterium *R. solanacearum*.

Problematic for the Study:

Background and context:

Ralstonia solanacearum is a devastating bacterial pathogen that causes bacterial wilt in a wide range of crop plants, including tomatoes, significantly affecting both yield and quality. Effective management of this pathogen is challenging due to its wide host range, persistence in soil and water, and capabilities for rapid dissemination and infection. Chemical control strategies not only have limited efficacy but also pose environmental and health risks due to the buildup of chemical residues in ecosystems and food. There is, therefore, a critical need for developing sustainable, biologically-based control strategies that could provide effective management of *R. solanacearum* without adverse environmental impacts.

Hypotheses:

1. Endophytic bacteria from *Euphorbia antiquorum* exhibit natural antagonistic properties against *R. solanacearum* due to competitive exclusion, antibiosis, or induction of host plant defenses.
2. Application of these endophytic bacteria to tomato plants will reduce incidence and severity of bacterial wilt compared to untreated controls.

Significance:

This study aims to uncover sustainable biological control agents that could be integrated into disease management programs for tomatoes affected by *R. solanacearum*. By identifying and utilizing bacterial endophytes from a novel plant source such as *E. antiquorum*, this research could contribute to agricultural sustainability and the reduction of chemical pesticide use. The findings may also provide insights into the complex interactions between endophytic bacteria, plant hosts, and bacterial pathogens, enhancing our understanding of plant microbiome dynamics.

In conclusion, this problematic frames the research within the broader context of sustainable agriculture and biological control of plant diseases, setting the stage for a detailed investigation into the potential of endophytic bacteria as biocontrol agents. The clear definition of objectives and hypotheses helps to guide the experimental design and potential applications of the research findings.

Research question:

What is the biological potential of the ten endophytic bacteria isolated from *Euphorbia antiquorum* in controlling *R. solanacearum* in tomato (*Solanum lycopersicum* L.), and how might these bacteria mechanistically inhibit the pathogen's growth and disease progression?

Main objective:

This study aims to evaluate the ability of ten bacilli isolated from cactus to induce resistance of tomato plant against *Ralstonia solanacearum*, which is responsible for bacterial wilt, and their ability to promote plant growth.

Specific objectives:

1. to evaluate the plant growth promoting potential and the antagonistic activity of selected Bacilli isolates in the presence of the isolated pathogen *R. solanacearum in vitro*.
2. to determine the endophytic ability of promising antagonistic agents and the relationship between the colonization rate and salicylic acid accumulation.
3. to evaluate the ability of the best biological control agents and their consortia to suppress bacterial wilt under controlled and opened field conditions.

LITERATURE REVIEW

Chapter 1: Literature review

I.1 The host plant: Tomato (*Solanum lycopersicum* L)

I.1.1 Origin and botanical description

Tomato (*Solanum lycopersicum* L.) is an herb belonging to the nightshade family, and it is the second most important vegetable crop after the potato (*Solanum tuberosum*). It is native to the Andes of South America (OCDE, 2017) and is generally cultivated as an annual plant. It can reach a height of more than 500 fixed and hybrid varieties with regular fruits of excellent taste quality but susceptible to disease (Blanca *et al*., 2012).

The growth habit of the plant varies from indeterminate to determinate and may reach up to 3 meters in height. The primary root may grow several meters in length. The stem is angular and covered by hairy and glandular trichomes that confer a characteristic smell. Leaves are alternately arranged on the stem. Leaves are covered by angular, hairy trichomes. The tomato fruit is globular or ovoid. Botanically, the fruit exhibits all of the common characteristics of berries (OCDE, 2017).

I.1.2 Taxonomy of the tomato Linnaei (1753)

The genus *Solanum* consists of approximately 1500–2000 species, including *Solanum lycopersicum* (Darwin *et al*., 2003). The classification of tomato as the genus solanum is maintained by several classical and modern botanists.

Kingdom: Plantae

Division: Angiosperm

Class: Magnoliopsida

Oder : Solanales

Family : Solanaceae

Genus : Solanum

Species: *Lycopersicum*

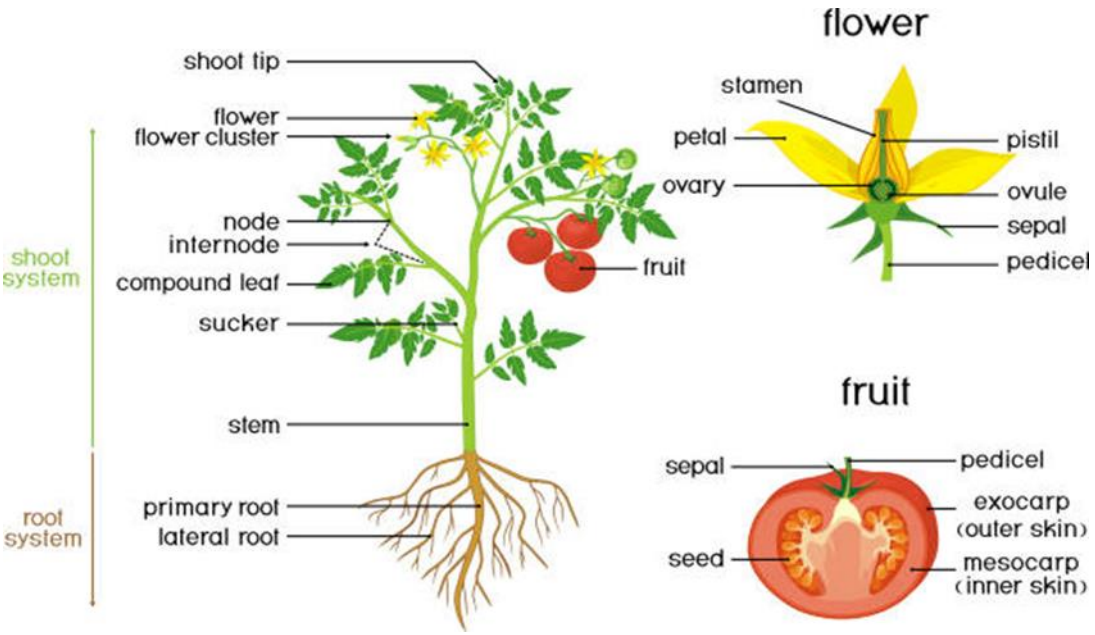

Fig. 1: Tomato plant with different parts (Kazakova, 2010)

I.1.3 Importance of tomato

I.1.3.1 Nutritional value

Tomatoes are now eaten freely throughout the world, and their consumption is believed to benefit the heart among other organs. They contain lycopene, one of the most powerful natural antioxidants (Nassarawa and Sulaiman, 2019). Tomato is termed “the most popular vegetable fruit”. It is cooked as a vegetable alone or in combination with many foods, in addition to being eaten crude when ripe. It is a fruit of good nutritional value because it is rich in vitamins (vitamin C) and other minerals, such as calcium, phosphorus and iron. Considering its low cost, it qualifies for inclusion in the daily diet of young and growing children (Yousuf *et al*., 2021).

Table 1: Average composition per 100g of raw tomato (red)

Constituents	**Quantities**
Provitamin A+	0.592 mg
Vitamin B1	0.057 mg
Vitamin B2	0.035 mg
Vitamin B3	0.530 mg
Vitamin B5	0.310 mg
Vitamin B6	0.100 mg

Vitamin B8	0.0040 mg
Vitamin B9	0.022 mg
Vitamin C	19 mg
Vitamin E	0.813 mg
Vitamin K	0.0056 mg
Boron	0.115 mg
Calcium	8.90 mg
Chloride	30 mg
Chrome	0.020 mg
Cobalt	0.0017 mg
Copper	0.057 mg
Iron	0.316 mg
Fluor	0.024 mg
Iodine	0.0011 mg
Magnesium	11 mg
Manganese	0.108 mg
Nickel	0.0058 mg
Potassium	235 mg
Selenium	0.0010 mg
Sodium	3.3 mg
Zinc	0.152 mg

I.1.3.2 Medicinal value and pharmacological properties

Traditionally, different plant parts of the tomato as well as juice and extract are being used orally, externally and internally to treat several health-related problems in several countries. In Fiji, a literature review suggests to using fresh fruit juice to induce vomiting in children suffering from food poisoning (Jafer, 2016). People use fresh juice extract to stop excessive bleeding from wounds (Jafer, 2016). Likewise, it is used externally in Greece to treat furuncles (Jafer, 2016). In Italy, it is used to cure scorpions and other insect bites (Jafer, 2016). Fresh tomato fruit is used for different purposes in several other countries. In Mexico, it is used externally as a febrifuge, whereas in the Philippines, the fresh fruit is used to treat edema in pregnant women (Jafer, 2016). Fresh fruit is used by Americans orally for kidney and liver problems, as a cathartic, and to maintain food digestion. In Cameroun, people use tomato fruit as a caustic (Jafer, 2016). In some studies, lycopene, especially in cooked tomatoes, has been

found to help prevent prostate cancer and improve the skin's ability to protect against harmful UV rays (Xin *et al*., 2016, Jessica *et al*., 2017).

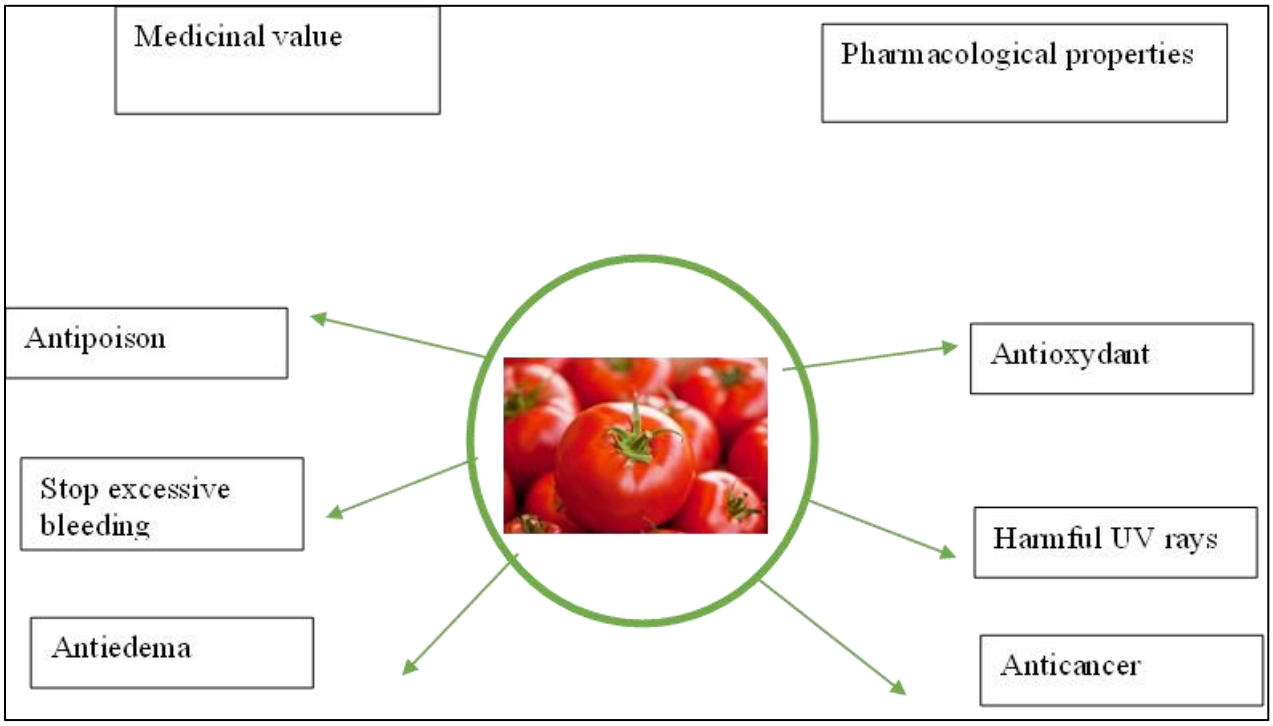

Fig. 2. Medicinal value and pharmacological properties of tomato fruit

I.1.2.3 Economic value

The cultivated tomato (*Solanum lycopersicum* L.) is the world's most highly consumed vegetable due to its status as a basic ingredient in a large variety of raw, cooked or processed foods. According to statistics from the Food and Agriculture Organization of the United Nations, world tomato production in 2020 amounted to 180, 301,750 million kg (FOASTAT, 2021). Almost 60% of world production comes from Asia, 13.3% from Europe, 8.7% from North America, 6.6% from Central America and South America, and 11.1% from Africa (FAOSTAT, 2021). In Cameroon, it has great dietary and economic importance, and it is a key input in agro-allied industrial products. Tomato production in Cameroon is estimated at 1.279.853 tons for a yield level of 12.1243 kg/ha (FAOSTAT, 2017). This value is much lower than the worldwide average yield (37.6004 kg/ha) (FAOSTAT, 2017), with an accompanying yield level of 12.286 $kg.ha^{-1}$ (FAOSTAT, 2021). Tomato cultivation represents a considerable source of income for many households, with 15000 francs indaily profit for the producer. A bulk of its production comes from the southwest region. Nevertheless, this production is much lower than the world mean (33.988 kg/ha). More despairing is the tomato population request, estimated at 1.008.000 tons too far higher than the production (FAO, 2015). This low production yield is directly

related to the ineffectiveness of the farmer's practices when facing the different constraints affecting the crop.

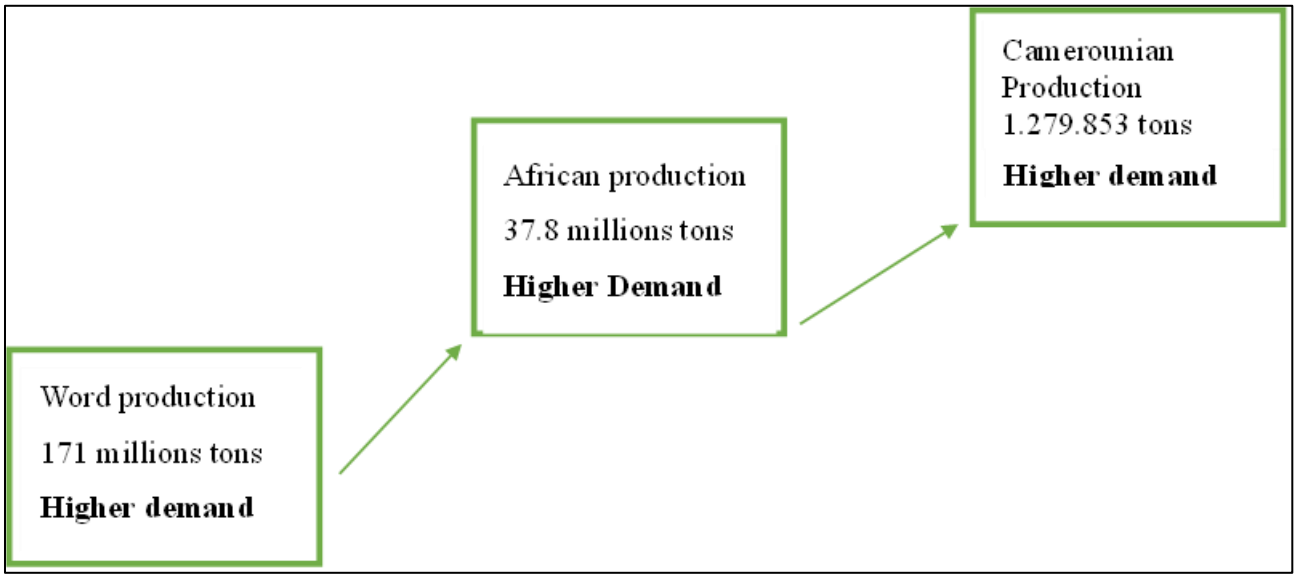

Fig. 3. Tomato production in the word, Africa and Cameroon and estimation of market need in 2020

I.1.3 Constraints related to tomato cultivation

Despite considerable efforts from farmers, tomato, which is grown as a major commercial crop, faces many biotic and abiotic challenges that contribute to its low yield (Ruchi *et al*., 2021, Rashmi *et al*., 2020, Kissoudis *et al*., 2015).

I.1.3.1 Abiotic constraints

Tomato cultivation is subject to many abiotic constraints, very often referred to as nonparasitic or physiological diseases. They are due to climatic phenomena, such as the succession of rain and sun, or excessive watering (Anonymous, 2011). Indeed, many publications have shown that excess sunlight degrades ascorbate, resulting in lower growth and therefore lower production yields. Similarly, Diakilia *et al*. (2017) showed that water stress is responsible for apical necrosis and has a negative impact on the quality of fruits.

I.1.3.2 Biotic constraints

From germination to harvest, tomato plants are prone to many diseases caused by various pathogens, including viruses, bacteria, fungi, nematodes and pests. Bacterial diseases are very devastating and can cause huge losses in the field (Shambhu *et al*., 2001). Bacterial

wilt caused by *Ralstonia solanacearum* is one of the most devastating nightshade diseases in the world, causing losses of up to 90% (Ravelomanantsoa et al., 2016). Mahbou (2011) cited this phytopathogen as the main constraint for growing tomatoes in the main production areas in Cameroon.

Fig. 4. Biotic and abiotic stresses mitigating tomato cultivation

I.2 Generalities on bacterial wilt

I.2.1 The causal agent: *Ralstonia solanacearum*

Ralstonia solanacearum is a Gram-negative bacterium causing bacterial wilt in crops of the solanaceous family, such as potato, tomato, pepper, and so on (Peeters *et al*., 2013). The first description of *R. solanacearum* occurred in 1908 by Smith (Li *et al*., 2014). The pathogen exists as a *Ralstonia solanacearum* species complex (RSSC) because of the presence of many strains that are genetically different (Prior *et al*., 2016). Previously, *R. solanacearum* strains were classified into 4 phylotypes, namely, phylotype I, phylotype II, phylotype III and phylotype IV. *Ralstonia solanacearum* is nonsporulating, aerobic and has rod-shaped cells with a length ranging from 0.5 to 1.5 µm. The attenuation index (AI) has been used to determine the pathogenicity of *R. solanacearum* (Liu *et al*., 2004). This index refers to the ratio of red spot diameter to the whole colony diameter (Zheng *et al*., 2014). The morphology of *R. solanacearum* colonies has been used to classify the pathogen into virulent, intermediate virulent, and avirulent strains (Liu *et al*., 2004). Accordingly, virulent strains have an AI of <0.65, avirulent strains have an AI of >0.75 and intermediate strains have an AI of 0.65 to 0.75. The colony characteristics of virulent strains include the presence of pink colonies at the center, irregular in shape, large white edge and greater mobility, whereas the intermediate virulent

colonies have a dark red spotted center, less mobile, surface humidity, and small white edge. The avirulent colony was round, less mobile, had a small or no white edge and had a dark red spot at the colony center (Fig5.) (Zheng *et al*., 2014).

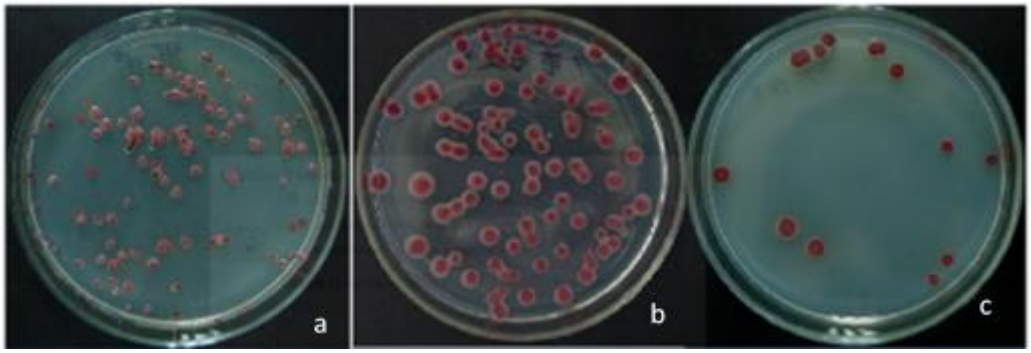

Fig. 5: Characteristics of *Ralstonia Solanacearum* strains a) Virulent strains b) Intermediate Virulence c) Avirulent strains (Zheng *et al*., 2014). Resolution: a, b, c 500 pixels

I.2.2 Bacteria wilt epidemiology

Soil-borne *R. solanacearum* is one of the most important plant bacterial pathogens (Mansfield *et al*., 2012; Claude *et al*., 2019). Many countries consider *R. solanacearum to* be a bioterrorism pathogen (Cellier *et al*., 2016). The disease is very destructive and causes fast and fatal crop wilting, subsequently resulting in yield losses. The level of damage is determined by the virulence strain, type of soil, climatic conditions, cropping pattern, and host (Elphinstone, 2005). Instance the disease causes 20–30% in ginger (Liu *et al*., 2005), 20-50% in chili, (Tan *et al*., 2014). 80 -100% in potato (Lemaga *et al*., 2001; Chen et al., 2005), 15-75% and up to 100% during extreme outbreaks in tobacco, 50–100% in peanut (Yu *et al*., 2011) and 10% to 80% in tomato (Singh *et al*., 2015; Wei et *al*., 2017).

In tomato, this bacterium can cause losses of up to 90% in the field (Dinesh et al., 2016). In Cameroon, this disease is reported in 4 of the 5 agro ecological zones (Sudano-Sahelian Zone, Bimodal Rainfall Forest Zone, Highlands Zone and Guinean High Savannah Zone). It is cited as the main constraint to the production of tomatoes in the Lekie (Obala, Okola) and Mbam (Bafia) divisions (Anonymous, 2002). In the highlands of West Cameroon (Foumbot. Dschang, Baham, Mbouda), considered to be the main vegetable patch in central Africa, bacterial wilt also represents a major constraint to the cultivation of nightshade (Mahbou, 2011) and other solanaceous crops.

I.2.3 *Ralstonia solanacearum*'s host range

Ralstonia solanacearum infects over 200 plant species, among which 50 are from dicot and monocot families (Elphistone, 2005). Some host-specific strains have been reported, such as potato (brown rot) and banana Moko strains (Peeters *et al*., 2013). Studies to identify host-specific related genes have not been successful (Cellier *et al*., 2012; Guidot *et al*., 2007). Different interactions between some Solanaceae crops (eggplant, tomato, pepper) and twelve *R. solanacearum* strains have been reported. A study on host–pathogen interactions revealed 6 phenotypes correlated with strain aggressiveness on the host (Lebeau *et al*., 2011). Being a flexible pathogen, *R. solanacearum* adapts easily to the environment and infects new hosts. *(R. solanacearum)* has been found in weeds, water, and wild species of Solanaceae crops (Wicker *et al*., 2007). There is a lack of clarity on the cause of the emergence of the new strains, but suggestions point to the vegetable and banana rotation programs practiced in Martinique.

I.2.4.1 Mechanism of infection and symptoms

Ralstonia solanacearum disseminates in agricultural fields through infested soil particles from the previous season, surface water and irrigation, infested farm equipment and tools, and latently infected planting materials. In the vicinity of a susceptible host, the bacteria are attracted toward root wounds and natural openings through the quorum sensing system and stick to the epidermal cell surface (Vasse *et al*., 1995). It has been reported that the pathogen produces esterases, endopolygalacturonase, methylesterase, and pectins to disrupt intercellular bonds and colonize the root epidermis, followed by the formation of biofilm microaggregates in the root endodermis (Mori *et al*., 2016; Gupta *et al*., 2018). Thereafter, the xylem-dwelling *R. solanacearum* releases extracellular polysaccharides, which are virulence factors that increase the viscosity of the xylem fluid, leading to the obstruction of water transport through the xylem vessels (McGarvey *et al*., 1999). There are three stages in the cycle of *R. solanacearum* infection, namely, colonization of the roots, cortical infection, and penetration of the xylem.

➢ Root colonization

The pathogen infects the host plant by recognizing certain signals, a process known as chemiotaxis. For the case of *R. solanacearum* it is done by recognition of the root exudates. At the roots of the host, *R. solanacearum* can penetrate through physical wounds and/or natural openings and attach to two precise root sites: the elongation zones of the roots and the armpits of the emerging or developed lateral roots (Belen *et al*., 2009).

➢ Cortical infection of plants roots

It begins at previously colonized sites and continues at the level of the internal cortex of the primary roots. The bacterium at this level forms large intercellular pockets, thus causing degeneration of the plant cells (Belen *et al*., 2009).

➢ Xylem penetration

Ralstonia solanacearum then multiplies exponentially within the xylem vessels and migrates into the stem, eventually reaching cell densities on the order of 10^{10} CFU/g of tissues. At this time, *R. solanacearum* forms biofilms to grip and move better in a vascular cylinder the displacement of *R. solanacearum* for the vascular cylinder involves the crossing of the endoderm (Belen *et al*., 2009). Once in the vascular cylinder, *R. solanacearum* infects the intercellular spaces of the vascular parenchyma adjacent to the xylem vessels, causing degradation of the cells of the surrounding parenchyma. The cell walls of plants are destroyed by the hydrolytic enzymes secreted by the bacteria. Within the xylem vessels, the pathogen travels throughout the stem to the upper parts of the plant, and it extensively produces exopolysaccharides in the water-conducting system in the plant, which causes wilting of the host due to clogging of vessels ((Belen *et al*., 2009). The plant dies, while the pathogen enters a saprophytic life in the soil or other environments, where it is expected to survive until it comes into contact with a new host (Saile *et al*., 1997).

It should be noted that virulence in this pathogen is enhanced by the presence of the type III secretion system (T3SS) (Coll and Valls, 2013). This system injects effector proteins into the cytosol, facilitating infection (Erhardt *et al*., 2010; Tampakaki *et al*., 2010).

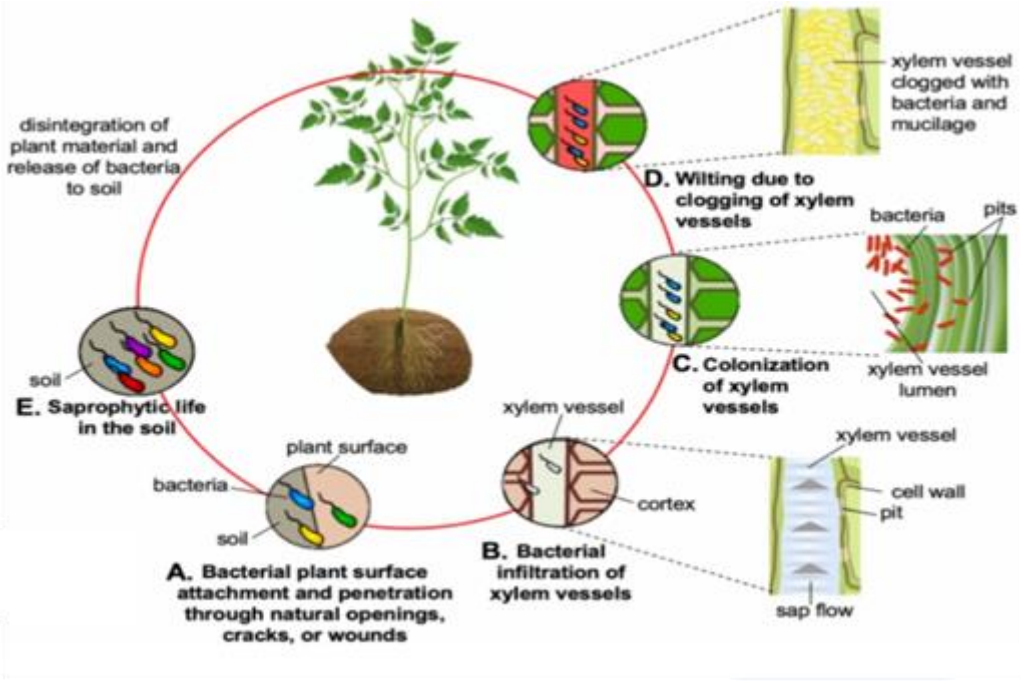

Fig. 6: *Ralstonia solanacearum* infection strategies within the intercellular space (Hikichi *et al*., 2017).

I.2.4.2 Bacterial wilt symptoms

There are several symptoms characterizing bacterial wilt in tomato. The most frequent symptoms are wilting (Fig. 7c), stunting, and yellowing (Fig. 7 a and b) of the foliage (Sebastien, 2012; Belen *et al*., 2009). In tomatoes in particular, the most classic manifestation is leaf epinasty, followed by the irreversible wilting of the plant. These symptoms are very often associated with the appearance of bulges and adventitious roots on the stem, resulting from the accumulation of acetic acid in plant cells (Belen *et al*., 2009). During the major infection, the vascular tissues of the stem take on a brown tint and the infestation of the stem can be visualized by the water test (fragment of the base of the stem placed in a volume of water) with the release of a whitish bacterial exudate.

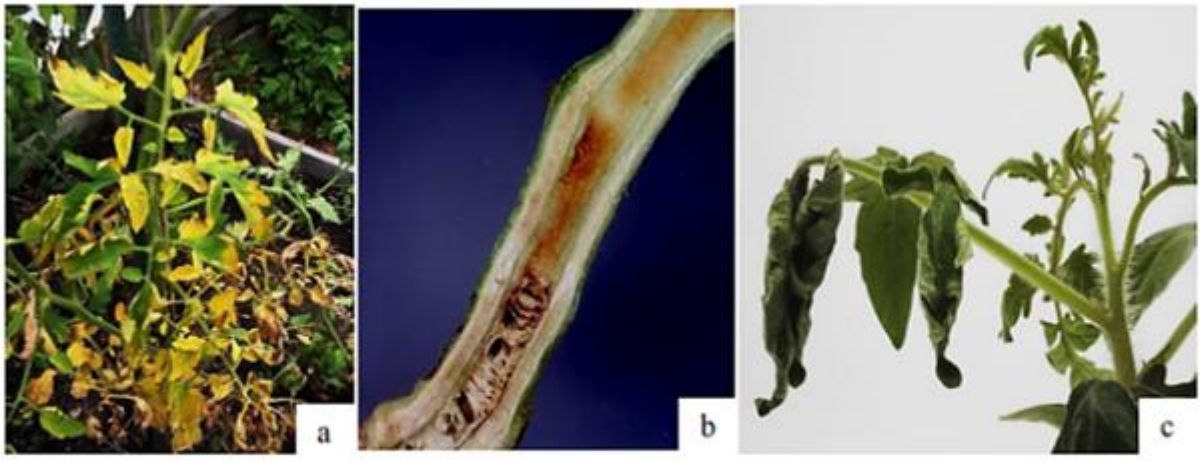

Fig : 7 : Symptoms of bacterial wilt due to *Ralstonia solanacearum* (a) Yellowing of leaves, (b) Vascular brown tint, (c) Leaf epinasty

I.3 Control methods for *Ralstonia solanacearum*

Because of its great capacity for dissemination and the great diversity of hosts it infects, *R. solanacearum* is a pathogen for which appropriate control strategies should be developed. These might include:

I.3.1 Use of healthy and high-quality seeds

Variety selection consisting of using seeds that are healthy and of high quality is currently one of the most commonly used methods for avoiding *R. solanacearum* impact. Applying this strategy in selecting many varieties of eggplants, peppers, tomatoes (Caribbean, Caracoli, Calinago), peanuts or tobacco have proven effective, but under specific environmental conditions (Ano *et al*., 2002). On the other hand, given the high number of strains of *R. solanacearum*, the host plants are only resistant for a short period of time.

I.3.2 Chemical control

Chemical control is the use of pesticides to fight against plant diseases. Synthetic pesticides such as acibenzolar (acibenzolar-S-methyl) (Blancard, 2013) are commonly used worldwide to control bacterial wilt (Blancard, 2013). However, despite being the most commonly used and effective method, they have a negative impact on the environment and human health and can lead to resistant species over time (Hiba, 2015). In Cameroon, no treatment is yet approved on the market against this pathology (MINADER, 2020). It is becoming necessary to propose alternative methods that are nontoxic and effective for the

control of *R. solanacearum*. The use of beneficial microorganisms would therefore be a promising choice.

I.3.3 Biological control

Biological control is the use of living organisms or natural substances to prevent or suppress plant diseases. Biological control agents are used to fight against phytopathogens by reducing their development and dissemination (Sebastien, 2012). A microorganism is considered a biocontrol agent if it should be able to control phytopathogens, favor the rapid growth and development of plants, and induce systemic resistance of plants against biotic and abiotic stresses (Babak *et al*., 2013). In this way, Jannat *et al*. (2020) have shown the ability of a biological product (*Bacillus subtilis*) to fight against Fusarium wilt of the roots and crown of tomato by inducing a reduction in the incidence of the disease by more than 95%. Thus, endophytic bacteria such as Bacillus, recognized as sources of molecules that are not dangerous for the environment, could constitute an effective and beneficial means of control to alleviate the chemical pesticides concerns, the ineffectiveness of cultural practice and varietal selection. However, strains of bacteria isolated from *Euphorbia antiquorum* living in extreme conditions (desert) have never been investigated to fight against the bacterial wilt caused by *R. solanacearum.* Studies have shown that desert endophytes have a greater capacity to increase nutrient uptake and plant resistance to drought, salt stress, and pathogen attack than endophytes living in normal conditions (Qiuwei and James, 2021).

I.4 Biocontrol mechanisms used by bacteria

I.4.1 Direct antagonism

I.4.1.1 Competition for ecological niches and nutrients

Once established in the rhizosphere, the microorganism of interest can first intervene favorably by depriving potentially infectious organisms of nutrients (carbon, oxygenated substrates, essential trace elements) and sparingly required for their development, reducing the number of habitable sites for microorganism pathogens and thus their growth (Carmona-Hernandez *et al*., 2019). Plant growth-promoting rhizobacteria (PGPR) are able to exclude other microorganisms (pathogens) from certain ecological niches where the production of root exudates is significant (Lugtenberg and Kamilova, 2009; Kamilova, 2005). The greatest interest has recently involved competition for iron. Under conditions of iron limitation, bacteria produce a range of iron-chelating compounds or siderophores with a very strong affinity for ferric iron.

These bacterial iron chelators have the capacity to sequester the supply of available iron in the rhizosphere by making it unavailable for pathogenic fungi and bacteria, thus limiting their growth (Haidar *et al*., 2017). Iron competition between *Bacillus velezensis* and pathogens has been clearly demonstrated (Tahir *et al*., 2017).

I.4.1.2 Production of extracellular enzymes

The involvement of enzymes in the inhibition of pathogens is referenced as one of the most important characteristics of mycoparasitism's use in biological control, but it blurs the distinction between parasitism and antibiosis. Several bacteria secrete lytic enzymes capable of degrading chitin, proteins, cellulose, hemicellulose, β-1-3-glucane and DNA, which are major constituents of the cell wall of microorganisms, thereby contributing to direct pathogen suppression (Meenu, 2013). Oana-Alima *et al*., 2015 characterized a group of *Bacillus strains*, substilis/amyloliquefaciens, that produce penzymes such as PR proteins, cellulases, proteases, lipases, amylase and decarboxylase that are involved in the damage and death of cells of *Pythium* and *Phytophtora* spp.

I.4.1.3 Antagonism by volatile compounds

Microbial strains capable of permanently producing organic volatile compounds (VOCs) with activity-inhibiting plant pathogens have recently received special attention (Liu *et al*., 2008). These antagonistic microorganisms include bacteria of the genera Pseudomonas and *Bacillus* as well as the nonpathogenic fungus Trichoderma. The production of volatile compounds by these soilborn microbes has been reported to promote plant growth, nematicidal activity, and induction of systematic acquire resistance (SAR) in the latter (Durrant and Xinnian, 2004). The researchers also found that VOCs produced by bacteria could also inhibit the germination of pathogens. Wei-Liang et al. (2020) demonstrated in their recent work that eight strains of *B. subtilis* and *B. amyloliquefaciens* are able to inhibit between 56 and 82% of the mycelial growth of *Fusarium solani* due to the VOCs they produce.

I.4.1.4 Antibiosis antagonism

Antibiotic antagonism is essentially based on the production of secondary metabolites by biocontrol agents of various kinds that have toxic effects on pathogens at low concentrations. It is probably the best-known and most used PGPR by Bacillus to limit the invasion of pathogens in the tissues of the host plant (Kohl *et al*., 2019). It consists of direct inhibition of pathogen growth via the production of metabolites and/or antibiotics (Stephanie, 2011; Munees and Mulugeta, 2013). Antibiotic production is a case of direct antagonism of phytopathogens

that plays a key role in biocontrol and depends on the efficient production of antibiotics. Certain Bacillus species, such as *B. subtilis* or *B. amyloliquefaciens*, can devote up to 8% of their genetic material to the synthesis of a wide range of antimicrobial compounds, including lytic enzymes, antibiotics, a range of (lipo) peptides synthesized according to nonribosomal mechanisms, and polyketides (Cawoy *et al*., 2015). Among these antibiotics, some, such as surfactins, are also involved in the colonization of roots and the formation of biofilms that allow bacteria to attack various substrates and with unfavorable environmental conditions.

I.4.2 Indirect antagonism/induction of resistance

Indirect antagonism involving defense reactions has been highlighted more recently and is currently the subject of many of the most promising research studies (Cawoy *et al*., 2014). Along with antibiosis, it is one of the most exploited in biological control against plant pathogens. It is now well demonstrated that plants respond to all stresses of biotic or abiotic origin with a cascade of signals that tend to induce their resistance. These defense reactions also manifest themselves both in response to a pathogen attack and to that of an antagonist, or so-called "voter molecules". PGPR (plant growth-promoting rhizobacteria) have already been shown to be able to stimulate the defense of plants. This "immunity" is initiated in principle following the perception of the plant by the so-called "elicitor" molecules produced by the beneficial microorganism. This phenomenon sequentially calls for the host's recognition of elicitors produced by agent inducers, the emission of a signal required to propagate the systemically induced state and the expression of defense mechanisms in the strict sense that limit the penetration of pathogens in plant tissue. ISR (Induced Systemic Resistance) is a systematic defense mechanism used by plant organs against various pathogens. According to Van and Bakker (1998). This is the case for tobacco and tomatoes, in which an accumulation of SA following treatment by *Pseudomonas aeruginosa* has been demonstrated

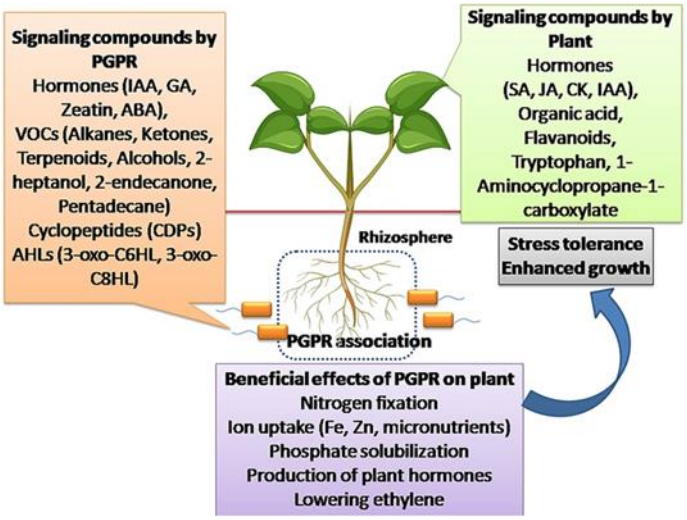

Fig. 8: Induction of resistance mediated by biological control agents. SA: salicylic acid, JA: jasmonic acid, CK: creatin kinase, IAA: indol acetic acid, ABA: abscisic acid, GA: gallic acid, PGPR: plant growth promoting rhizobacteria.

1.5 Bacillus as biocontrol agents against bacterial wilt in tomato

Many research teams around the world have invested in the research and use of bacteria with biocontrol potential and plant growth promoters; Sivaskthi et al. (2014° evaluated the antagonistic potential of Pseudomonas and Bacillus spp. isolated from the tomato rhizosphere to promote the growth of tomato plants while reducing the severity (40%) of wilt (*R. solanacearum*). The effects of these bacteria were attributable to the production of indole acetic acid (IAA) and hydrogen cyanide (HCN) and the solubilization of phosphorus. Sara et al. (2020) showed the in vitro antagonistic potential of *Bacillus amyloliquefaciens* to inhibit the virulence factors (production enzyme, biofilms and exopolysaccharide) of *R. solanacearum*. Dinesh et al. (2016) showed the antagonistic potential of *Bacillus amyloliquefaciens* DSBA-11 and DSBA-12 against *R. solanacearum* with a plant protection rate of 82.05%. Similarly, Devappa *et al*. (2022) evaluated the antagonistic potential of polyketide antibiotic production by *Bacillus* spp. isolated from rhizospheric soil against *R. solanacearum*.

MATERIALS AND METHODS

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Antagonistic microorganisms

The antagonistic bacterial strains used in this work were obtained from the core collection of the Biocontrol Agents Unit, Laboratory for Phytobiochemistry and Medicinal Plants Studies, University of Yaoundé I-Cameroon. The strains (Table 2) were previously isolated from the aerial parts, roots, and seeds of a wild *Euphorbia trigona* MILL plant grown in the Far North Region of Cameroon (5’ 13.4988’’ N; 15° 0’ 53.3952’’E). The collection took place in November 2018 at Maroua during the dry season. It is noteworthy that this locality is strongly influenced by the nearby Sahara Desert, having a 9-month dry season per year (up to 45 °C) and extremely erratic (400 to 900 mm) rainfall patterns.

Pure cultures were stored at -20 °C and -80 °C in medium containing 50% glycerol and 50% Lurea broth for further use. The identities of the isolates were confirmed by sequencing the 16S rDNA gene (Eke *et al*., 2019).

Table 2: Sources of biological control agents (BCAs)

Bioagent	**Organ (*E. antiquorum*)**	**NCBI accession**
Bacillus amyloliquefaciens CBa_BFL2	Leaves	MH788970
Bacillus amyloliquefaciens CBa_RA37	Roots	MH788971
Brevibacillus brevis CBb_RA14	Roots	MH788977
Bacillus cereus CBc_LPR8	Roots	MH788973
Bacillus velezensis CBv_BE1	Seeds	MH788975
Bacillus amyloliquefaciens CBa_BFL1	Leaves	MH788972
***Lysinibacillus* CBa_LPR19**	Leaves	MH7889
***Bacillus xiamenensis* CBx_LPR2**	Roots	MH788987
Lysinibacillus fusiformis CBl_LPR11	Leaves	MH788986
Bacillus megaterium CBm_RR10	Roots	MH788974

2.1.2 Assay materials, reagents, equipment and study site

The materials and reagent used in our study were mostly made of: culture media (Lurea Bertani Broth, Muller Hinton Agar, Nutrient Broth), Petri dishes, micropipettes, water bath, light microscope, microplate reader spectrophotometer (infiniteM200 TECAN), plastic cups, five-liter plastic buckets, incubators, streptomycin as positive control, Pikovskaya's agar medium, Shenker reagent , Nessler's reagent, Salkowski's reagent for in vitro experiment. The study site was in Yaounde, at the top of the Ngoa-Ekelle hill, at a place called plateau Atemengue of the Yaounde III subdivision, Mfoundi division more precisely behindthe Institute for Demographic Reseach and Training . The geographical coordinates of the 32 Northern Hemispheres are 777 873 m from the central meridian and 426826 m from the equator.

2.1.3 Plant material

Plant material was constituted by healthy seeds of *solanum lycopersicum* variety RIO GRANDE TM328 used during pot and field assays (Fig. 9 a and a'). These were obtained from a seed company (SEEM'S AGRO) and were used to have nurseries (Fig. 9b).

Fig. 9: *S. lycopersicum* purchased from farmer house (Yaounde) (a); seeds (a'), and germinated seedlings nursery (b).

2.2 Methods

2.2.1 Isolation and pathogenicity assay of *Ralstonia solanacearum*.

2.2.1.1 *R. solanacearum* isolation

Wilted tomato plants collected from local commercial farms in Bagangte, (West, and Cameroon). The stems were cut into 1 to 2 cm pieces, and the lower ends were immersed in sterile water to allow bacterial ooze out. An aliquot of the resulting suspension was transferred

to Nutrient Broth (NB: Himedia, India) and allowed to grow overnight (28 °C). Thereafter, a diluted (10^{-8}) aliquot was plated onto Chlorure Tetrazolium agar (TZC) medium and incubated for 48 hours at 28 °C. Afterwards, colonies with an irregular viscous appearance with a pink center (chosen British or American) and white border were selected and purified by streak culture on fresh medium. Ultimately, the Biovar of the *R. solanacearum* isolates was determined based on their capability to oxidize hexose alcohols on basal medium and utilize disaccharides (Hayward, 1964).

2.2.1.2 Pathogenicity testing

The virulence of the *R. solanacearum* isolates was confirm following the method described by Hoque and Mansfield. (2005). Healthy tomato seedling (Var. Rio Grande) pregerminated for 3 weeks on sterile garden soil were transplanted into sterile half-liter plastic pots filled with garden soil as substrate. Thereafter, the *R. solanacearum* isolates to be screened were mass multiplied by subculture in Muller Hinton Broth (MHB) medium under constant stirring (150 rpm). Fifty (50) milliliters of 10^{-8} diluted *R. solanacearum* suspensions were poured around the hypocotyls of the test plants. Control seedlings received an equal volume of sterile distilled water. Fifteen (15) replicates were considered for each *R. solanacearum* isolate. One month after inoculation, the percentage of wilted seedling was recorded per isolate using the scale of (He, 1983; Hoques and Manfied, 2005), and the most pathogenic agent was selected for downstream activities.

2.2.2 Screening of endophytic bacterial strains against the most virulent *R. solanacearum*

To evaluate the antagonist activity of the 10 biological control agents under study with the aim of selecting the best agent, antagonistic activity tests were carried out.

2.2.2.1 Antibacterial propriety of *Bacillus spp* against the most virulent *R. solanacearum*

Evaluation of the antagonistic potential of endophytes was performed as described by Maryam *et al*. (2017) In brief, single colonies of the pathogen (*R. solanacearum*) and bioagents were cultured overnight in Luria Bertani broth (LBB, Himedia) medium under constant stirring (150 rpm, 28 °C). The cultures were centrifuged (10000 rpm, 5 min), and the pellets obtained were resuspended in saline solution (NaCl, 0.09%) to obtain inoculum solutions concentrated at 10^8 cells/ml. For the confrontation assay, 500 µl of the inoculum suspension of *R. solanacearum* was inoculated onto LBA (Luria Bertani Agar) to obtain uniform bacterial lawns. Plates were kept to rest for 15 min, and after that, triplicate wells (Ø = 6 mm) were drilled in the medium in which 50 µl of each antagonist was poured. Negative control wells were filled

with 50 µl of a sterile saline solution. The plates were sealed and incubated at 28 °C for 48 hrs. The inhibitory activity of antagonistic bacteria was materialized through a clear halo around the wells. Inhibition diameters were measured, and the activity was expressed in millimeters (mm).

2. 2.2.2 Bioactivity of culture filtrate of *Bacillus* species at different times

A loopful of each bioagent at log-phase was inoculated in 500 ml Erlenmeyer flasks containing 100 ml sterile LBB. The flasks were incubated under constant stirring (150 rpm; 28 °C) for 5 days. Thereafter, 5 ml subsamples were taken on the 1st, 2nd, 3rd, 4th and 5th days post incubation, and the filtrates were obtained by successive filtration through Whatman filter paper N°1 and centrifugation at 10000 rpm for 15 min. The supernatants were passed through filtration membranes (Millipore, 0.22 µm), and the antibacterial properties of the resulting culture filtrates were evaluated according to CLSI, 2008 (standard procedure). In fact, sterile LBB medium of each antagonist was first distributed in triplicate in 96-well microliter plates. The first-line wells were then filled with 100 L of stock solutions of each culture filtrate (4 mg/mL). After thorough mixing, a twofold serial dilution was performed by consecutive transfer of 100 µL of the mixture into subsequent wells. A 100 µL suspension of *R. solanacearum* at 10^8 CFU/mL was inoculated in the wells except for the sterility mock, which consisted of a blank without *R. solanacearum* and negative controls made up of *R. solanacearum* free from the inhibitor. The plates were incubated at 28 °C for 48 hours. The antibacterial activity was expressed in terms of the minimum inhibitory concentration (MIC), which is the lowest filtrate concentration exhibiting complete inhibition of *R. solanacearum.* The assay was performed twice, and the means were used to separate the bioagents.

2.2.2.3 Antibacterial activity of ethyl acetate extracts

One milliliter (1 ml) of each antagonistic strain set at 10^8 cells/ml was inoculated in 10 ml of LBB and incubated for 5 days (200 rpm, 25 °C). The resulting culture was separated using the liquid–liquid partition method with equal volumes of ethyl acetate. The ethyl acetate fraction was harvested and evaporated using a rotary evaporation system (BUCHI, Switzerland). The MIC of the resulting ethyl acetate crude extracts was determined on *R. solanacearum* using the broth microdilution method as described above.

2. 2.2.4. Salicylic acid (SA) production

Salicylic acid is a plant immune response regulator, and its synthesis is often triggered in response to pathogen attacks. The ability of the bacterial antagonists to produce SA was screened following the protocol described by Meyer et al. (1992). Ten microliters of bacterial

suspension at 1.5×10^8 CFU/ml was inoculated in 5 ml of succinate medium and incubated for 48 h under constant stirring (100 rpm à 30 °C). The suspensions were centrifuged (6000 g; 5 min), and 4 ml aliquots of the supernatants were acidified (HCl, 1 N). Salicylic acid was then extracted by liquid–liquid partition using chloroform, and the organic solvent was evaporated using the rotary evaporator system (BUCHI, Switzerland). SA was then revealed in the medium with $FeCl_3$. The absorbance read-out of the purple iron-salicylic acid complex developed upon addition of 5 µl ferric chloride ($FeCl_3$) in chloroform extracts were made at 527 nm using microplate reader spectrophotometer (infiniteM200 TECAN). The SA content was determined against a standard calibration curve of pure SA ($R^2 = 0.96$) and expressed in µg/ml.

2.2.3 Metabolomic profiling of BCAs culture extracts using High Performance Liquid Chromatography coupled with Mass Spectrometry (HPLC/MS)

To search for secondary metabolites that could be responsible for antibacterial activities, LC/MS analysis of ethyl acetate extracts from contrasted treatments was carried out on a Shimadzu CLASS-VP V6.14 SP1 (USA) HPLC apparatus incorporating a UV detector. The analytes were determined at room temperature on an analytical column (Diamonsil C18, 150 × 4.6 mm, i.d., 5-µm particle size) (Dikma Technologies, Beijing, China). The mobile phase consisted of the solvents (A) 1% (v/v) aqueous phosphoric acid and (B) methanol using a gradient elution of 10-30% (B) at 0-10 min, 30-35% (B) at 10-15 min, 35-60% (B) at 15-25 min, 60-80% (B) at 25-35 min, and then returning to the initial condition for a 5 min (35-40 min) re-equilibration, with a total run time of 40 min. The mobile phase was passed under vacuum through a 0.45 µm membrane filter before use. The analysis was carried out at a flow rate of 1 ml/min with the detection wavelength set at 253 nm. A molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPS Documentation/) on the GNPS website (http://gnps.ucsd.edu).

2.2.4 *In vitro* plant growth promoting (PGP) traits

2.2.4.1 Assay for ammonia (NH_3) production

Ammonia is closely connected to plant growth and immunity. Among others, it provides the necessary building blocks to synyhetize most of the defense related secondary metabolites and is central for NO production whose role in plant pathogen. We have thus assessed the capacity of our biocontrol agents to produce ammonia which will be beneficial for the plant.

Freshly grown antagonists were inoculated at log phase into 5 ml peptone water (10%) and incubated at 30 °C for 48 h as described by Cappuccino and Sherman (1992). After

incubation, 0.5 ml of Nessler's reagent was added. After thorough mixing, the development of yellow–brown coloration indicated ammonia production. The OD read-out of the yellow–brown complex was made at 450 nm using a microplate reader (infinite M200, TECAN). The concentration of ammonia produced was estimated using a standard curve of $(NH_4)_2SO_4$ in the concentration range of 1-1 µmol/ml ($R^2 = 0.96$).

2.2.4.2 Assay for phosphate solubilization

Phosphorus is one of nutrients essential for plant growth. Its functions cannot be performed by any other nutrient and an adequate supply of phosphorus is requirired for optimum growth and reproduction of plant. Very often present in the soil but unavailable to the plant, we evaluated the ability of bioagents to solubilize phosphorus to make it available to the plant.

The beneficial bacterial strains were further tested for their ability to solubilize inorganic phosphate using the procedure described by Pikovskaya (1948). Briefly, autoclaved (121 °C, 15 min) Pikovskaya's agar medium, made up of 2.5 g calcium phosphate ($CaHPO_4$), 13 g glucose 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4$-$7H_2O$, 0.5 g yeast extract, 0.2 g KCl, 0.0002 g $MnSO_4$, 0.0002 g $FeSO_4$-$7H_2O$, 10 g dextrose and 15 g agar per liter (pH=7.2), was poured in Petri dishes (90 mm). A spot-inoculation bacterial strain (5 mm Ø) was cultured on the center of the plates and incubated at room temperature for 3 days. The solubilization of phosphate was checked by a clear halo around the colony.

2.2.4.3 Siderophore production

Iron is involved in many crucial metabolic processes and therefore required to maintain cells in a healthy state. Iron can accept and donate electrons easily, making it a cofactor of choice for many enzymes. Under iron deficiency cells showsaffected growth. To obtain iron from environment, the most widespread strategy developed bymicroorganisms involves siderophores secretion uptake. We have thus assessed the capacity of our biocontrol agents to produce siderophores which are small molecules with high affinity for iron Fe (III).

2.2.4.3.1 Carboxylic siderophores

A loopful of bacterial antagonist was inoculated in 5 ml of sterile LBB and incubated under orbital shaking (150 rpm, 25 °C) for 18 hours. The slurry was filtered (Millipore, 0.22 µm), and 400 µl acetate buffer and 200 µl Shenker reagent (1 ml of copper sulfate $CuSO_4$ 250

µM) were added to a 200 µl aliquot of each supernatant. A decrease in the blue cuprous complex developed by carboxylic siderophores was monitored by OD read-out at 280 nm spectrophotometry (Infinite M200 TECAN). Siderophore production percentage was determined by formula (1) (Payne, 1994).

$$\% \text{ Siderophore production} = \frac{\mathbf{ABSref - ABSts}}{\mathbf{ABSref}} x\ 100\ (\mathbf{1})$$

Where ABSref is the absorbance of the reference (simple medium) and ABSts is the absorbance of the test sample (Schwyn and Neilands, 1987).

2.2.4.3.2 Hydroxamate and catecholate siderophores

Hydroxamate and catecholate siderophores were determined as follows: 1 ml ferric chloride solution (2%) was added to the 18-hour culture filtrate prepared above. A color change from red to purple indicated hydroxamate siderophore production. Both forms of siderophores, hydroxamate and catecholate, were quantified spectrophotometrically (infinite M200 TECAN) at 450 nm and 495 nm, respectively (Neilands, 1982). Hydroxamate and catecholate siderophore production, expressed as a percentage, was determined by formula (1) above (Payne, 1994).

2.2.4.6 Indol acetic acid (IAA) production

Indol acetic acid is a powerful growth hormone produced naturally by plants. They are found in shoot and root tips and promote cell division, stem and root growth. We evaluated the ability of bioagents to produce indol acetic acid which will futher help the plant in its growth.

The capability of the bioagent to produce IAA was assessed as per the procedure proposed by Goswani et al. (2013). Briefly, the strains were grown for 3 days in LBB tubes supplemented with 0 or 5% glucose and 500 µg/ml L-tryptophan. Then, 5 ml of each culture was centrifuged at 9000 × g for 20 min, and 2 ml of Salkowski's reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) were added to an equal volume of supernatant. The mixture was then incubated in darkness for 25 min. The pink coloration developed was spectrophotometrically measured at 530 nm (infinite M200 TECAN), and IAA was quantified by extrapolation on an IAA standard curve prepared at a concentration range of 10-100 µg/ml.

2.2.5 Screening for hydrolytic enzyme production

Hydrolytic enzymes are involved in inhibiting the growth of pathogens through hydrolysis of their cell wall, proteins, and DNA and in the colonization and development of the plant. We assessed the ability of bioagents to produce certain hydrolytic enzymes.

2.2.5.1 Cellulase activity

Cellulase is an enzyme which is involved in the degradation of the the cell wall of pathogen and also in the colonization of plants by degradation of pectocellulose wall.

The ability of the bioagents to produce and release cellulase was assessed by inoculating a loopful of each antagonist in M9 minimal salt medium containing 20 g cellulose and 1.2 g yeast extract per liter.The plates were incubated at 28 ± 2 °C for 8 days and flooded with aqueous Congo red solution (0.3%). The flooded plates were allowed to rest for 20 min at room temperature (Rt). Clear halos formed around the colonies were signs of cellulose hydrolysis and thus cellulase production. The experiment was performed in triplicate and repeated twice. The enzymatic activity index (EAI) was calculated as described by (Ramos et al., 2014):

$$\mathbf{EAI} = \frac{\mathbf{a} + \mathbf{b}}{\mathbf{a}} (\mathbf{2})$$

With a representing the colony diameter, and b representing the halo diameter.

2.2.5.2 Protease activity

Protease plays a significant role in the lysis of cell wall of phytopathogens by breaking down major proteins of phytopathogenes into peptide chain and thereby destroy their capacity of pathogen's protein to act on plant cells.

The ability of the bioagents to synthesize proteases was assessed by spreading inoculation of each antagonist on skim milk agar (casein 0.5%, yeast extract 0.25%, dextrose 0.1%, skin milk powder 2. 8% and agar 1.5%) and incubation for 24 h at 28 °C. The appearance of clear zones around the inoculation spot marked positive protease activity (Abbas and Leila, 2011). The experiment was performed in triplicate and repeated twice. The enzymatic activity index (EAI) was calculated as described above.

2.2.5.3. Amylase activity

During seed germination, amylase plays an important role in hydrolyzing the endosperm starch into metabolizable sugars, which provide the energy for the growth of roots and shoots.

For amylase production, a loopful of each antagonist was inoculated on starch-agar plates containing 1% starch and 2% agar, followed by incubation at 28 ± 2 °C for 48 h. After incubation, the plates were flooded with a 1% iodine solution for 5 min and washed with distilled water to remove the excess dye (Bahadure et al., 2010). The amylase activity (clean halo) was measured and expressed in mm. The experiment was performed in triplicate and repeated twice. The enzymatic activity index (EAI) was calculated as described above.

2.2.6 Phytotoxicity test

Prior to the application of the *Bacillus* strains on planting material, their potential toxic effects toward plant cells were screened on detached tobacco (*N. tabacum*) leaves (Granada and Sesqueira, 2011). A micrusyringe was used to inject 10 µl of bacterial cell suspension (10^8 cells/ml) into tobacco leaves. Control (negative control) leaves were injected with sterile distilled water (SDW). Both controls and test leaves were incubated at room temperature for 24 h. Bacterial candidates causing chlorotic and/or necrotic zones around the inoculated points were considered pathogenic and were excluded from forthcoming tests.

2.2.7. Tomato seed biopriming assay

Healthy tomato seeds (RIO Grande TM328) with no cracks or any visible deformation were surface sterilized with 3% sodium hypochlorite for 10 min and rinsed thrice with autoclaved distilled water. Seeds were air dried in the hood, and the surface sterilized and dried seeds were seeded in Petri plates provided with sterile wet tissue paper. Antagonistic bacterial suspensions were prepared at 0.5 Mac faland a total of 10^8 CFU/ml and 5 ml inoculum of each antagonist were poured on the plates (Prachi et al., 2020). For the control, the inoculum was replaced with sterilized distilled water. Five plates of 20 seeds each were used per treatment. Germinated seeds were counted daily for 15 days. The germination index (2) (GI) and mean germinated time (2) (MET) were calculated as reported by Eke *et al*. (2019).

$$GI = \left[\frac{\text{No of germinated seeds}}{\text{Days of first count}}\right] + \ldots + \left[\frac{\text{No of germinated seeds}}{\text{Days of last count}}\right] \quad (3)$$

$$MET = \sum nidi / n \quad (4)$$

2.2.8 Assessment of endophytic performances of promising bacterial strains

2.2.8.1 Spontaneous antibiotic multiresistant mutant generation

The ability of the BCAs to produce multiresistant mutants has been evaluated on Luria Berthani Agar (HIMEDIA, India) amended with 100 µg/ml ampicillin, chloramphenicol, ciprofloxacin, or rifampicin and to isolate spontaneous antibiotic multiresistant mutants (Sophia et al., 2021). Briefly, 50 mh of 18-hour (mid-log phase) bacterial suspension from each antagonist was centrifuged (8000 g, 2 min), and the cell pellets were resuspended in 5 ml of saline (0.09% NaCl) solution. An aliquot (100 µL) of the resulting suspension was plated on LBA incorporated with the abovementioned antibiotics. Distinct colonies (ground glass appearance, cream hemolytic colonies, with irregular edges) were harvested and streaked onto freshly prepared LBA medium amended with the combination of the three antibiotics (Sheoran *et al*., 2016). The obtained antibiotic multiresistant mutants were stored in antibiotic-amended Luria Berthani broth at -20 °C.

2.2.8.2 Tomato inoculation with multi-resistant mutant BCAs and gnotobiotic growth

Two (2) weeks-old tomato plantlets (Cv. Rio Grande) grown in plastic pots (0.5 kg) containing double-sterilized (121 ºC, 20 min) garden soil were used. Overnight cultures (LBA) of antibiotic multi-resistant mutants, were washed by suspending the colonies in saline (0.09% w/v) solutions followed by centrifugation (10000 rpm, 5 min). The process was repeated twice, and the cell pellet was re-suspended in fresh saline and adjusted to 0.25 x 10^8 0.5 x 10^8, 0.75 x 10^8, 10^8, 2 x 10^8, and 4 x 10^8 CFU/ml with a sufficient amount of saline solution. 100 ml of each inoculum were poured around the collar region of young plantlets. Control plants received SDW instead of inoculum. The bacterized and mock plants were grown under axenic conditions with constant watering. Twenty-one (21) days post inoculation (dpi), endogenous bacterial population size was determined.

2.2.8.3 Reisolation and quantification of endophytic competent candidates

Two (2) week-old tomato plantlets (Cv. Rio Grande) grown in plastic pots (0.5 kg) containing double-sterilized (121 °C, 20 min) garden soil were used. Overnight cultures (LBA) of antibiotic multiresistant mutants were washed by suspending the colonies in saline (0.09% w/v) solutions followed by centrifugation (10000 rpm, 5 min). The process was repeated twice, and the cell pellet was resuspended in fresh saline and adjusted to 0.25 x 10^8, 0.5 x 10^8, 0.75 x 10^8, 10^8, 2 x 10^8, and 4 x 10^8 CFU/ml with a sufficient amount of saline solution. One hundred

milliliters of each inoculum was poured around the collar region of young plantlets. Control plants received SDW instead of inoculum. The bacterized and mock plants were grown under axenic conditions with constant watering. Twenty-one (21) days post inoculation (dpi), endogenous bacterial population size was determined.

2.2.8.4 Time-dependent evaluation of salicylic acid biosynthesis in colonized seedlings.

- **Tracking of salicylic acid**

The tracking of salicylic acid was performed at the end of each day (day one to day seven) by the method of Meyer *et al*. (1993). The plant material infected with each strain was grown and introduced into tubes containing 5 ml of succinate medium. After incubation with stirring (100 rpm à 30 °C) for 48 h, the whole was centrifuged at 6000 × g for 5 min. Four milliliters (4 ml) of supernatant were acidified with a solution of HCl (1 N) to obtain a pH of 1.5-2. Salicylic acid was extracted from 4 ml of chloroform by rota vaporization. Four milliliters (4 ml) of water and 5 µl of ferric chloride were added to the extracted salicylic acid. The absorbance of the purple iron-salicylic acid complex was measured at 527 nm using a spectrophotometer. The amount of salicylic acid produced in the culture was deduced from the standard calibration curve expressed in µg/ml. The tracking of SA was correlated with the tracking of colonization, which was always performed in 7 days.

- **Tracking of plant tissue colonization**

The same plant used for the tracking of SA has been used to track colonization at the same time and on the same date using the previous protocol of colonization (Sheoran *et al*., 2016).

2.2.8.5 Box PCR fingerprinting for the confirmation of colonization

The oligonucleotide was used to compare the BOX-PCR profiles of the wild and reisolated BCAs.The amplification mixture contained 2 µM BOX primer, Dntps, 10 mM Tris-HCL, 50 mM KCl, 2.5 mM $MgCl_2$, 1.25 µM *taq* DNA polymerase, and as template DNA, 100 µM DNA from reisolated antibiotic-resistant and wild-type *B. velezensis* CBv_BE1, *B. amyloliquefaciens* CBa_BFL2 and *B. amyloliquefaciens* CBa_RA37 strains. Amplification was performed with the following Thermal Cycler program: (i) initial denaturation step of 7 min at 95 °C, (2) 30 cycles of 1 min at 94 °C, 1 min at 53 °C and 8 min at 65 °C with a final elongation step of 15 min at 65 °C. PCR amplification products were detected by electrophoresis (Sambrook and David, 2001) and visualized under UV light. Images were taken using a

BioPrint apparatus. One KB DNA ladder was used as a standard. Amplifications were performed at least twice in separate assays to ensure the reproducibility of the patterns, and only bands common to the replicated amplifications were scored. DNA fingerprints (band patterns) of wild-type and antibiotic-resistant mutant BCAs were then compared.

2.2.9 Validation of the best biocontrol agents and compatibility assessment

The tested *Bacillus* candidates were ranked on the basis of their performances, and those encompassing most of the traits (antagonism, hydrolytic enzymes, and HCN production, growth promotion tests, biofilm formation, nontoxicity toward tobacco cells and tomato seeds, and seedling colonization) were selected and taken further in the experiments. The best bacterial candidates on the ranking board were then screened for their abilito cohabitate in the same niche. One hundred microliter suspensions of overnight bacterial cultures were inoculated into LBA medium to form a bacterial lawn. Then, the other bacteria were drop-inoculated (10 µl) at equidistant points on the same plate and vice versa and incubated (28 °C) for three days. Four replications were prepared per bacterium. An inhibition halo eventually observed at the interface of the two bioagents was proof of incompatibility (John *et al*., 2007).

2.2.10 *In* planta (pot and field) assay

2.2.10.1 Seed pregermination and inoculation with biocontrol agents

Multicompetent compatible Bacillus strains were used to challenge *R. solanacearum in planta.* In fact, tomato seeds were surface sterilized and pregerminated in plastic trays for 5 days as described above. On the other hand, bacteria were grown overnight in LB broth under orbital shaking (150 rpm). Medium-free cells (10.000 rpm, 5 min) were resuspended in saline solution (0.09% NaCl), and the concentration was adjusted to 10^8 CFU/mL. Pregerminated seeds were dipped in each solution overnight. Control seeds were soaked in SDW. The seeds, both primed and unprimed, were planted in plastic pots filled with sterile garden soil and watered when necessary.

2.2.10.2 Pathogen preparation and tomato seedling inoculation

A single clean colony of the highly pathogenic *R. solanacearum* isolate (Rs5) was grown in MHA (28 °C, 48 h) under constant stirring (150 rpm). The culture was resuspended in sterile saline to a final cell load of 1. 10^8 CFU/mL. For plant infection, young tomato seedlings (14 dpt) were carefully uprooted and rinsed with SDW. The root tips of the plantlets were thereafter excised with sterile scissors and dipped in the previously prepared pathogen suspension for 30

min (Ji et al., 2014). The infected plantlets were transplanted into their respective pots. Negative control seedlings were dipped in the same volume of SDW. The treatments applied were codified as follows: (1) Control: uninoculated seedlings (neither *Bacillus* sp nor *R. solanacearum* Rs5), (2) Rs5: seedlings infected with *R. solanacearum* Rs5 alone, (3) BFL2_Rs5: seedlings primed by *Bacillus amyloliquefaciens* CBa_BFL2 codification and infected with *R. solanacearum* Rs5, (4) BE1_Rs5: seedlings primed by *Bacillus velezensis* CBv_BE1 and infected with *R. solanacearum* Rs5, and (5) RA37_Rs5: seedlings primed with *Bacillus amyloliquefaciens* CBa_RA37 and challenged with *R. solanacearum* Rs5. (6) BFL2_BE1_Rs5: seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2 and *Bacillus velezensis* CBv_BE1 and infected with *R. solanacearum* Rs5, (7) RA37_BE1_Rs5: seedlings dually primed with *Bacillus amyloliquefaciens* CBa_RA37 and *Bacillus velezensis* CBv_BE1 and infected with *R. solanacearum* Rs5, (8) BFL2_RA37_Rs5: seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2 and *Bacillus amyloliquefaciens* CBa_RA37 and infected with *R. solanacearum* Rs5. The test pots were arranged in a semicontrolled pot following a randomized complete block design (RCBD) with ten (10) replications per treatment.

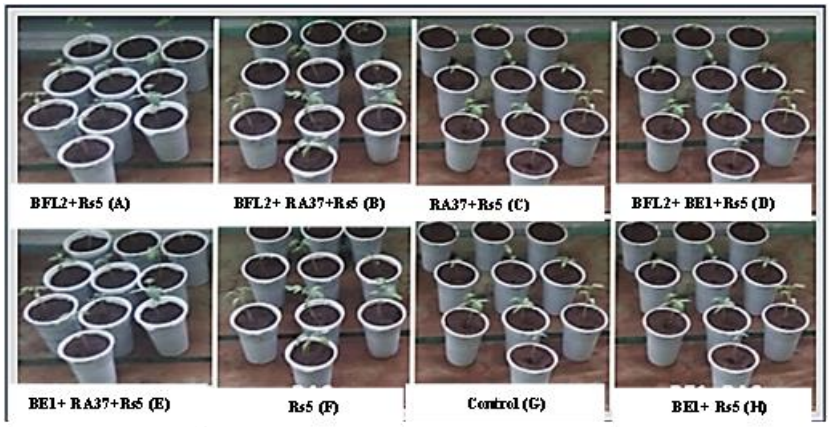

Fig. 10: Experimental design of the pot experiment consisting of eight treatments

(A) plantlets primed with *B. amyloliquefaciens* CBa_BFL2 alone and grown on *R. solanacearum* Rs5 infected soil. (B) Tomato seedlings dually inoculated with *B. amyloliquefaciens* CBa_RA37 and *B. amyloliquefaciens* CBa_RA37 and challenged with *R. solanacearum* Rs5. (C) plantlets primed with *B. amyloliquefaciens* CBa_RA37 alone and grown on *R. solanacearum* Rs5 infected soil. (D) Tomato seedlings dually inoculated with *B. amyloliquefaciens* CBa_BFL2 and *B. velezensis* CBv_BE1 and challenged with *R. solanacearum* Rs5. (E) Tomato seedlings dually inoculated with *B. amyloliquefaciens* CBa_RA37 and *B. velezensis* CBv_BE1 and challenged with *R. solanacearum* Rs5. (F) *R. solanacearum* Rs5 infected plants without antagonist. (G) Seedling not inoculated seedlings (control) by *Bacillus* spp or *R. solanacearum* Rs5. (H) Plants primed with *B. velezensis* CBv_BE1 alone and grown on *R. solanacearum* Rs5 infected soil.

2.2.10.3 Disease and agromorphological parameter records

After 7, 14, 21 and 28 dpt, the percentage of wilted plants (PWP) was recorded per treatment. Likewise, the wilting severity (WS) for each treatment was further calculated as per the 1 to 5 disease rating scale reported by He (1983) with slight modifications as follows: 1: no visible wilting on the leaf system, 2: one wilted leaf, 3: two to three wilted leaves with no yellowing, 4: four or more wilted leaves with slight leaf chlorosis, and 5: overall wilted leaves or total plant dead (Fig. 11). The WS was therefore calculated using the formula below (4):

$$\mathbf{WS\ (\%) = 100\left[\sum nivi/VN\right]} \quad (5)$$

Where: n = number of plants displaying similar disease severity score; vi given disease score in relation to ni; N = Total number of examined plants per treatment; V= highest wilting severity (A to E).

Fig. 11. Bacterial wilt (disease severity) rating scale

Later, the area under the disease progress curve (AUDPC) was calculated per treatment. The AUDPC was hence determined as a function of time f(tk) and thus evaluated at a cumulative count of each single recording period (tk) (Simko et Hans, 2011)

$$\mathbf{AUDPC} = \left[y_1 \times \frac{t_2 - t_1}{2}\right] + \left[\sum_{i=2}^{n-1}\left(y_i \times \frac{t_{i+1} - t_{i-1}}{2}\right)\right] + \left[y_n \times \frac{t_n - t_{n-1}}{2}\right] \quad (6)$$

Where y_1, y_2 and y_n are the wilting index on day one, day two and last day of observation, respectively, and t_1, t_2, t_{n-1} and t_n are the times of the first, second, and second to the last day of disease estimation, respectively.

Twenty-eight days posttransplantation, plantlets were carefully uprooted by immersing the pots in a 20-liter water bucket to prevent root damage. The aboveground and underground

parts were separated, and the root and shoot lengths were measured and expressed in centimeters. The plant parts were then oven-dried, and dry masses of the aboveground and underground parts were recorded in grams.

2.2.10.4 Extraction and assessment of oxidizing enzymes

During environmental stresses, the generation of ROS (reactive oxygen species) is enhanced, consequently cause lipid peroxidation, inhibition of enzyme activities, oxidation of proteins, deterioration of nucleic acids and leads to programmed cell death, hence cause damage to cells and finally death. Antioxidant defense system including both enzymatic antioxidants like superoxide dismutase (SOS), guiacol peroxidase (GPX), ascorbate peroxidase ((APX) play major role for the amelioration of theses ROS from the cells. They are essential for detoxification of ROS during stress.

Extraction was performed as reported by Aliaksandr (2020). One gram of fresh plant tissue was homogenized in a prechilled mortar and pestle at 4 °C with a 10 ml special mixture containing 50 mM phosphate buffer pH 7.4, 1 mM EDTA, 1 g polyvinylpyrrolidone (PVP) and 5% (V/V) Triton X-100 under ice-cold conditions. The homogenate was centrifuged at 10000 × g for 20 min at 4 °C, and the supernatant was used for quantification of guaiacol peroxidase (GPX), superoxide dismutase (SOD) and phenylalanine ammonia lyase (PAL) activities.

2.2.10.4.1 Assay of peroxidase (GPX) specific activity

The intracellular level of H_2O_2 is regulated by several enzymes, the foremost important of which are catalase and peroxidases participating within the fine regulation of ROS concentration through the cell.

Peroxidase activity was determined following the method of Bestwick et al. (1998). Peroxidase activity was measured in a reaction mixture consisting of supernatant (1 ml) and guaiacol as a substrate. Three milliliters of a reaction mixture consisting of (100 mM sodium phosphate buffer, pH 7.0 and 20 mM guaiacol). The increase in absorbance at 470 nm was measured spectrophotometrically after 20 µl H_2O_2 was added. Enzyme activity was defined as a change in the optical density/g fresh weight/min.

2.2.10.4.2 Assay of superoxide dismutase (SOD) specific activity

Superoxide dismutase has been proposed to be essential in plant stress resistance and provides the first line of defense against the harmful effects of elevated levels of reactive oxygen species (ROS).

The activity of total superoxide dismutase was determined according to Aliaksandr (2020). The activity was assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). In a test tube, 200 µl of enzyme supernatant and 300 µL of buffer solution (50 mM k-phosphate buffer 7.8 and 0.1 mM EDTA) were added to a 3.5 mL O^{2-} generator mixture (14.3 Mm methionine, 82.5 µM NBT and 2.2 µM riboflavin). The test tube was shaken and placed 30 cm under the direct lamp for 10 min. The measurements were taken at a wavelength of 560 nm. Blanks and controls were run in the same way but without illumination and enzyme, respectively. The superoxide dismutase activity unit is defined as the amount of protein in milligrams required to cause 50% inhibition of the reduction of nitrotroblue tetrazolium (NBT) at 560 nm under the assay conditions.

2.2.10.4.3 Assay of phenylalanine ammonia lyase (PAL)- specific activity

The enzyme phenylalanine ammonia lyase (PAL) catalyzes deaminating reaction of the amino acid phenylalanine from the primary metabolism into the important secondary phenylpropanoid metabolism in plants.

PAL-specific activity was measured following the method of Ross and Sederoff (1992). The assay mixture, containing 100 µl of plant extract, 500 µl of 50 mM Tris HCl (pH 8,8), and 600 µL of 1 mM/L phenylalanine, was incubated for 60 min at room temperature, and the reaction was arrested by adding 2 N HCl. The assay mix was extracted with 1.5 ml of toluene by vortexing for 30 sec. Toluene was recorded after centrifugation at 1000 rpm (CRU-5000 centrifuge ITC) for 5 min. The absorbance of the toluene phase containing trans cinnamic acid was measured at 290 nm against the black of toluene. Enzyme activity was expressed as nmol trans cinnamic released $min^{-1}.g^{-1}$ fresh weight.

2.2.11. Confirmation of the *in planta* best biological control consortia

The best combination obtained in the pot (RA37/BFL2) was used under semicontrolled conditions in the pot and in the open field to challenge *R. solanacearum.*

2. 2.11.1 Preformulation of the best consortium

Biocontrol agents were grown at 20 °C in 250 mL flasks, each containing 100 ml of Lurea Bertani broth (LB) liquid medium and shaking at 1000 rpm for 10 min. A talc-based powder formulation of endophyte bacteria was prepared according to the protocol described by Vidhyasekaran *et al.* (2003). Briefly, carboxymethylcellulose (10 g) was mixed with talc powder (1 kg), and the pH was adjusted to 7 by adding calcium carbonate. The mixture was autoclaved at 121 °C for 20 min on each of 2 consecutive days. A bacterial suspension (150 ml) containing $6.6x10^9$ CFU was added to the mixture and mixed well under sterile conditions (Durga and Rajagopal, 2000). The formulation was sealed in polythene bags and stored at room temperature (25± 2 °C).

2.2.11.2 Shelf life evaluation of formulation

Biocontrol agents were grown at 20 °C in 250 mL flasks, each containing 100 ml of Lurea Bertani broth (LB) liquid medium and shaking at 1000 rpm for 10 min. A talc-based powder formulation of endophyte bacteria was prepared according to the protocol described by Vidhyasekaran et al. (2003)Briefly, carboxymethylcellulose (10 g) was mixed with talc powder (1 kg), and the pH was adjusted to 7 by adding calcium carbonate. The mixture was autoclaved at 121 °C for 20 min on each of 2 consecutive days. A bacterial suspension (150 ml) containing $6.6x10^9$ CFU was added to the mixture and mixed well under sterile conditions (Durga and Rajagopal, 2000). The formulation was sealed in polythene bags and stored at room temperature (25± 2 °C).

2.2.11.3 Preparation of *R. solanacearum* and streptomycin

A single clean colony of the highly pathogenic *R. solanacearum* isolate (Rs5) was grown in LB (28 °C, 48 h) under constant stirring (150 rpm). The culture was resuspended in sterile saline conditions to achieve a cell load of 1. 10^8 CFU/ml (Singh *et al*., 2018).

The positive control used was streptomycin (Sigma China Co. Shanghai, China), 200 µg/ml (Hancheng *et al*., 2015). Preparation was performed with a mixture of 20 g of streptomycin powder in 100 g of talc powder.

2.2.12. Confirming the efficacy of the talc- based formulation

2.2.12.1 Seed disinfection and bacterization.

The tomato seeds were coated with each of the formulations (biocontrol agents and positive control) and pregerminated in trays containing sterile soil. To serve as controls, some parts of the seeds were germinated without treatment. The treated and untreated seeds were pregerminated in sterile soil for 3 weeks.

2.2.12.2. Seedling infection with *R. solanacearum* and experimental layout

An inoculum of *R. solanacearum* adjusted to 10^8 CFU/ml was used to inoculate seedlings. Control seeds were soaked in SDW. The primed and unprimed plants were sown in five-liter buckets of nonsterile growing medium and watered when necessary.

The experiment was conducted in a completely simple design (CSD) with four treatments and seven replicates for 3 months. The treatments consisted of the followings:

- Control (1): uninoculated seedling (neither *Bacillus* sp nor *R. solanacearum* R_s5)
- R_S5 (2): seedlings infected with *R. solanacearum* R_S5 alone
- Ps_R_S5 (3): seedlings primed by streptomycin and infected with *R. solanacearum* R_S5
- BFL2_RA37_R_S5 (4): seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37 and infected with *R. solanacearum* R_S5 (5)

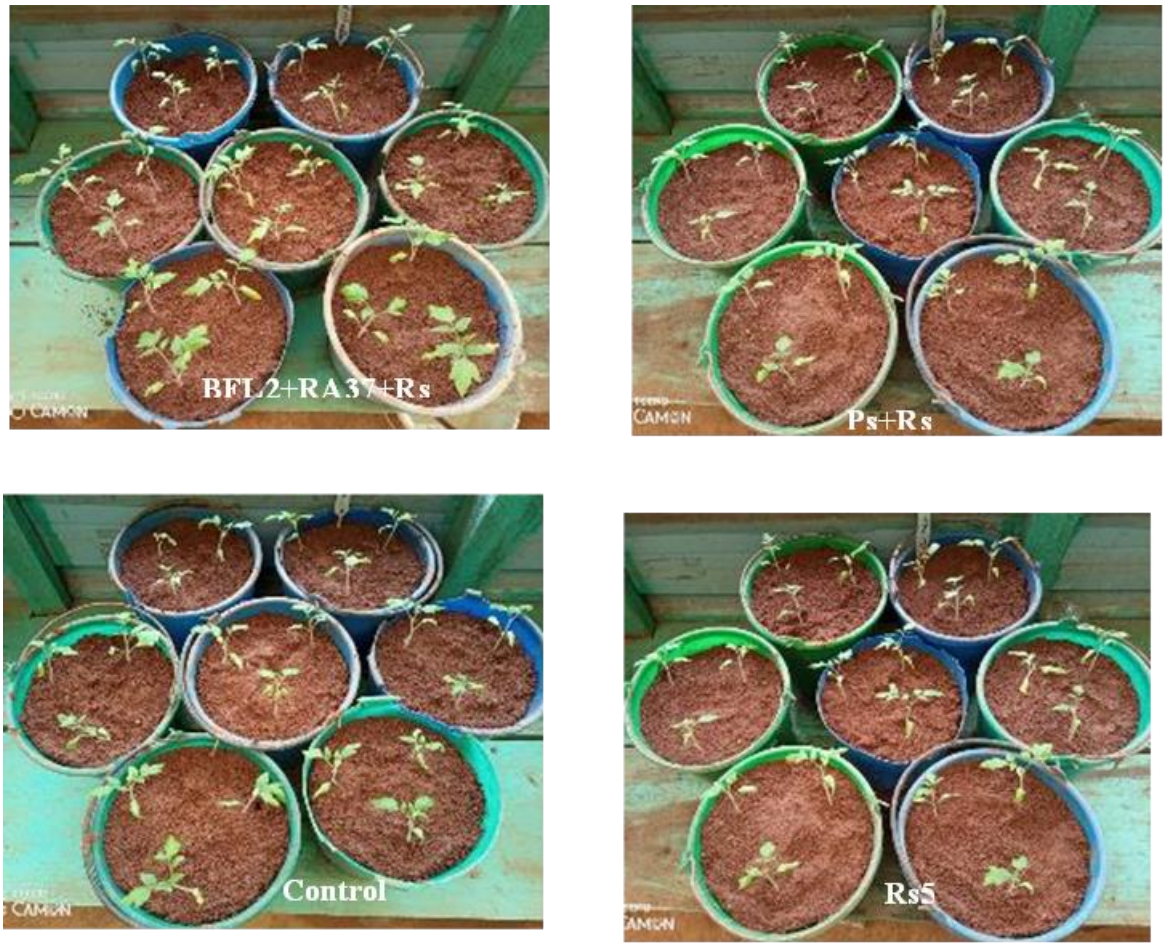

Fig. 12: Experimental design with the best treatment.

BFL2+RA37+R_S5: Seedlings dually primed with *Bacillus* amyloliquefaciens CBa_BFL2, *Bacillus* amyloliquefaciens CBa_RA37 and infected with *R. solanacearum* R_S5 (5), Ps+R_S5: Seedlings primed by streptomycin and infected with *R. solanacearum* R_S5, Control: uninoculated seedling (neither *Bacillus* sp nor *R. solanacearum* R_s5), R_S5: seedlings infected with *R. solanacearum* R_S5 alone

2.2.12.3 Data recorded

- **Growth parameters**

Plant parameters such as plant height (cm), leaf number per plant and treatment, were measured using a leaf area meter. All data were analyzed to evaluate test significance.

All data were analysed for evaluating test significance.

- **Disease parameters (incidence and severity)**

Data were taken at 7-day intervals to calculate disease incidence and severity by using the following formula and scale (Sunil et al.; 2017).

$$\%\ \textbf{Disease incidence} = \frac{\textbf{Number of infected plants}}{\textbf{Total number of plants}} \boldsymbol{x}\ \mathbf{100}\ (7)$$

The severity of disease was evaluated visually on tagged plants along the plot and recorded as the percentage of plant parts (tissues) affected.

- **Yield parameter**

The date of first flowering, date of 50% flowering (when half of the plants have flowered, we note the date of 50% flowering), average weight of fruit, fruit number per plant, weight of fruit, and yield of fruit (t/ha) weredetermined according to Sunil *et al*. (2017).

2.2.13 Field experiment

2.2.13.1. Description of study site

The study site was in Yaounde, at the top of the Ngoa-Ekelle hill, at a place called plateau Atemengue of the Yaounde III subdivision, Mfoundi division. The geographical coordinates of the 32 Northern Hemispheres are 777 873 m from the central meridian and 426826 m from the equator. The edaphic cover is made of ferrallitic soil rejuvenated with erosion and reworked, intimately associated with brown mineral soil and poorly developed soils (Valerie, 1995). The vegetation consists mainly of grasses. Hydrophilic herbaceous formations grow in swampy lowlands. We noted the presence of shurbs, fruit trees, and ornamental plants and cultivable areas. The Guinean climate is equatorial with an average of 1500 and 1600 mm of rain per year divided into 4 seasons: a long dry season from mid-November to mid-March, a long rainy season from mid-March to the end of June, a short dry season from July to August, and a short rainy season from September to mid-November. The city's climate is characterized by moderate warmth and high humidity, with temperatures fluctuating between 28°C to 34°C. These temperatures make Yaoundé a relatively warm city year-round, with a very mild temperature variation across the seasons, typical of many equatorial cities. The consistency in temperature paired with seasonal changes in precipitation defines the overall climate of the area.

2.2.13.2 Determination of soil properties

Certain physical properties of soil were evaluated two weeks before transplanting. Three steel core samples collected from 5 to 15 cm below each plot were used for the evaluation of bulk density, porosity and gravimetric water content after placement of samples in an oven set at 100 °C for 24 h. Total porosity was calculated using a particle density of 2.65 mg/m^{-3}.

Samples were obtained from 5 to 15 cm below each plot at 3 sites per plot at the beginning of harvest to determine soil chemical properties. The samples were composited, air-dried, and passed through a 2 mm sieve before making the determinations. The organic matter content was determined using a dichromate oxidation method. Available phosphorus was determined calorimetrically after Bray-P1 extraction (Bray and Kurtz, 1945). Exchangeable calcium, potassium, and magnesium were extracted with ammonium acetate and determined on a flame photometer, and total nitrogen (N) was determined by the Kjeldahl digestion method (Jackson, 1958).

2.2.13.3 Land preparation

The chosen field experimentation was prepared as described below.

Fig. 13: Land ground work. The field was cleared using a cutlass, and grasses were raked and dumped at the border of the field. The soil was tilled using the hoe prior to insecticide treatment.

2.2.13.4. Soil treatment

The soil was treated with the insecticide ACETAMIPRID (optimal$_{20}$ sp). The solution was prepared by diluting two caps of oriba filled with insecticide in 15 liters of water for an area of 500 m^2 and left for 7 days before planting to eliminate pests and avoid insect infestation.

Fig. 14: Soil treatment. Insecticide (ACETAMIPsurface sprayedsp)) was surface sprayed to prevent insect invasion.

2.2.13.5 Transplantation

Holes of fifteen centimeters depth and ten centimeters width were made at a rate of four holes per row and four rows per bion. A string was used to respect the alignment. The soil was infested by adding 100 ml of the bacterial cell suspension (1.10^8 CFU/ml) of *R. solanacearum* around the stem base of each plant except the control (Cremashi et al., 2012)**.** The tested treatments were arranged in a randomized complete block design with 16 replicates. The whole plowed plot consisted of 4 complete blocks.

Fig. 15: Experimental holes for transplantation: Holes (width; 10 cm; depth: 15 cm) were designed on the surface of treated experimental field for tomato seedling planting.

Fig. 16: Transplanted plants in the pre-treated field. Tomato was transplanted according to the experimental design (arrows).

2.2.13.6 Field design and treatments

The experimental layout was a randomized complete block design (RCBD), and the blocks were labeled A, B, C and D (Fig.17). Each block was divided into four experimental units for a total of 16 units, and each treatment was replicated four times. The different units of the field layout were measured using a measuring tape and line, and each treatment had an area of 2 m^2. The beds in the treatments were raised to a height of 25 cm, and the spacing between treatments was 0.5 m. Each microplot corresponds to a treatment and contains four lines at intervals of 0.50 m from each other. The spacing between two plants was 15 cm. The total dimension of the experimental setup was 60 m^2. Treatment consisted of the followings:

- Control (1): uninoculated seedling (neither *Bacillus* sp nor *R. solanacearum* R_s5)
- R_S5 (2): seedlings infected with *R. solanacearum* R_S5 alone
- R_S5_M (3): seedlings primed by streptomycin and infected with *R. solanacearum* R_S5
- BFL2_RA37_R_S5 (4): seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, and *Bacillus amyloliquefaciens* CBa_RA37 and infected with *R. solanacearum* R_S5 (5)

The layout was as shown below.

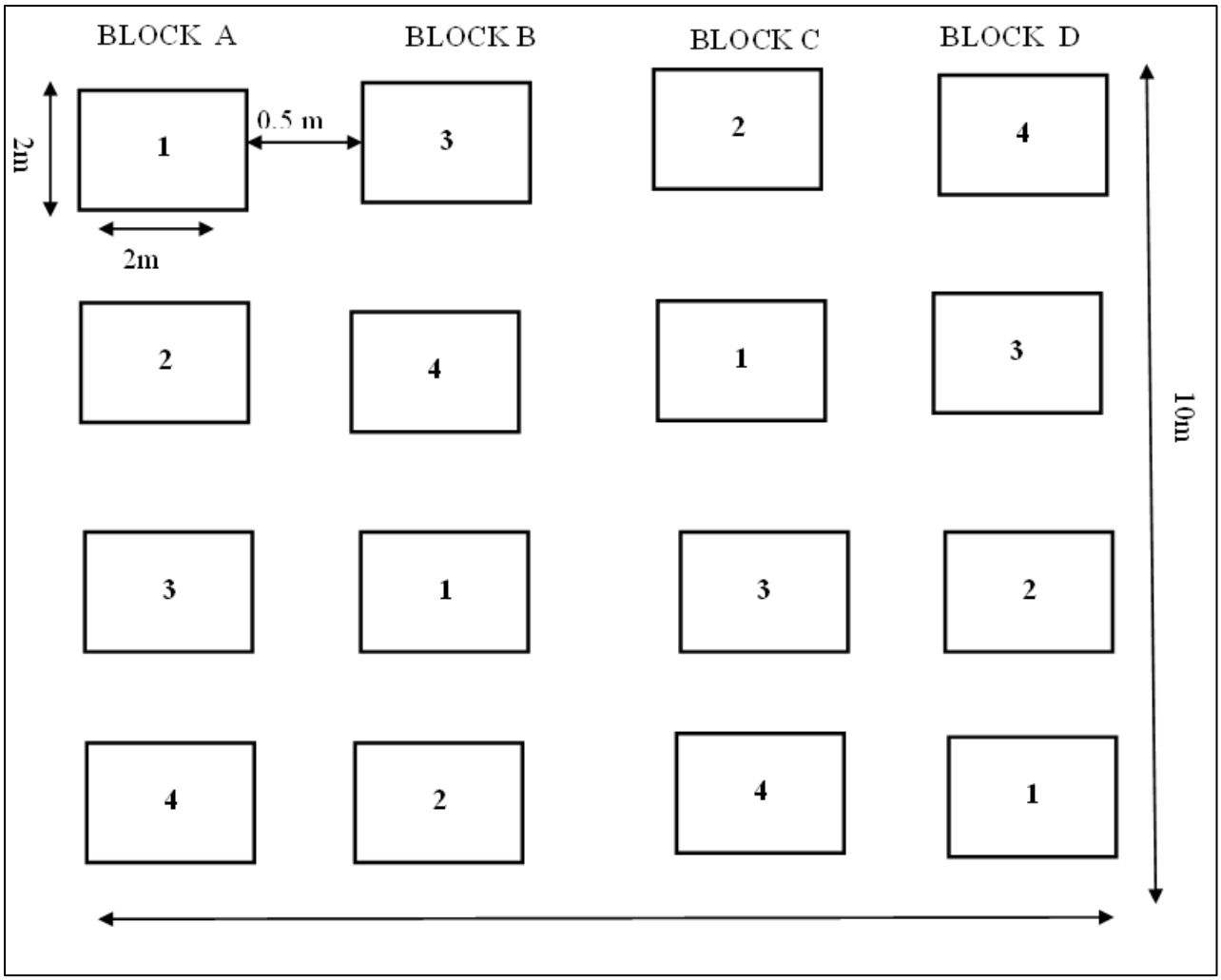

Fig. 17: Experimental layout. The experimental layout was a randomized complete block design, and the blocks were labeled A, B, C and D. Each block was divided into four experimental units for a total of 16 units, and each treatment was replicated four times. Each treatment had an area of 2 m^2. The beds in the treatments were raised to a height of 25 cm, and the spacing between treatments was 0.5 m.

2.2.13.7 Parameters recording.

2.2.13.7.1 Disease and growth parameters (incidence and severity)

Plant parameters such as plant height (cm), leaf number per plant and treatment, and leaf surface area were measured using a leaf area meter. All data were statistically analyzed to evaluate test significance.

Disease intensity

Data were taken at 7-day intervals to calculate disease incidence and severity by using the following formula and scale (Sunil *et al*., 2017):

$$\text{Disease incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} x\ 100 \quad (8)$$

The severity of disease was estimated visually on tagged plants along the plot and recorded as the percentage of plant parts (tissues) affected.

$$\text{Percentage severity index} = \sum \left(\frac{\text{Numerical rating}}{\text{Number of plants rated}} \times \text{Max score of scale} \right) \times 100 \quad (9)$$

2.2.13.7.2 Yield parameters

The date of first flowering (from the transplantation day, the day where the first flower appears is considered as the date of the first flowering), date of 50% flowering (when half of the plants have flowered, we noted it as the date of 50% flowering), fruit maturity time, average weight of a fruit, fruit number per plant, weight of fruit, and yield of fruit (t/ha) were determined (Sunil *et al*., 2017). The production yield was determined for all the treatments at the end of the experiment and expressed in kg per hectare.

2.2.14 Data analyses

Raw data were normalized and subjected to an analysis of variance (ANOVA). For assays involving colony counting, the values were log transformed prior to analyses. The generated mean values were pairwise compared using the Newman-Keul post hoc test. The Pearson test was used to study the relationship between parameters when needed. The Sigmaplot 11.0 statistical package was used for the analyses, and the significance threshold of the overall analyses was set at 5%.

RESULTS

Chapter 3: Results

3.1 Results

3.1.1 Isolation of *R. Solanacearum* and pathogenicity test

During the isolation process, a distinctive white streaming bacterial ooze flowing out of stem pieces of wilted tomato plants was observed. Isolation allowed the growth of diverse bacteria with distinct morphologies, of which viscous bacteria with pink centers and white borders were selected as putative *R. solanacearum*. Biochemical characterization such as solubility in 3% KOH, was utilized to further ascertain the identity and to discard false positives. A total of nine (Rs1 to Rs9) *R. solanacearum* isolates were recovered from wilted tissues and their virulence was checked toward young tomato seedlings in thenursery. The results (Fig. 18) indicated that all 9 *R. solanacearum* successfully induced wilting symptoms in *S. lycopersicum* at varying intensities. For instance, the percentage of wilted plants (PWP) ranged from 37.2 to 100%. The isolate Rs5 (PWP=100 %) was the most virulent amongthe others and was thus selected for further experiments.

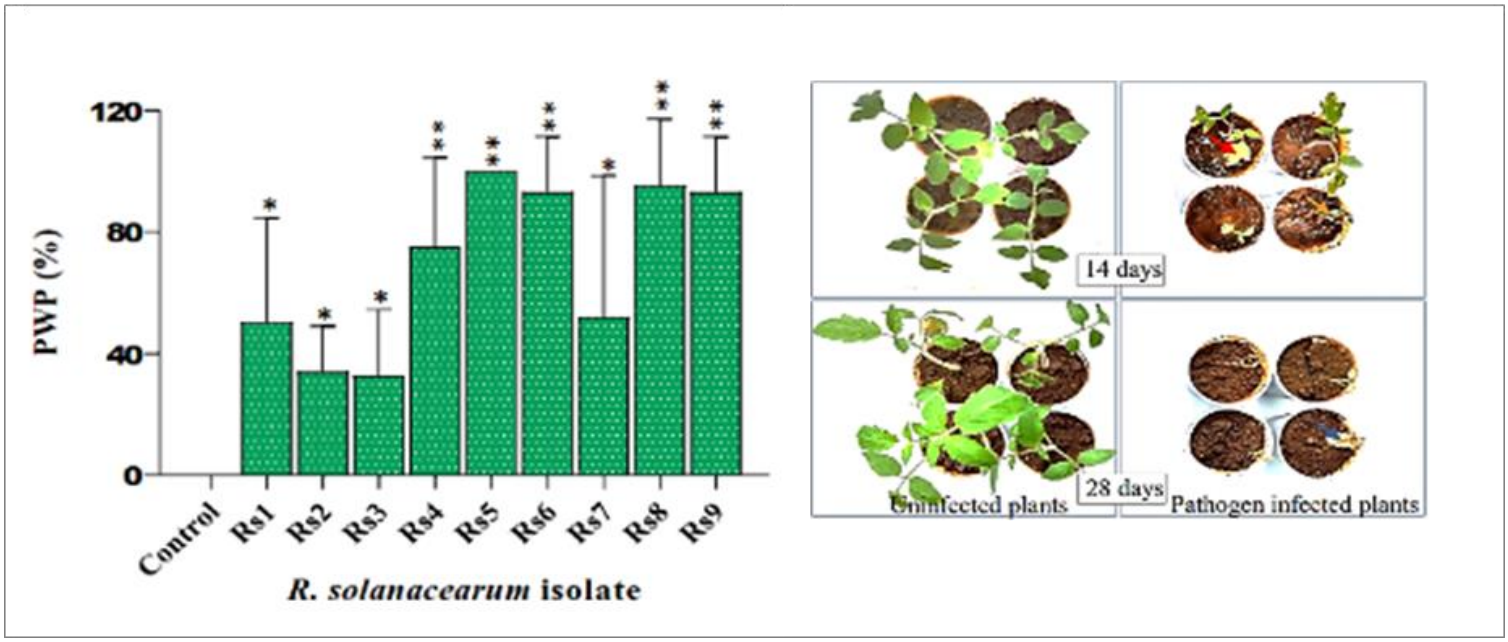

Fig. 18: Differential pathogenic power of *R. solanacearum* isolates toward *S. lycopersicum* var Rio Grande at 14 and 28 days post infection.

Mean values (bar charts) bearing star are significantly different ($p \leq 0.05$) as determined by the Tukey's HSD post hoc test. Bar charts bearing one (*) or two stars (**) are significantly different from the control at p= 0.01and p= 0.001 respectively. PWP: the percentage of wilted plants.

3.1.2 Antagonistic potential of Bacillus sp against the most virulent *R. solanacearum* in dual culture

To examine the antibacterial effects of the tested Bacillus species vis-a-vis *R. solanacearum* Rs5, the latter were analyzed by the well diffusion method upon which the inhibition diameters were drawn around the wells. Among the ten (10) endophytic bacteria screened, six strains, including *Bacillus amyloliquefaciens* CBa_RA37, *Bacillus velezensis* CBv_BE1, *Bacillus amyloliquefaciens* CBa_BFL2, *Lysinibacillus* CBa_LPR19, *Bacillus megaterium* CBm_RR10 and *Bacillus amyloliquefaciens* CBa_BFL1 inhibited *Ralstonia solanacearum* growth with inhibition diameters of 41, 35, 38, 43, 15 and 26 mm, respectively (Fig. 20). The strains *Bacillus xiamenensis* CBx_LPR2, *Bacillus cereus* CBc_LPR8, *Lysinibacillus fusiformis* CBl_LPR11, and *Brevibacillus brevis* CBb_RA14 were inactive.

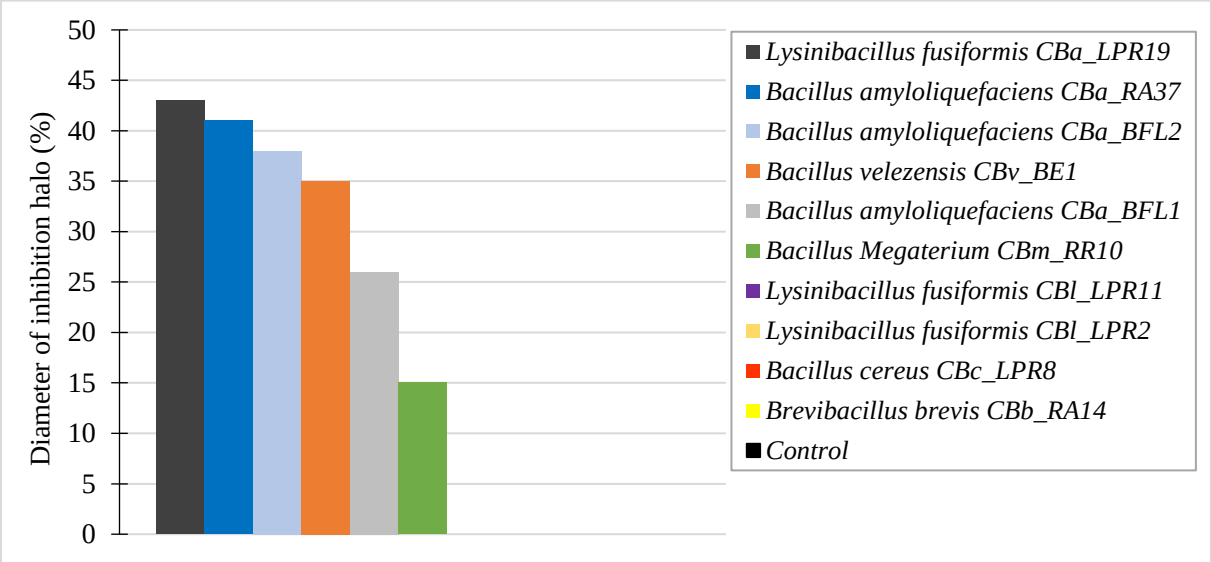

Fig. 19: *R. solanacearum* growth inhibition diameters obtained with the antagonistic Bacillus spp. in dual culture

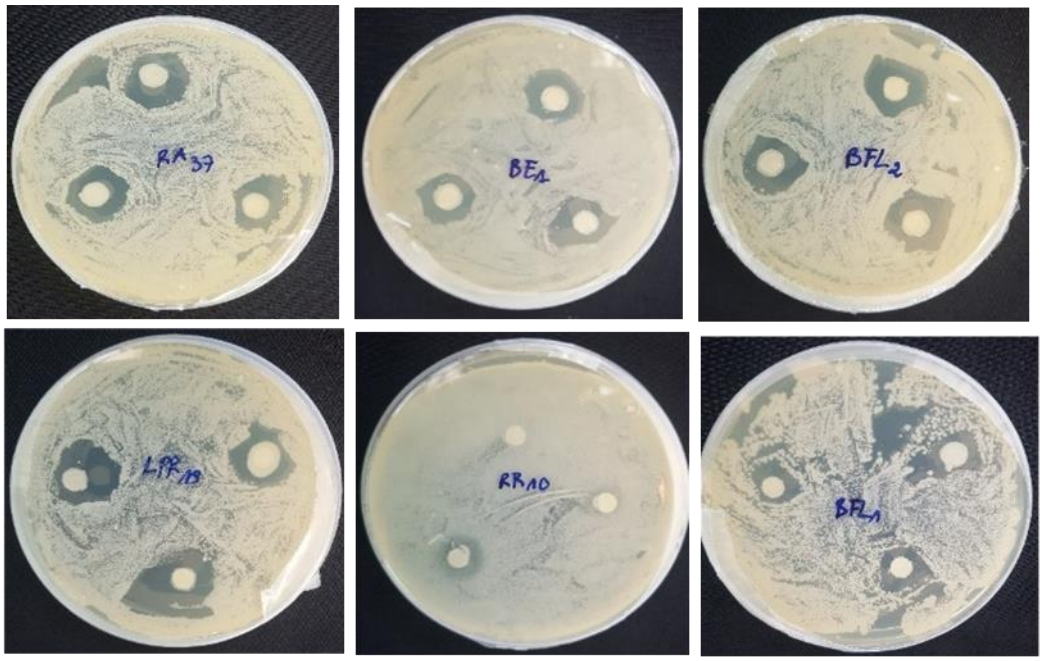

Fig. 20: *R. solanacearum* growth inhibition by antagonistic *Bacillus* spp. in dual culture.

RA37: Petri dish infected with *R. solanacearum* Rs5 and *Bacillus amyloliquefaciens* CBa_RA37, BE1: Petri dish infected with *R. solanacearum* Rs5 and *Bacillus velezensis* CBv_BE1, BFL2: Petri dish infected with *R. solanacearum* Rs5 and *Bacillus amyloliquefaciens* CBa_BFL2, LPR19: Petri dish infected with *R. solanacearum* Rs5 and *Lysinibacillus fusiformis* CBa_LPR19, RR10: Petri dish infected with *R. solanacearum* Rs5 and *Bacillus megaterium* CBm_RR10, BFL1: Petri dish infected with *R. solanacearum* Rs5 and *Bacillus amyloliquefaciens* CBa_BFL1.

3.1.3 Antibacterial potential of antagonis culture filtrate and extracts against the most virulent *R. solanacearum*

Culture filtrates from the selected antagonistic bacteria that exhibited promising confrontation potential were investigated for their ability to inhibit the growth of *R. solanacearum*. Prior to this, the effect of the bacterial culture time on activity was investigated to select the appropriate culture time required for the endophytes to elicit optimal levels of antibacterial potency (Fig 21). From the results achieved, extracts obtained between 24 and 72 h culture did not exhibit considerable activity at up to 1000 µg/ml, except for the 72 h culture extract from BE1, which exhibited weak activity (MIC of 1000 µg/ml). However, extracts obtained from 96-120 h cultures showed more promising activity (MIC from 15.62-1000 µg/ml), with the best MIC values obtained with extracts from the 120 h cultures (15.65-125 µg/ml). The most active endophytic extracts were from *B. Velezensis* CBv_ BE1 (MIC of 15.625 µg/ml) and *B. amyloliquefaciens* CBa_BFL2 (MIC of 31.25 µg/ml). However, a dramatic activity loss was observed when bacteria were cultured for 144 h, with a 16- and 32-fold activity reduction for extracts from BE1 and BFL2 and a complete loss of potency for the remaining extracts.

Therefore, the 120 h culture time was selected as the appropriate duration mfor the production of antibacterial metabolites by selected endophytic strains. Thus, the ethyl acetate extracts obtained from 5-day cultures of these bacteria exhibited very promising activity levels against *R. solanacearum* (Table 2), with MIC values ranging from 7.812 to 62.50 µg/ml, with the extract from *B. amyloliquefaciens* CBa_RA37 (7.812 µg/ml) being the most active, followed by *B. velezensis* CBv_BE1 and *B. amyloliquefaciens* CBa_BFL1 (MIC of 15.625 µg/mL). Of note, the ethyl acetate extracts from all six bacterial cultures exhibited improved antibacterial activity levels compared to the culture filtrates. This could be due to the ability of the solvent to solubilize and concentrate the active principles.

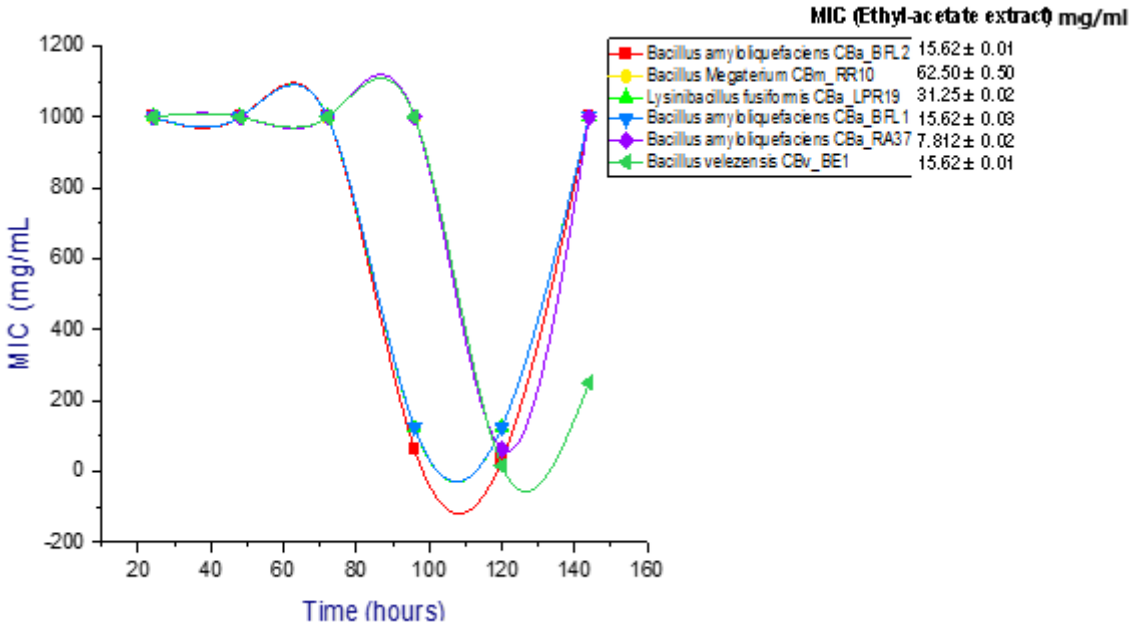

Fig. 21: Variation in MIC of the filtrates and ethyl acetate extracts of endophytic bacteria with respect to culture duration.

The experiment was performed in duplicate and repeated twice. Data are expressed as the mean values ± standard deviations (SD).

3.1.4 Chemical composition of the extracts of the three best biocontrol agents

The dereplication of the 3 extracts coming from the three best strains strains (*B. amyloliquefaciens* CBa_BFL2, *B. velezensis* CBv_BE1 and *B. amyloliquefaciens* CBa_RA37) revealed a variability of compounds in each of the extracts. The extract of RA37 had the most compounds (99) followed by BE1 (96) and BFL2 (93). The molecular formulas of all these compounds have been determined. However, only the names of fifteen compounds for BE1, sixteen for BFL2 and twelve for RA37 were determined using the GNPS library. The compound cyclo (L-Phe-Dpro) was the most commonly compound present in the tree differents extracts.

Five common compounds (Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-[(4-hydroxyphenyl)methyl], Linoleic acid, cyclo(L-Val-L-Pro), music acid, (2S,8R)-8-hydroxy-2-[(1S)-1-hydroxyheptyl]-2,3,4,6,7,8-hexahydrochromen-5-one were found at in extracts BE1 and BFL2 (Table 3). Several of these compounds have already shown biological activity. The observation of several similar compounds in the RA37 and BFL2 extracts would explain the fact that the best treatment is the consortium CBa_RA37+CBa_BFL2.

Table 3: Compounds identified in extracts from promising BCAs

Compounds name (LibraryID)	Molecular formular	Bacterial strain		
		BE1	BFL2	RA37
Acamprosate| 3-acetamido-1-propanesulfonic acid	$C_5H_{11}NO_4S$	✓		
1 Cyclo(proline-leucine)		✓		
11-dioxododecanoic acid	$C_{12}H_{20}O_4$	✓		
Mucic acid	$C_6H_{10}O_8$	✓		
Azelaic acid	$C_9H_{16}O_4$	✓		
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-[(4-hydroxyphenyl)methyl]-	$C_{10}H_{12}N_8O_2$	✓	✓	
Linoleic acid		✓	✓	
Phytol, mixture of isomers|3,7,11,15-Tetramethyl-2-hexadecen-1-ol|(E,7R,11R)-3,7,11,15-tetramethylhexadec-2-en-1-ol		✓		
Cyclopentasiloxane, decamethyl		✓		
1-O-b-D-glucopyranosyl sinapate|1-O-Sinapoyl-beta-D-glucose|1-O-Sinapoyl beta-D-glucoside|[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] (E)-3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-en		✓		
Austinoneol				

7b,9-Dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-5-oxo-1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-9aH-cyclopropa[3,4]benzo[1,2-e]azulen-9a-yl acetate		✓		
Acamprosate| 3-acetamido-1-propanesulfonic acid		✓		
cyclo(L-Val-L-Pro)		✓		✓
Cyclo(proline-leucine)		✓		
phenazine-1-carboxylic acid		✓		
Mucic acid		✓		✓
cyclo(L-Phe-D-Pro)		✓	✓	✓
(2S,8R)-8-hydroxy-2-[(1S)-1-hydroxyheptyl]-2,3,4,6,7,8-hexahydrochromen-5-one	$C_{16}H_{26}O_4$		✓	✓
Glycerol 1-myristate			✓	
Phytol,mixture of isomers|3,7,11,15-Tetramethyl-2-hexadecen-1-ol|(E,7R,11R)-3,7,11,15-tetramethylhexadec-2-en-1-ol			✓	
(E)-10-(3,5-dihydroxy-6-methyloxan-2-yl)oxyundec-2-enoic acid			✓	
Cyclopentasiloxane, decamethyl			✓	
beta-D-Glucopyranoside, 4-hydroxy-2-(hydroxymethyl)phenyl, 6-benzoate			✓	
2-(4-hydroxyphenyl)-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2,3-dihydrochromen-4-one			✓	
Cyclo(leucylprolyl)				✓
3-Hydroxyoctadecanoic Acid	$C_{14}H_{30}N_6$			✓

14-(hydroxymethyl)-5,9-dimethyltetracyclo[11.2.hexadecan-5-ol	$C_{14}H_{26}N_6O$	✓
(E)-10-(3,5-dihydroxy-6-methyloxan-2-yl)oxyundec-2-enoic acid	$C_{29}H_{32}O_2$	✓
Cyclopentasiloxane, decamethyl	$C_{16}H_{19}ClN_2O_6$	✓
beta-D-Glucopyranoside, 4-hydroxy-2-(hydroxymethyl)phenyl, 6-benzoate		✓
2-(4-hydroxyphenyl)-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2,3-dihydrochromen-4-one	$C_{19}H_{30}N_2O_8$	✓
7b,9-Dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-5-oxo-1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-9aH-cyclopropa[3,4]benzo[1,2-e]azulen-9a-yl acetate	$C_{19}H_{33}N_3O_8$	✓

The metabolomic profiling of the ethyl acetate extracts of baccili cultures using HPLC/MS revealed 288 secondary methabolites. Briefly, the sensitivity threshold enabled the identification of 17 compounds in BE1 extract, 10 in the BFL2 extract and 12 in the RA37 extract. Cyclo(L-Phe-D-Pro) was identified in the three analyzed extracts (BE1, BFL2 and RA37) while pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-[(4-hydroxyphenyl)methyl]-, linoleic acid and cyclo(L-Phe-D-Pro) were found in BE1 and BFL2 extracts, cyclo(L-Val-L-Pro) and mucic acid in BE1 and RA37 extracts and (2S,8R)-8-hydroxy-2-[(1S)-1-hydroxyheptyl]-2,3,4,6,7,8-hexahydrochromen-5-one in the BFL2 and RA37 extracts. These shared secondary metabolites are likely to be implicated in the potency of the Baccilus strains.

3.1.5 Ammonia, siderophores and phosphate solubilization

Selected antagonist bacteria were also evaluated for their growth promotion properties. Characteristics such as their ability to solubilize phosphorus, and produce ammonia and siderophores were investigated. Among the six bacteria, four endophytic bacteria *Bacillus velezensis* CBv_BE1 (6.0 mm), *Bacillus amyloliquefaciens* CBa_RA37 (4.0 mm), *Bacillus*

amyloliquefaciens CBa_BFL2 (3.0 mm), and *Bacillus megaterium* CBm_RR10 (1.5 mm) solubilized phosphate by forming halo around the inoculation zone (table 4). All six were able to produce ammonia with concentrations ranging from 0.568 to 0.829 µmol/mL. The best strain was *Bacillus amyloliquefaciens* CBa_RA37 (0.829 µmoL/mL) followed by *Bacillus velezensis* CBv_BE1 (0.761 µmol/ml) and *Bacillus amyloliquefaciens* CBa_BFL2 (0.751 (table 4). We also noted that all antagonist agents were able to produce the three types of siderophores. *Bacillus velezensis* CBv_BE1, *Bacillus amyloliquefaciens* CBa-RA37, and *Bacillus amyloliquefaciens* CBa-BFL2 produced the highest amount (100%) of hydroxamate and catecholate type siderophores, while for the carboxylic siderophores, *Bacillus velezensis* CBv_BE1 was by far the best producer (69.13%).

Table 4: Quantitative estimation of phosphate solubilization, ammonium and siderophores production

BCA	Phosphate (mm)	Ammonium (µmol/ml)	Siderophores (%)		
			Catecholate	Hydroxamate	Carboxylic
***B. velesensis* CBv_BE1**	6.0	0.761	100	100	63.13
***B. amyloliiquefaciens* CBa_BFL2**	3.0	0.751	100	100	27.96
***B. amyloliquefaciens* CBa_BFL1**	0.0	0.71	89.75	77.85	6.22
***B. amyloliquefaciens* CBa_RA37**	4.0	0.829	100	100	20.20
***Lysinibacillus* CBa_LPR19**	0.0	0.532	44.58	61.02	7.18
***B. megaterium* CBm_RR10**	1.5	0.607	55.43	44.84	19.26

BCA: Biocontrol agent; BE1: *B. velezensis* CBv_BE1; BFL2: *B. amyloliquefaciens* CBa_BFL2; BFL1: *B. amyloliquefaciens* CBa_BFL1; RA37: *Bacillus amyloliquefaciens* CBa_RA37; RR10: *Bacillus megaterium* CBm_RR10; LPR19: *Lysinibacillus* CBa_LPR19. Please indicate if there is significant differences among the variables examined

3.1.6 Screening for IAA and SA production

The ability of selected endophytic bacteria to produce phytohormones including indole acetic acid (IAA) and salicylic acid (SA), was also investigated (Fig. 22). Five out of six bacterial strains were able to produce SA, with *B. velezensis* CBv_BE1 (29.53 µg/ml) followed by *B. amyloliquefaciens* CBa_RA37 (26.98 µg/ml) being the most active. Regarding their ability to produce IAA, *B. velezensis* CBv_BE1 was by far the best producer (25.32 µg/ml).

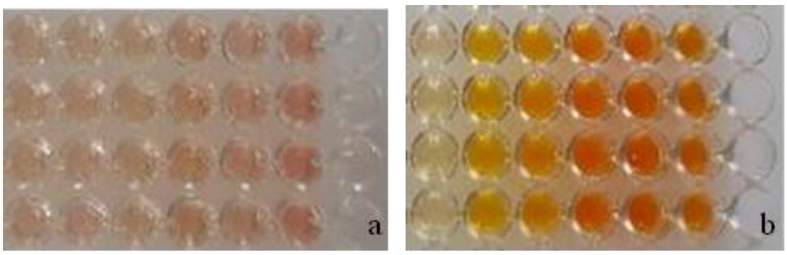

Fig. 22: Qualitative estimation of indol acetic acid (a) and salicylic acid (b) produced by selected *Bacillus* species.

Pink color indicates the formation of indol acetic color and orange color the formation of siderophores

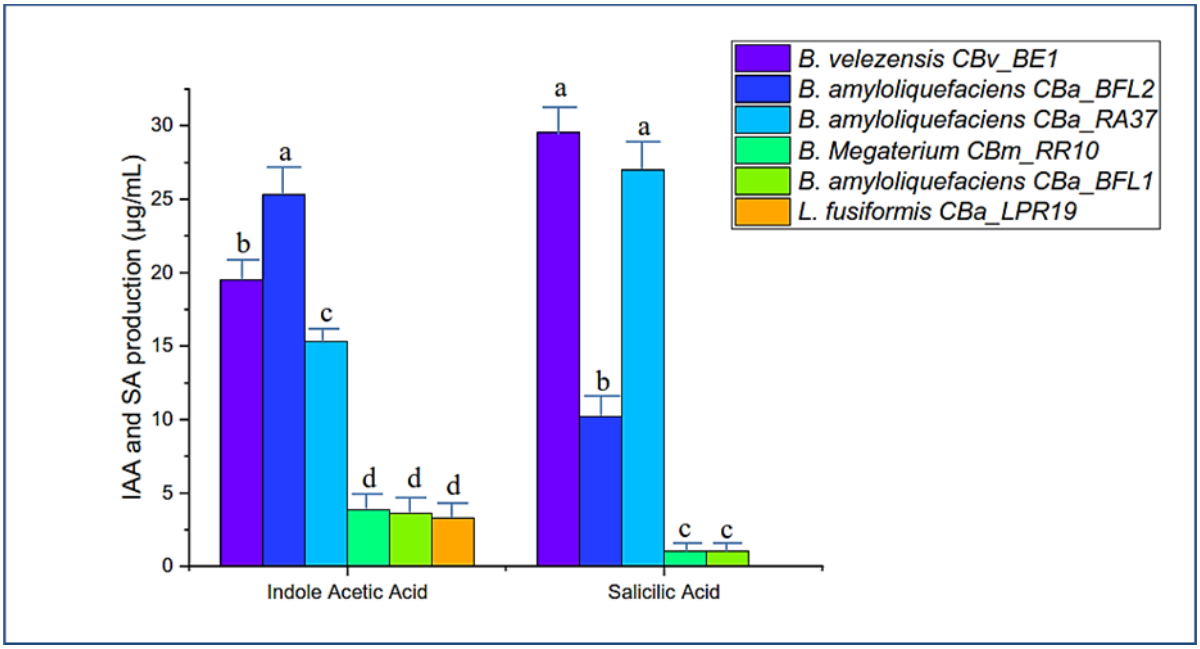

Fig. 23: Quantitative estimation of salicylic acid and indole acetic acid production by the bacterial endophytes.

Bar charts (IAA or SA) bearing different letters denote significantly different effects at $p \leq 0.05$, according to Tukey's *post-hoc* test; C: Control: Treatment with all the reagents in the absence of biocontrol agents).

3.1.7 Hydrolytic enzyme prodution

The ability of selected bacteria to produce hydrolytic enzymes was investigated. The results showed that all six agents could synthetize and release amylase, cellulose, and proteases. Regarding the proteases and cellulase production, the best endophytes were *Bacillus amyloliquefaciens* CBa_BFL2 (1.72 and 2.00), *Bacillus velezensis* CBv_BE1 (1.54 and 2.50) and *Bacillus amyloliquefaciens* CBa_RA37 (1.41 and 2.11). *Bacillus velezensis* (2.33) followed by *Bacillus amyloliquefaciens* CBa_RA37 (2.16) were the most interesting amylase producers. Overall, all the most potent endophytes belonged to the genus Bacillus.

Fig. 24: Different hydrolytic enzyme production in specific medium in the order of amylase, cellulase, protease respectively. The observation of halos around the inoculation spots materialized a positive result.

Table 5: Enzymatic index of hydrolytic enzymes

Bacteria strains	**Proteolytic activity**	**Amylasic activity**	**Cellulasic activity**
		Enzymatic index	
B. velesensis **CBv_BE1**	1.54±0.01[b]	2.33±001[c]	2.50±0.01[d]
B. amyloliquefaciens **CBa_BFL2**	1.72±0.01[b]	1.89±0.00[b]	2.00±0.01[c]
B. amyloliquefaciens **CBa_BFL1**	1.33±0.04[a]	1.92±0.02[b]	1.55±0.03[b]
B. amyloliquefaciens **CBa_RA37**	1.41±0.01[a]	2.16±0.01[c]	2.11±0.02[c]
B. megaterium **CBm_RR10**	1.00±0.03[a]	1.29±0.01[a]	1.06±0.04[a]
Lysinibacilllus **CBa_LPR19**	1.15±0.06[a]	1.43±0.02[a]	1.15±0.02[a]
P values	0.001	0.002	0.001

BE1: *B. velezensis* CBv_BE1; BFL2: *B. amyloliquefaciens* CBa_BFL2; BFL1: *B. amyloliquefaciens* CBa_BFL1; RA37: *Bacillus amyloliquefaciens* CBa_RA37; RR10: *Bacillus megaterium* CBm_RR10; LPR19: *Lysinibacillus* CBa_LPR19; Ø: Diameter of clear halos formed around the colonies indicating hydrolytic activity.

Mean values within each column give the difference between the strains ($P \leq 0.005$) as given by Turkey's HSD post hoc test. Mean values within each column superscripted by the same letter (a, b, c, or d) are not signifcantly diferent ($P \leq 0.05$).

Table 6: General ranking of endophytic bacteria based on their antagonist properties against *R. solanacearum* and their plant growth-promoting traits.

Code	BCA	Antagonistic activity						PGP traits				Total	Rank
		DC	EX	CF	CE	P	A	IAA	SA	PS	NH3	Sd		
B.	*velesensis* CBv_BE1	4	3	3	3	2	3	3	3	5	3	4	**36**	**1**
B.	*amyloliquefaciens* CBa_RA37	5	4	1	2	2	3	3	2	3	4	4	**33**	**2**
B.	*amyloliquefaciens* CBa_BFL2	4	3	2	3	2	2	2	2	3	2	4	**29**	**3**
B.	*amyloliquefaciens* CBa_LPR19	5	2	1	1	1	1	2	0	0	1	2	**16**	**4**
B.	*megaterium* CBm_RR10	2	1	1	1	1	1	2	1	1	1	2	**14**	**5**
B.	*amyloliquefaciens* CBa_BFL1	2	1	1	1	1	2	1	1	0	1	2	**13**	**6**
B.	*xiamenensis*CBx_LPR2	0	0	0	0	0	0	1	0	1	2	1	**5**	**7**
B.	*brevis* CBb_RA14	0	0	0	0	0	0	2	0	0	2	1	**5**	**8**
B.	*Cereus* CBc_LPR8	0	0	0	0	0	0	1	1	0	1	2	**5**	**9**
L.	*fusiformis* CBl_LPR11	0	0	0	0	0	0	1	0	0	1	1	**3**	**10**

BCA: Biocontrol agent; DC: Dual culture; EX: Ethyl acetate extract; CF: Culture filtrate; CE: Cellulase activity; P: Proteolytic activity; A: Amylase activity; IAA: Indole acetic acid; SA: Salicylic acid; PS: Phosphate solubilization; NH3: Ammonia production; Sd: Siderophore production

3.1.8 Hypersensitivity of biological control agents

After inoculation of BCAs into tobacco tissues known for their sensitivity to any pathogen, no hypersensitivity reaction (absence of aureole around the inoculation point) was observed.

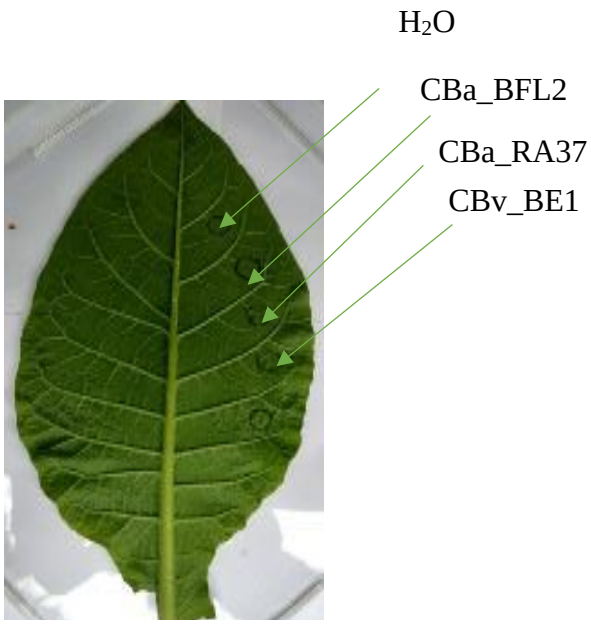

Fig. 25: Tobacco leaf in the presence of BCAs after 24 hours of incubation

BE1= *B. velezensis* CBv_BE1, RA37= *B. amyloliquefaciens* CBa_RA37, BFL2= *B. amyloliquefaciens* CBa BFL2 and H_2O= water stérile

3.1.9 Seed germination mediated by selected endophytic bacteria

Three of the best antagonist agents (Table 7) exhibiting interesting growth promotion properties were evaluated *in vitro* for their ability to stimulate the germination of tomato seeds. Compared to the germination index (GI) and germination rate (GR) of the control (Gr/Gi 33.69/8.81), all three bacteria exhibited better potency, with *B. velezensis* CBv_BE1 (Gr/Gi 114.59/16.03) being the best, followed by *B. amyloliquefaciens* CBa_BFL2 (Gr/Gi 107.33/15.1) and *B. amyloliquefaciens* Cba_RA37 (Gr/Gi 89.6/14.16). These three bacteria were further submitted to *in planta* studies to investigate their ability to protect tomato plants from *R. solanacearum* deterrent effects.

Table 7: Induced germination index and germination rate of potent biocontrol agents (CBv_BE1, CBa_RA37, CBa_BFL2)

Biological control agents	GR	GI
B. amyloliquefaciens CBa_BFL2	107.33	15.10
B. amyloliquefaciens CBa_RA37	80.7179	14.16
B.s velezensis CBv_BE1	114.59	16.03
Control	33.694	8.81

GI= Germination index; GR= Germination rate; Control= Plants that received no biological control agent but wate

3.1.10 Endophytic competence

3.1.10.1 Production of antibiotic-resitant bacteria

Table 8 shows that at a concentration of 100 µg/L, all the three strains generated strains with the capacity to resist chloramphenicol, ampicillin, ciprofloxacin and rifampicin. The best resistance was observed at a concentration of 200 µg/ml with rifampicin.

Table 8: Resistance to antibiotics.

	B. velezensis CBv_BE1	*B. amyloliquefaciens* CBa_BFL2	*B. amyloliquefaciens* CBa_RA37
Chloramphenicol (50 µg/mL)	+	+	+
(100 µg/mL)	-	+	+
(200 µg/mL)	-	-	-
Am (50 µg/mL)	+	+	+
(100 µg/mL)	+	+	+
(200 µg/mL)	-	-	+
Ci (50 µg/mL)	+	+	+
(100 µg/mL)	+	+	+
(200 µg/mL)	-	-	-
Ri (50 µg/mL)	+	+	+
(100 µg/mL)	+	+	+
(200 µg/mL)	+	+	+

Ch : Chloramphenicol ; Am : Ampicillin ; Ci : Ciprofloxacin ; Ri : Rifampicin ; (+) test positif : (capacité à produire les résistants) (-) incapacité à produire les résistants

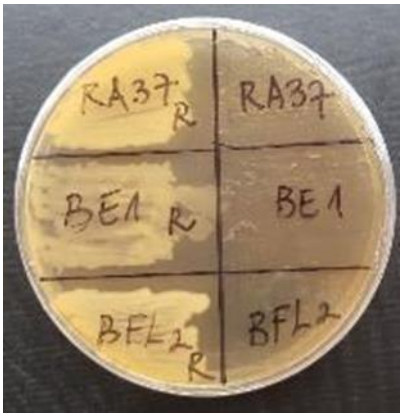

Fig. 26: Growth of resistant bacterial strains on rifampicin 200 µg/ml against nonresistant strains.

RA37: *Bacillus amyloliquefaciens* Cba_RA37, RA37r: *Bacillus amyloliquefaciens* Cba_RA37 resistant to rifampicin at the concentration 200 µg/ml. BE1: *Bacillus velezensis* CBv-BE1, $BE1_r$: *Bacillus velezensis* CBv-BE1resistant to rifampicin at the concentration of 200 µg/ml. BFL2: *Bacillus amyloliquefaciens* Cba_BFL2, $BFL2_r$ *Bacillus amyloliquefaciens* Cba_BFL2 at the concentration 200 µg/ml.

3.1.10.2 Colonization

Interestingly, all the shortlisted antagonists have successfully colonized tomato plants despite being isolated from a different host (Fig.27). The endogenous endophyte population enumerated in the 21th day old tomato seedlings culminated at approximately log10 CFU = 4.29 cells per gram fresh weight. In addition, it was also noted that the endophyte population size did not significantly change as the inoculum concentration increased ($p > 0.01$).

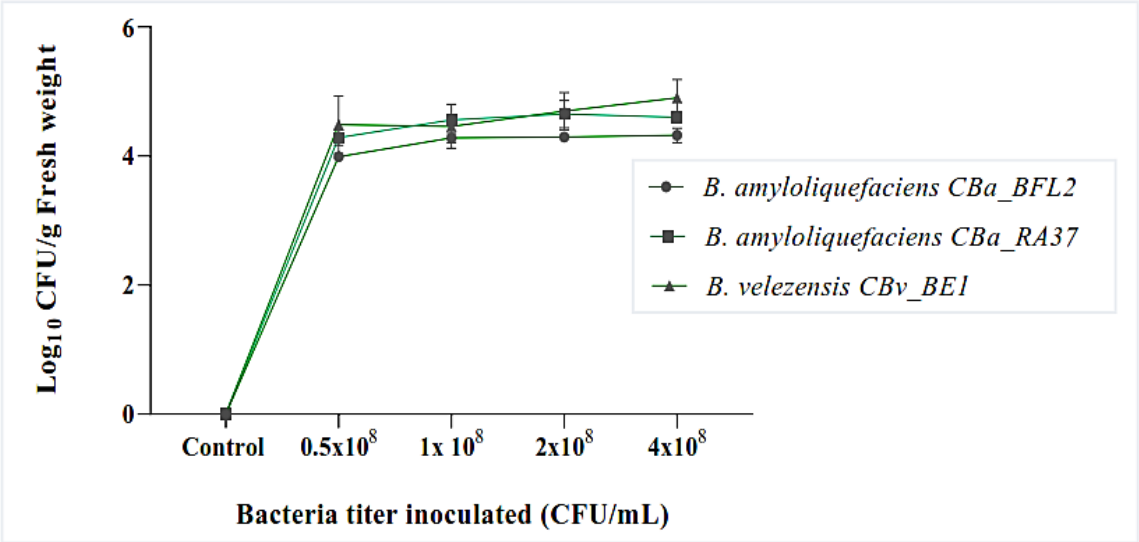

Fig. 27: Bacterial endophytic competence and population size in young tomato tissues as affected by increasing inoculum size at 21 dpi.

Each represented value is the mean and standard error of the mean values of two different assays tested in triplicate each.

We can see according this picture that isolate and reisolate strains are identical

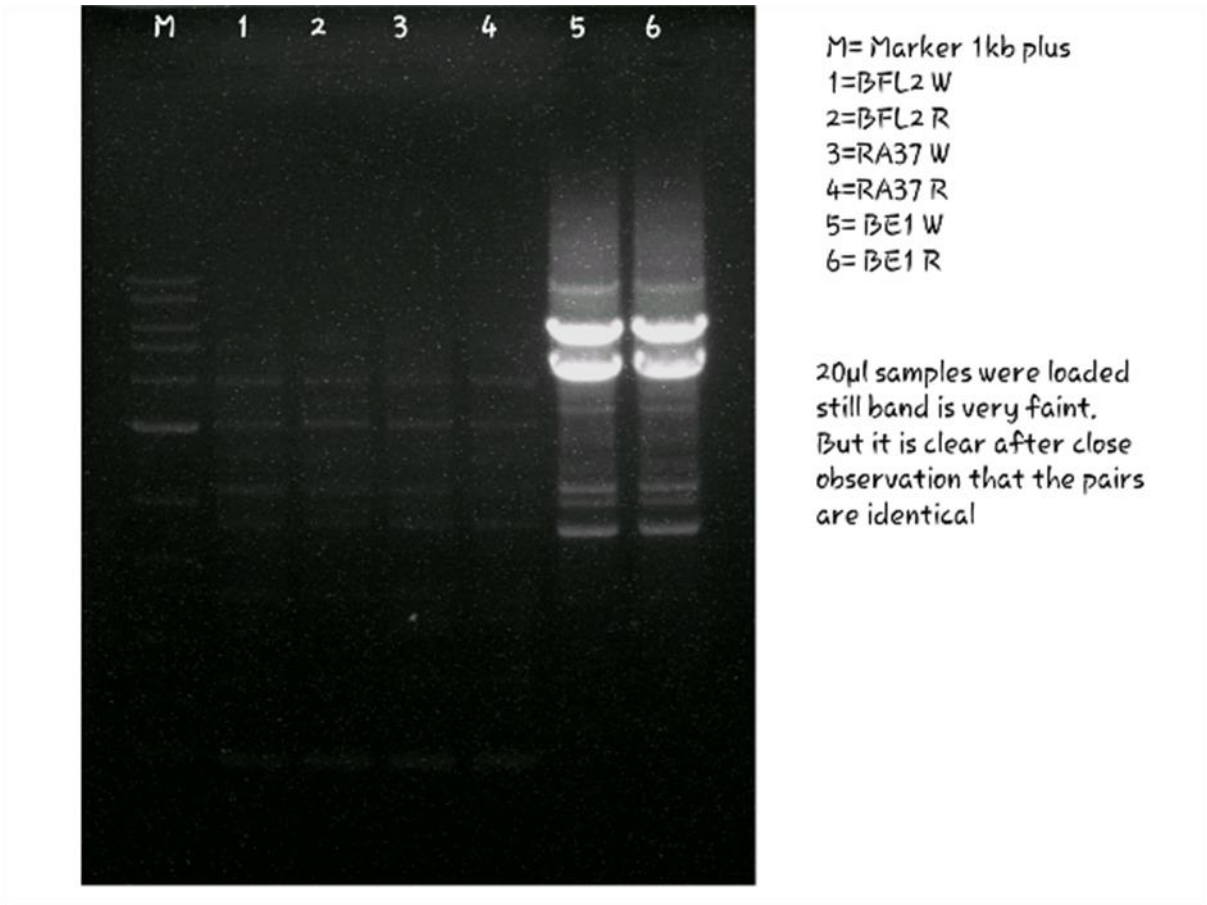

Fig. 28: Box_PCR fingerprint of wild and reisolated antibiotic-resistant BCAs.

(1-2), (3-4) and (5-6): band patterns of wild and reisolated CBa_BFL2, CBa_RA37, and *B. velezensis* CBv_BE1, respectively.

3.1.11 Salicylic acid and colonization relationship

Figures 15 shows that there is a relationship between the quantity of salicylic acid produced and the colonization time by endophytic bacteria. We observed that until the fourth day of colonization by BCAs, the amount of salicylic acid continued to increase until reaching a peak on the fifth day. Nevertheless, from the fifth day of colonization the quantity of salicic acid decreases as the quantity of microorganisms remains constant.

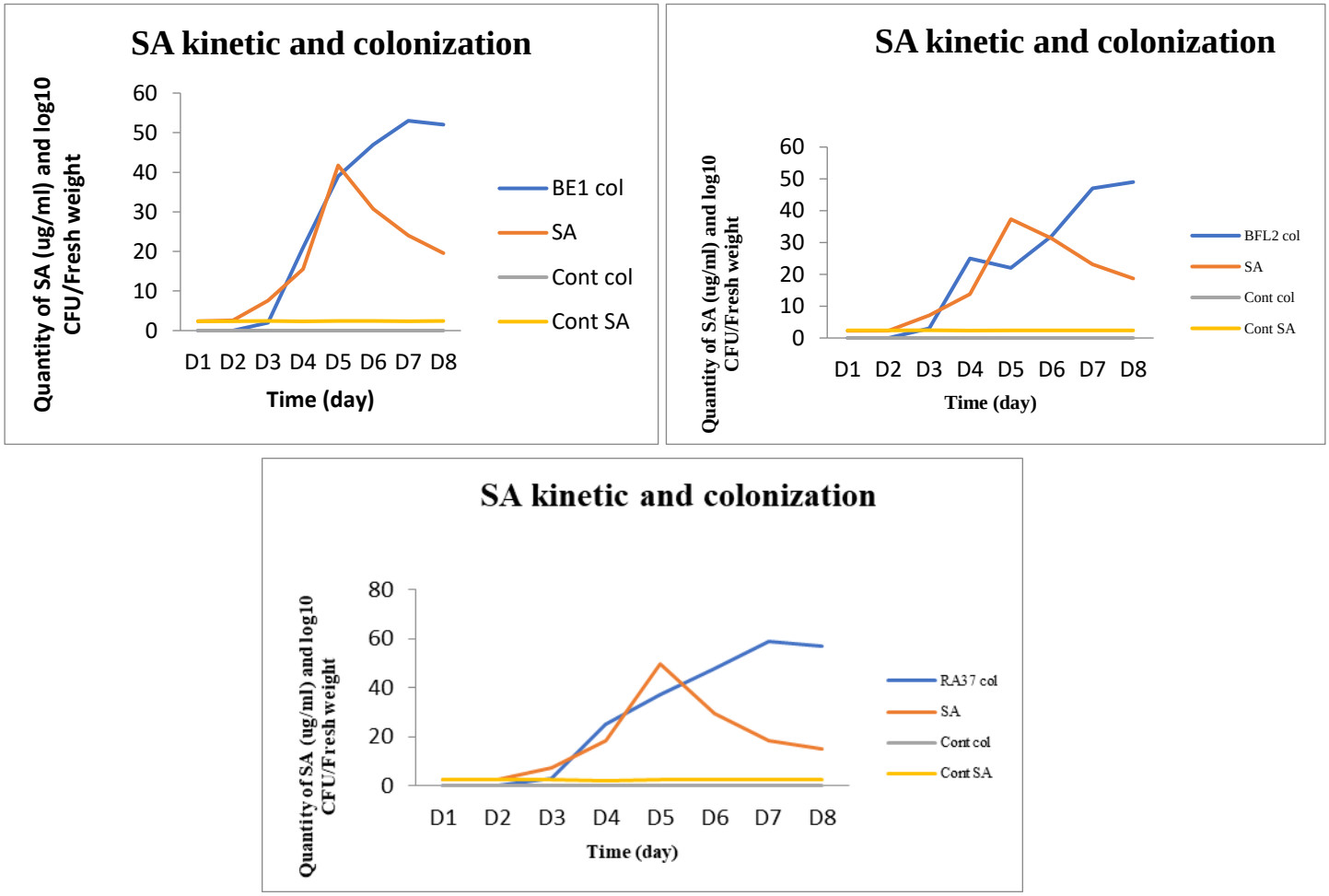

Fig 29: Evolution of colonization (a) and salicylic acid production (b) of biological control agents.

RA37: Salicylic acid kenetic mediated by the colonization of *Bacillus amyloliquefaciens* CBa-RA37, BFL2: Salicylic acid kenetic mediated by colonization of *Bacillus amyloliquefaciens* CBa-BFL2, SA: Salicylic acid kenetic mediated by plants control.

3.1.12 Bacterial wilt suppression under pot conditions

3.1.12.1 Impact of seedling bacterization on wilt incidence and severity

Three (3) Bacillus strains (Bacillus amyloliquefaciens CBa_RA37, Bacillus amyloliquefaciens CBa_BFL2 and Bacillus velezensis CBv_BE1), selected based on their individual *in vitro* biocontrol traits (table 9) were tested either singly or in consortium for their ability to dwarf off the destructive effects of the highly pathogenic *R. solanacearum* Rs5 in tomato seedlings under pot conditions. Obviously, typical symptoms such as seedling stunting, wilting of the youngest leaves at the end of the branches at earlier stages, brown staining of vascular rings and general wilting and yellowing occurring at later stages leading to plant collapse and death in severe cases were evidenced upon pathogen infection. However, when

challenged with bacterial antagonists, the disease establishment and progress dropped significantly ($p<0.01$) compared to plant emerging from pots infected with the pathogen alone (Rs5-treated plants). The PWP ranged from 100% to 10%, an the consortium among *B. amyloliquefaciens* CBa_RA37 and *B. amyloliquefaciens* CBa_BFL2 being the most protective as it could prevent 90% of plants from becoming infected by the pathogen (PWP = 10) at the final (28 dpi) count (Fig.30). In the case of successful infection, the wilting severity (WS) was significantly reduced at 28 dpi when the pathogen was challenged with the bioagents and their consortia, culminating in an 89% protection rate with the CBa_BFL2+CBa_RA37- treated plants (Fig. 16 and 17) which performed better than the other consortia and single treatments (Fig.16).

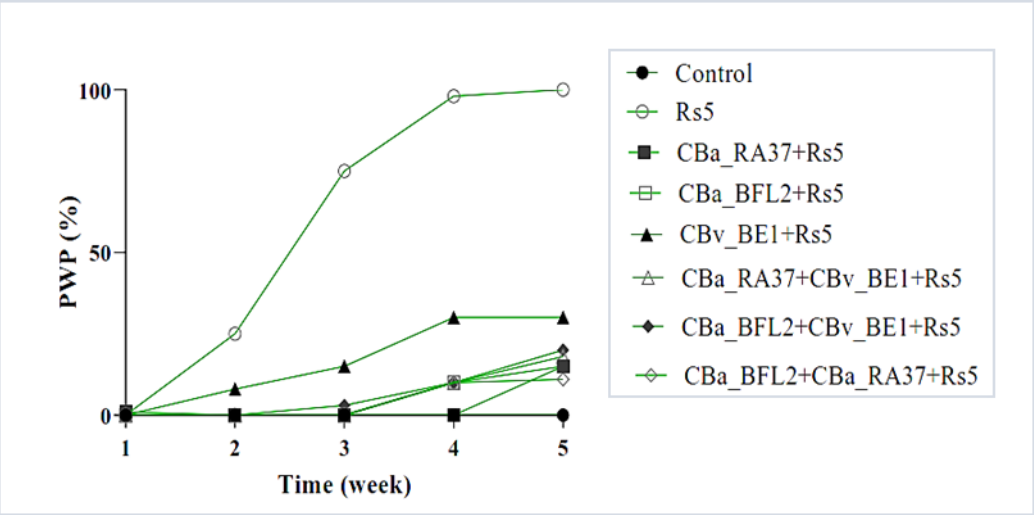

Fig. 30: Variation of the percentage of wilted tomato plantlets as a function of time and antagonistic bacterial priming. Standing plants were counted six weeks after *R. solanacearum* Rs5 infection and expressed as percentages of the total number of assayed plants (n=15). Data are the mean values of 15 replicates per treatment.

Control: Uninoculated seedlings (Neither Bacillus sp nor *R. solanacearum* Rs5), Rs5: Seedlings infected with *R. solanacearum* Rs5 alone, BFL2_Rs5: Seedlings primed by *Bacillus amyloliquefaciens* CBa_BFL2 and infected with *R. solanacearum* Rs5, BE1_Rs5: Seedlings primed by *Bacillus velezensis* CBv_BE1 and infected with *R. solanacearum* Rs5, RA37_Rs5: primed with *Bacillus amyloliquefaciens* CBa_RA37 and challenged with *R. solanacearum* Rs5. BFL2_BE1_Rs5: Seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2 and *Bacillus velezensis* CBv_BE1 and infected with *R. solanacearum* Rs5, RA37_BE1_Rs5: Seedlings dually primed with *Bacillus amyloliquefaciens* CBa_RA37, and *Bacillus velezensis* CBv_BE1 and infected with *R. solanacearum* Rs5, BFL2_RA37_Rs5: Seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, and *Bacillus amyloliquefaciens* CBa_RA37, and infected with *R. solanacearum* Rs5.

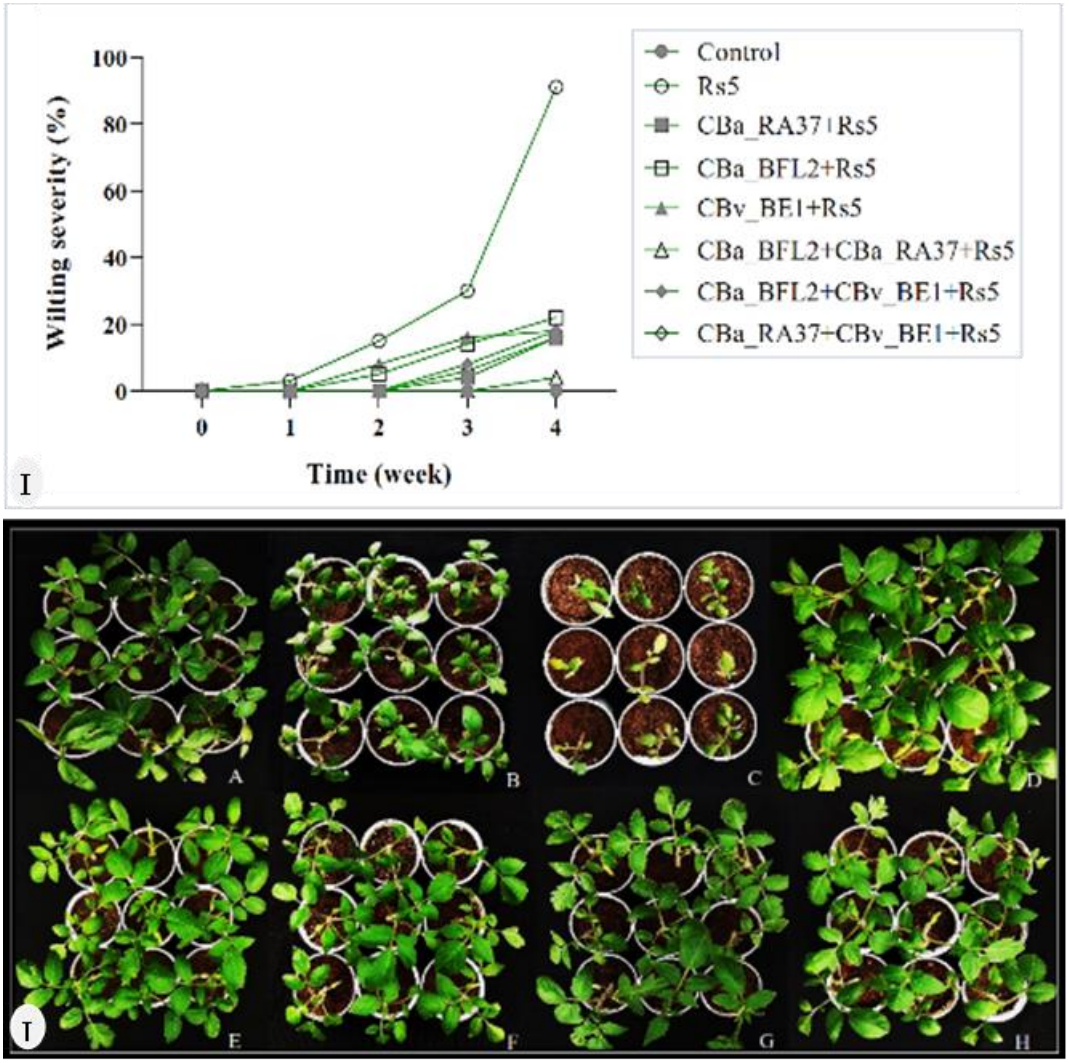

Fig. 31: Biological control of bacteria wilt and enhanced growth performance in tomato seedlings under pot conditions by use of selected cacti Bacillus antagonists and their consortia **(I)**; Differential phenotypes of aboveground parts and disease suppression in tomato seedlings at 21 dpi. **(II)**

(A) Tomato seedlings dually inoculated with *B. amyloliquefaciens* CBa_RA37 and *B. velezensis* CBv_BE1 and challenged with *R. solanacearum* Rs5. (B) Seedling not inoculated seedlings (control) by *Bacillus* spp or *R. solanacearum* Rs5. (C) *R. solanacearum* Rs5 infected plants without antagonist. (D) Plantlets coinoculated with *B. amyloliquefaciens* CBa_RA37 and CBa_BFL2 and infected with *R. solanacearum* Rs5. (E) plantlets primed with *B. amyloliquefaciens* CBa_RA37 alone and grown on *R. solanacearum* Rs5 infected soil. (F) Plants primed with *B. velezensis* CBv_BE1 alone and grown on *R. solanacearum* Rs5 infected soil. (G) Plantlets coinoculated. The values presented in Fig. 8I were taken on weekly basis and were generated from fifteen replicates at each recording date.

Fig. 32: Highlight of the outstanding biocontrol status of the consortium application of B. *amyloliquefaciens* CBa_RA37 and CBa_BFL2 under *R. solanacearum* Rs5 pressure.

Stress-free control seedlings (left). Witltered and stunted tomato seedlings grown on *R. solanacearum* Rs5 infested substrate (middle). Heightier and more vigorous plantlets binary primed (*B. amyloliquefaciens* CBa_RA37 and CBa_BFL2) emerging from *R. solanacearum* Rs5 infested soil (righ).

3.1.12.2 Area under the disease progress curve (AUDPC)

To quantitatively summarize the disease evolution with time of the overall treatment over the experimental period, AUDPC was calculated by the trapezoidal integration method. This parameter transforms the disease intensity under each treatment into a measurable area across the whole experimental period. The results revealed that the AUDPC ranged from 14 to 556. 5. When compared to Rs5-treated plants alone (AUDPC = 14), the dual inoculation of seedlings with CBa_RA37 + CBa_BFL2 had the best cumulative disease suppression potential over time with AUDPC = 14 and the binary inoculation of the seedlings with CBa_RA37 + CBa_BFL2 exerted the best cumulative disease suppression potential over time (AUDPC = 14) compared to Rs5-treated plants alone. (AUDPC=556.5).

3.1.12.3 Effect of bacterial priming on tomato growth parameters under *R. solanacearum* Rs5 infection

The growth performance of bacterial primed tomato seedlings was assessed under *R. solanacearum* Rs5 suppressive effects (Table 9). Notably *R. solanacearum* Rs5 infestation resulted in a drastic reduction in overall growth parameters, culminating at up to 66% and 86% reductions in shoot and root dry matter respectively, compared to unstressed (uninfected and unprimed control) plants. Interestingly, plants primed with the biocontrol agents, resulted in a significant enhancement of seedling vigor, which ranged from 157 to 392%. Binary priming with *B. amyloliquefaciens* CBa_BFL2 and CBa_RA37 performed better than the other BCAs by exhibiting as high as 229%, 392%, 157% and 319% more shoot length, root length, and root and shoot dry weights, respectively (Fig. 31).

Table 9: Changes in tomato growth parameters as influenced by bacterial priming and challenged by the *R. solanacearum* Rs5 pathogen.

Treatment	Agro-morphological parameters and AUDPC				
	SL (cm)	RL (cm)	RW (g)	SW (g)	AUDPC
Control	10.7 ± 0.8^{b}	16.7 ± 2.0^{bc}	0.5 ± 0.1^{b}	0.82 ± 0.2^{b}	nd
Pathogen (Rs5)	4.8 ± 0.5^{a}	4.1 ± 0.9^{a}	0.07 ± 0.01^{a}	$0.62 \pm$	556.5
CBa_RA37+Rs5	13.7 ± 0.7^{c}	18.4 ± 1.5^{c}	0.48 ± 0.1^{b}	2.2 ± 0.22^{d}	56
CBa_BFL2+Rs5	12.2 ± 1.1^{b}	14.7 ± 2.4^{b}	0.94 ± 0.14^{d}	2.0 ± 1.1^{c}	175
CBv_BE1+Rs5	12.3 ± 1.2^{b}	18.3 ± 2.2^{c}	0.91 ± 0.1^{d}	2.2 ± 0.4^{d}	175
CBa_RA37+CBv_BE1+Rs5	13.3 ± 1.4^{bc}	15.2 ± 1.6^{b}	0.71 ± 0.01^{bc}	1.6 ± 0.1^{c}	49
CBa_BFL2+CBv_BE1+Rs5	13.5 ± 1.3^{bc}	14.8 ± 2.0^{b}	0.81 ± 0.12^{c}	1.9 ± 0.1^{c}	56
CBa_BFL2+CBa_RA37+ Rs5	15.8 ± 1.5^{d}	20.2 ± 1.9^{d}	1.2 ± 0.2^{e}	2.6 ± 0.3^{e}	14
P values	0.001	0.002	0.001	0.001	

Legend: SL (Shoot length), RL (Root length), RW (Root weight), SW (Shoot weight). Control (uninoculated seedlings), Rs5 (*R. solanacearum* Rs5 infected plants without antagonist). CBa_RA37+Rs5 (plantlets primed with *B. amyloliquefaciens* CBa_RA37 alone and grown on *R. solanacearum* Rs5 infected soil).CBa_BFL2+Rs5 (Plantlets primed with *B. velezensis* CBv_BE1 alone and grown on *R. solanacearum* Rs5 infected soil). CBv_BE1+Rs5 (plantlets primed with *B. velezensis* CBv_ Means values within each column superscripted by the same letter are not significantly different ($P < 0.01$) as given by the Tukey's HSD post hoc test.BE1 alone and grown on *R. solanacearum* Rs5 infected soil). CBa_RA37+CBv_BE1+Rs5 (seedlings primed with both *B. amyloliquefaciens* CBa_RA37, *B. velezensis* CBv_BE1 and challenged with *R. solanacearum* Rs5). CBa_BFL2+CBv_BE1+Rs5 (Plantlets co-inoculated with *B. amyloliquefaciens* CBa_BFL2, *B. velezensis* CBv_BE1 and challenged with *R. solanacearum* Rs5). CBa_BFL2+CBa_RA37+ Rs5 (plantlets coinoculated with *B. amyloliquefaciens* CBa_BFL2, CBa_RA37 and challenged with *R. solanacearum* Rs5). The letters a, b, c, d, e. represent the results of statistical analysis carried out between the different treatments and control and nd: not determined.

3.1.12.4 Bacterial priming enhances defense-related enzyme specific activity under *R. solanacearum* Rs5 challenge

The specific activity of selected defense-related enzymes of tomato plants primed or not with biocontrol bacteria and challenged with *R. solanacearum* Rs5 was studied (Fig. 33). It was evident that *R. solanacearum* Rs5 infection substantially triggered the synthesis of peroxidases (GPX) and superoxide dismutase (SOD), as well as the phenylpropanoid pathway through phenylalanine ammonia lyase, marking the activation of plant defense machinery. A significantly ($p < 0.05$) lower activity of the overall enzymes was also noticed upon the antagonist challenge, as referred as pathogen control (Rs5). A comparison of the responses to single and dual biocontrol agent applications revealed no statistical variation. The Pearson model depicted positive and significant correlations between the activities of GPX ($r = 0.85$; p

= 0.006), PAL (r = 0.77; p =0 .02) and SOD (r = 0.72; p = 0.05) and wilt severity. Meanwhile, the higher the disease the greater the activity of defense enzymes.

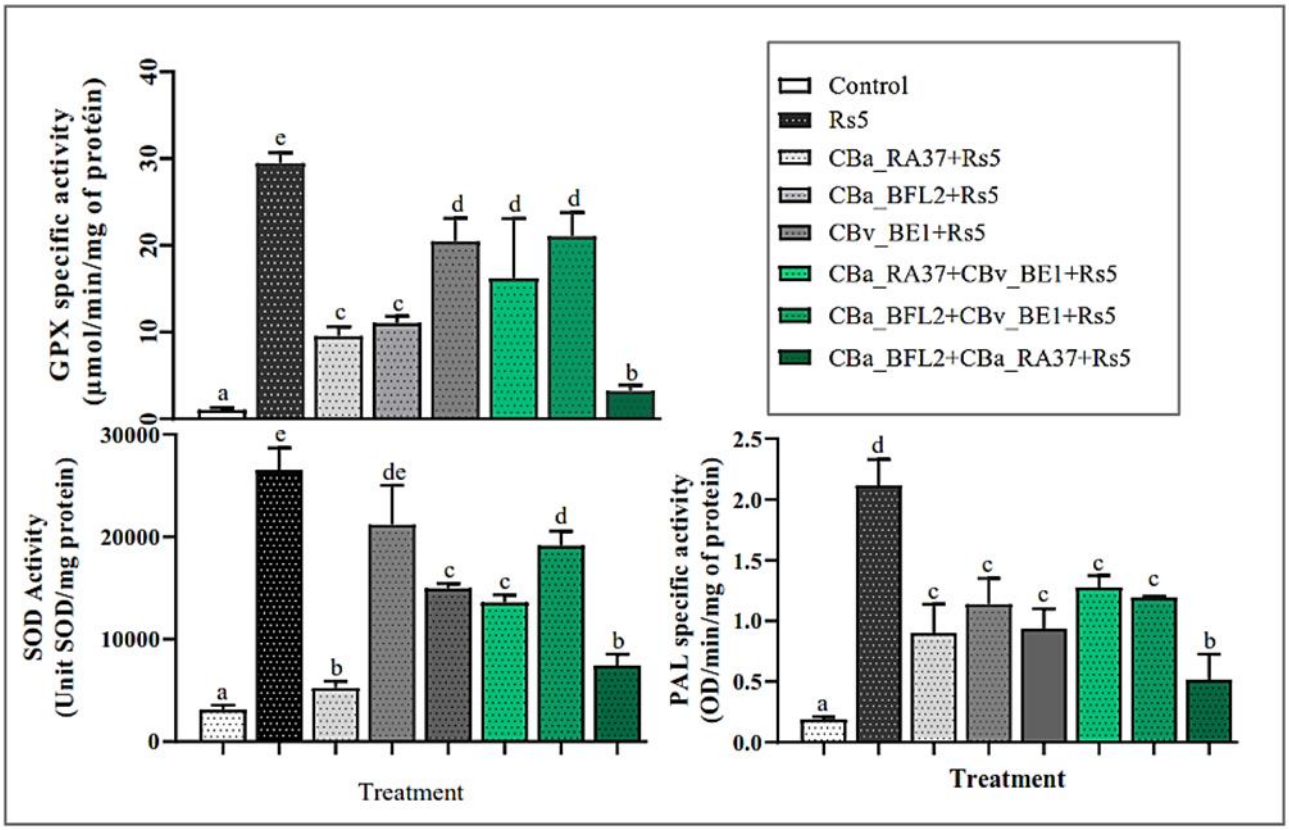

Fig. 33: *Bacillus* antagonist challenge.

Control (uninoculated seedlings), Rs5 (*R. solanacearum* Rs5 infected plants without antagonist). CBa_RA37+Rs5 (plantlets primed with *B. amyloliquefaciens* CBa_RA37 alone and grown on *R. solanacearum* Rs5 infected soil).CBa_BFL2+Rs5 (plantlets primed with *B. velezensis* CBv_BE1 alone and grown on *R. solanacearum* Rs5 infected soil). CBv_BE1+Rs5 (plantlets primed with *B. velezensis* CBv_BE1 alone and grown on *R. solanacearum* Rs5 infected soil). CBa_RA37+CBv_BE1+Rs5 (Seedlings primed with both *B. amyloliquefaciens* CBa_RA37, *B. velezensis* CBv_BE1 and challenged with *R. solanacearum* Rs5). CBa_BFL2+CBv_BE1+Rs5 (plantlets co-inoculated with *B. amyloliquefaciens* CBa_BFL2, *B. velezensis* CBv_BE1 and challenged with *R. solanacearum* Rs5). CBa_BFL2+CBa_RA37+ Rs5 (plantlets co-inoculated with *B. amyloliquefaciens* CBa_BFL2, CBa_RA37 and challenged with *R. solanacearum* Rs5). Mean values (bar charts) bearing different letter are significantly different ($p \leq 0.05$) as determined by the Tukey's HSD post hoc test.

3.1.13 Bacterial wilt suppression by the talc-based formulation Cba_RA37/Cba_BFL2) in the pot and field

3.1.13.1 Sheft live of biopesticide

Study of the life stability of the formulated biopesticide shows that the microbial load remains constant for three months and begins to drop at the end of the third month until becoming inactive in the ninth month.

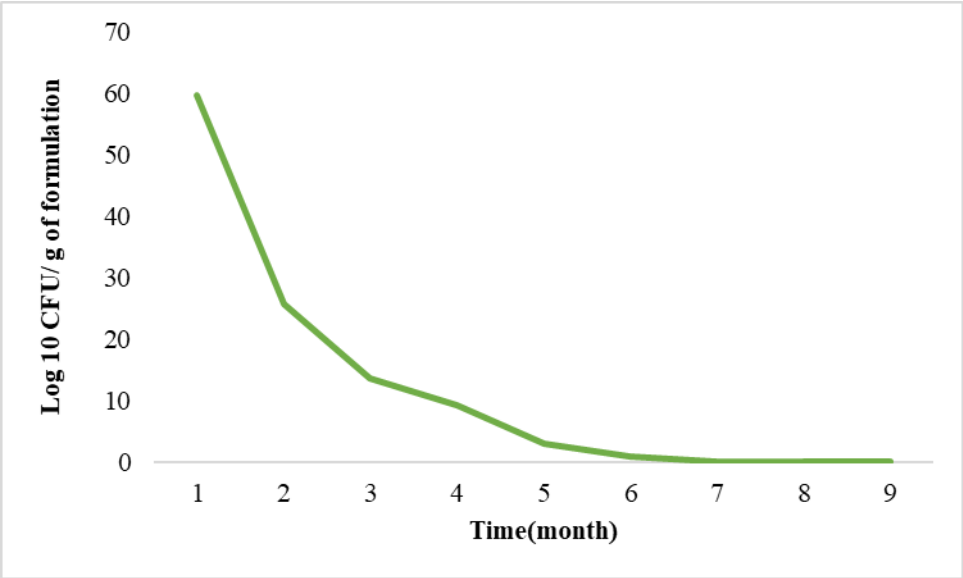

Fig. 34: Shift live of formulated biopesticide. The viability of biopesticide was assessed over a period of time. We found that after nine months the biopesticide is not longer active.

3.1.13.2 Evaluation of wilt incidence and severity in the pot

The best treatment (CBa_BFL2+CBa_RA37) selected based on its activity was tested for its ability to dwarf off the destructive effects of the highly pathogenic *R. solanacearum* Rs5 in tomato seedlings under pot and field conditions up to production on nonsterilized soil. Obviously, typical symptoms such as seedling stunting, wilting of leaves, brown staining of vascular rings, general wilting, and yellowing occurring at later stages, leading to plant collapse and death in severe cases, were caught to evaluate the incidence and severity of wilt disease (Fig. 31). However, when challenged with a consortium of bacteria, the disease establishment and progress dropped significantly ($p<0.01$) compared to plants emerging from pots infected with the pathogen alone (Rs5-treated plants). The PWP ranged from 100% to 12%, with the consortium among *B. amyloliquefaciens* CBa_RA37 and *B. amyloliquefaciens* CBa_BFL2 being the most protective compared to the positive control (streptomycin), as it could prevent 88% of plants from becoming infected by the pathogen (PWP = 8) against 15% of plants becoming infected by the pathogen (PWP = 85) upon the final (90 dpi) count.

3.1.13.3 Soil chemical characteristics

The soil chemistry characteristics of the study site are presented in Table (10). The recorded water pH value (7.46) indicated that the soil was moderately basic. The cation Exchange capacity (CEC) values of the soil in the different areas were very low, ranging from 4.64 to 7.91 $cmol^{+}\ kg^{-1}$respectively. The available phosphorus and potassium were at 4.2 mg kg^{-1} and 110 mg kg^{-1}. The different sources of azote were 38.11 $mg.kg^{-1}$ for soluble nitrate

nitrogen (N-NO_3) and 6.42 mg.kg^{-1} for soluble ammonium nitrogen N-NH_4. The C/N ratio was relatively low and varied between 0,044 and 0.216.

Table 10: Chemical characteristics of the soil

Sample	Depth	pH_{H2O}	EC	Total soluble salts	N-NO_3	N-NH_4	$N_{mineral}$ (N-NO_3 + N-NH_4)	P_{AL} (P mobil)	K_{AL} (K mobil)	C_{org}	H
	cm	-	mS/cm	g/100g	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	%	%
Soil	0-20	7.46	0.0823	0.02634	38.11	6.42	44.53	4.02	110	1.69	2.91

Soluble nitrate nitrogen (N-$N0_3$), soluble ammonium nitrogen, (N-NH_4), phosphorus (mobile form) P_{AL}, mineral nitrogen (N), , potassium (mobile form), K_{AL}, organic carbon C_{org} Humus content, H,

3.1.13.4 Evaluation of wilt incidence and severity in the open field

As for the pot experiment, the results showed that the treatment of tomato plants by pathogenic *R. solanacearum* Rs5 alone showed a PWP increase over time, and on the 90th day, 95.31% of induction of the disease by the latter was observed. BCAs (CBa_BFL2+CBa_RA37) significantly reduced disease progression with a 25% incidence. Treatment with the positive control was ineffective, with an induction of the disease of 87.5%. Similarly, the treatment of tomato plants by RAS alone, showed 100% severity after 90 days of inoculation (Fig. 35). In addition, treatment by the consortium CBa_BFL2+CBa_RA37, in the presence of *R. solanacearum* significantly reduced the progression of the disease, with a percentage reduction of 87.5%. Treatment by the consortium was more active than treatment with the positive control, which showed a severity of 87.5%.

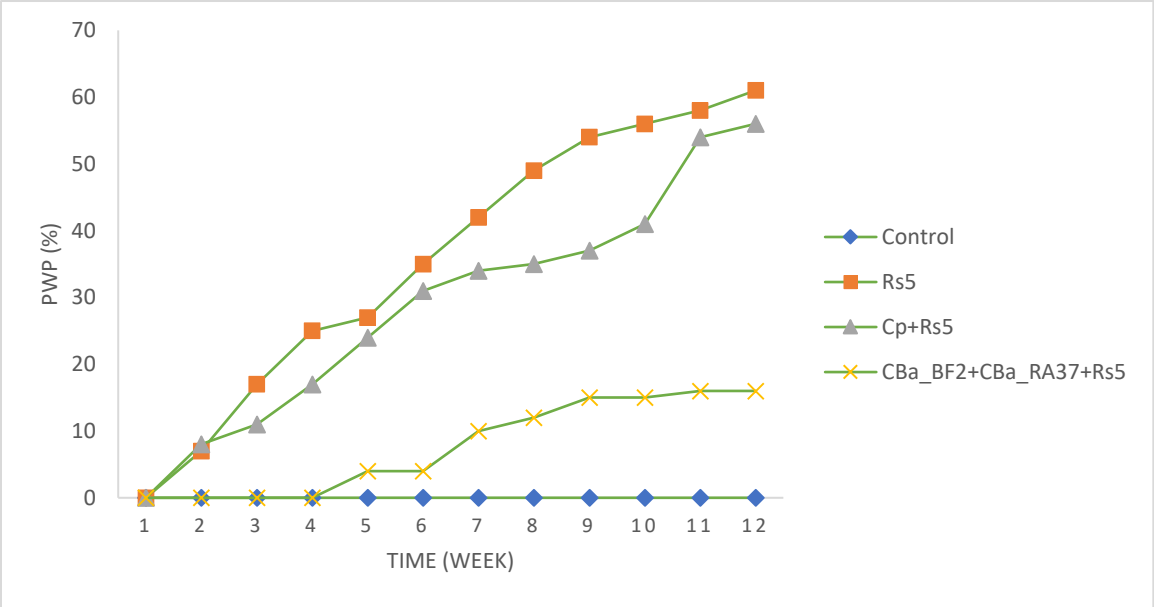

Fig 35: Variation in the percentage of wilted tomato plantlets as a function of time and antagonistic bacterial priming. Standing plants were counted twelve weeks after *R. solanacearum* Rs5 infection and expressed as percentages of the total number of assayed plants (n=64).

The data are the mean values of 64 replicates per treatment. Control: Uninoculated seedlings (neither *Bacillus* sp. nor *R. solanacearum* Rs5), Rs5: seedlings infected with *R. solanacearum* Rs5 alone, Cp_Rs5: seedlings primed by streptomycin and infected with *R. solanacearum* Rs5, BFL2_RA37_Rs5: seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37, and infected with *R. solanacearum* Rs5.

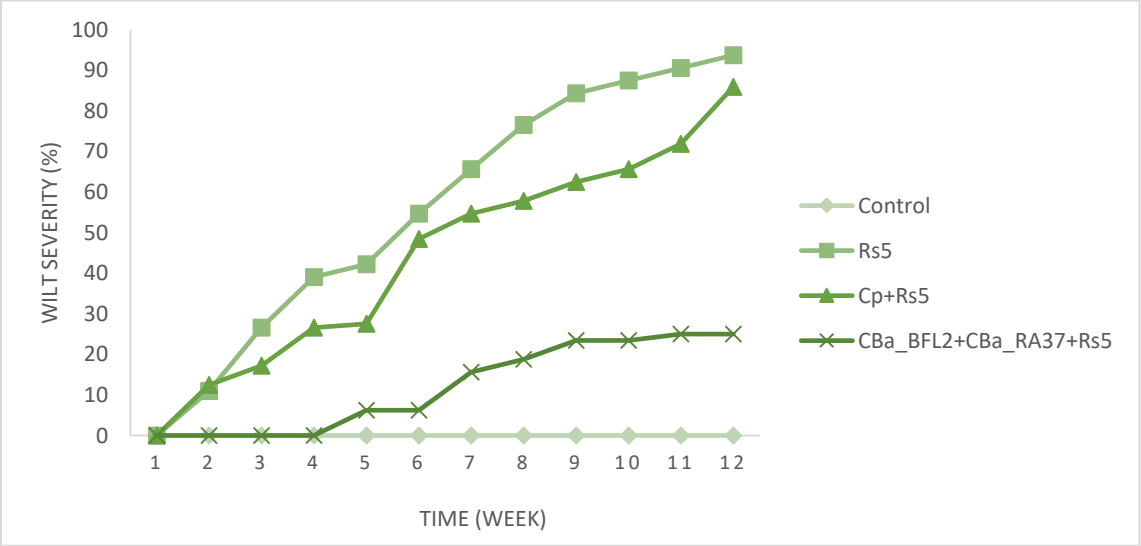

Fig 36: Biological control of bacterial wilt and enhanced growth performance in tomato seedlings under field conditions by use of a selected cacti Bacillus antagonist: Differential phenotypes of aboveground parts and disease suppression in tomato seedlings at 90 dpi. The values presented were taken on a weekly basis and were generated from fifteen replicates at each recording date.

Control: Uninoculated seedlings (Neither Bacillus sp nor *R. solanacearum* Rs5), Rs5: Seedlings infected with *R. solanacearum* Rs5 alone. CBa_RA37+CBa_BFL2+Rs5: Seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus velezensis* CBa_RA37 and infected with *R. solanacearum* Rs5, Cp+Rs5: seedlings primed with streptomycin (positive control) and infected with *R. solanacearum* Rs5.

Fig. 37: Biological control of bacteria wilt and enhanced growth performance in tomato seedlings under field conditions by use of selected the best treatment (consortia CBa_RA37+CBa_BFL2+Rs5).

Control: Uninoculated seedlings (Neither Bacillus sp nor *R. solanacearum* Rs5), Rs5: Seedlings infected with *R. solanacearum* Rs5 alone. CBa_RA37+CBa_BFL2+Rs5: Seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus velezensis* CBa_RA37 and infected with *R. solanacearum* Rs5, Cp+Rs5: seedlings primed with streptomycin (positive control) and infected with *R. solanacearum* Rs5.

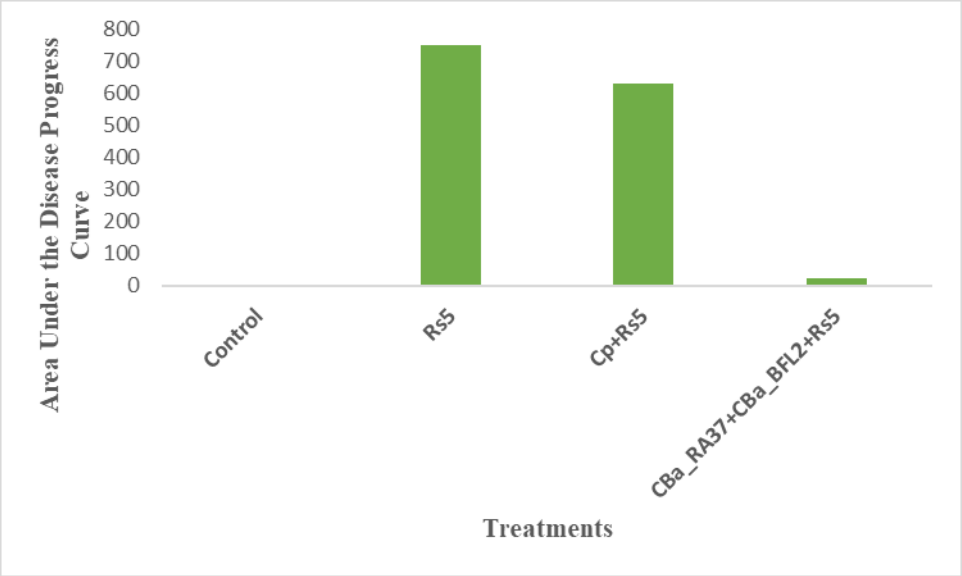

Fig. 38: Variation of AUDPC depending on the treatments.

Control: Uninoculated seedlings (Neither Bacillus sp nor *R. solanacearum* Rs5), Rs5: Seedlings infected with *R. solanacearum* Rs5 alone. CBa_RA37+CBa_BFL2+Rs5: Seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus velezensis* CBa_RA37 and infected with *R. solanacearum* Rs5, Cp+Rs5: seedlings primed with streptomycin (positive control) and infected with *R. solanacearum* Rs5.

3.1.13.5 Effects of treatments on agronomic growth parameters of *S. lycopersicum* in a pot

Figure 39 shows variations in aerial lengths, the number of branches per plant, the number of leaves per plant, the diameter of the snares, and the percentage of flowering. It appears that regardless of the parameter that was evaluated, treatment by the consortium of control agents was the best compared to treatment in the presence of *R. solanacearum* alone and treatment with positive control.

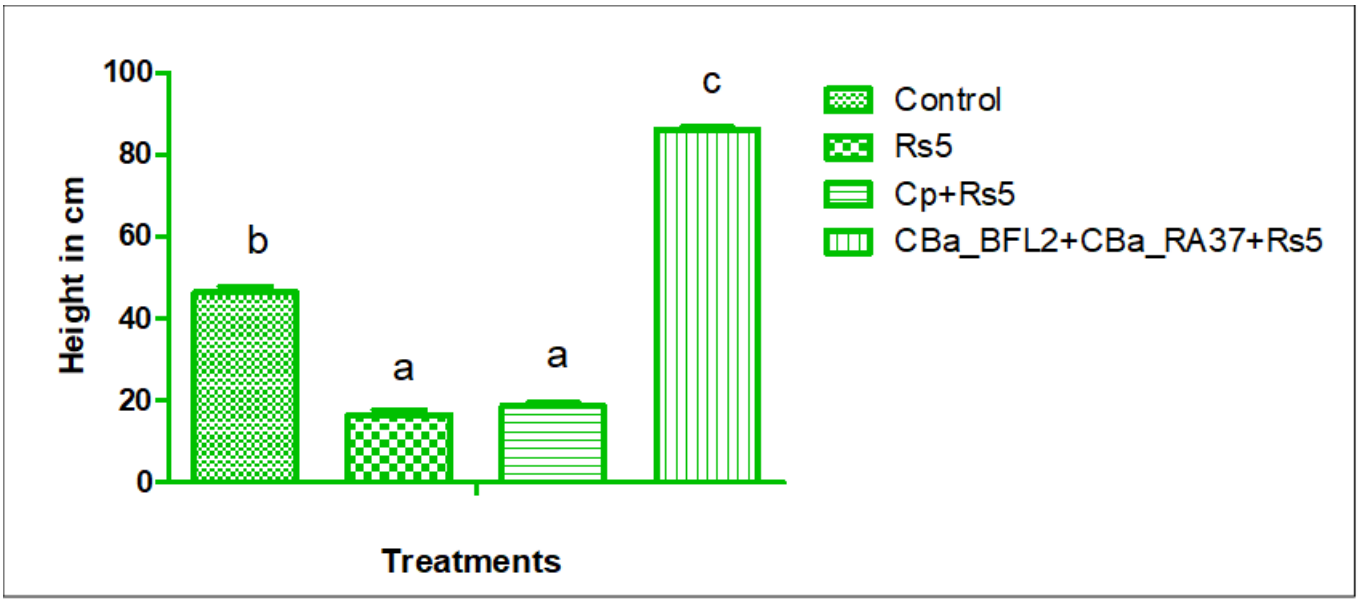

Fig. 39: Variation of height plant as function of treatment.

Standing plants were measure twelve weeks after *R. solanacearum* Rs5 infection and expressed in cm. Control: Uninoculated seedlings (Neither Bacillus sp nor *R. solanacearum* Rs5), Rs5: Seedlings infected with *R. solanacearum* Rs5 alone, Cp_+Rs5: Seedlings primed by streptomycin and infected with *R. solanacearum* Rs5, BFL2_+RA37_+Rs5: Seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37, and infected with *R. solanacearum* Rs5. Mean values (bar charts) bearing different letter are significantly different ($p \leq 0.05$) as determined by the Tukey's HSD post hoc test.

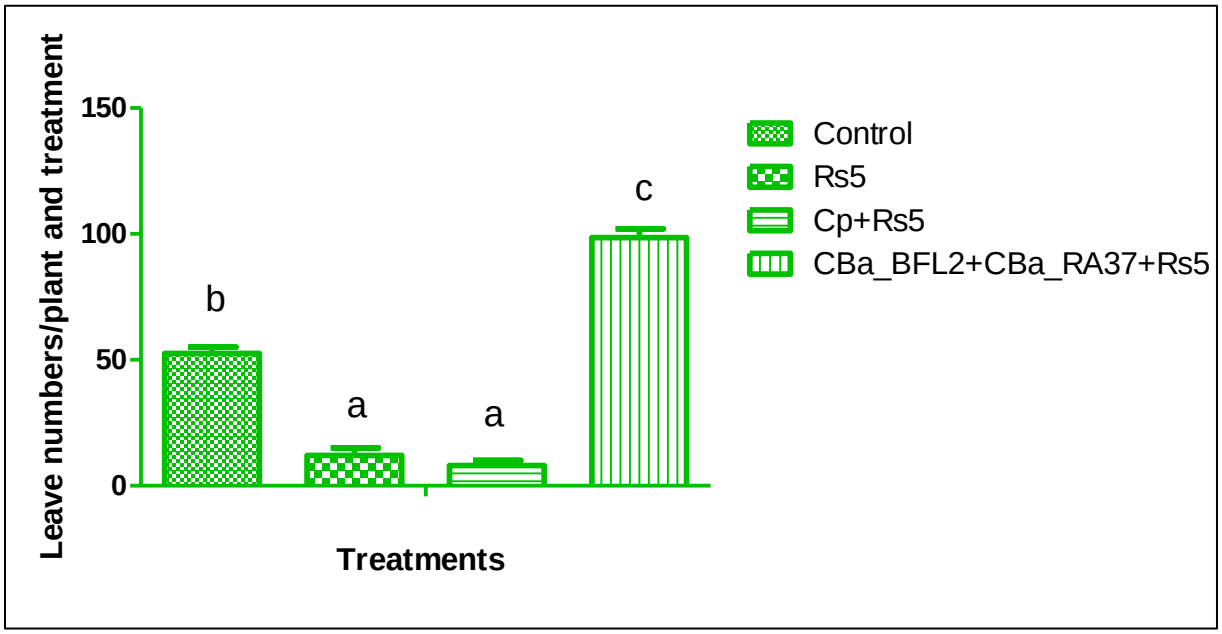

Fig. 40: Variation of leave number per plant as a function of treatment.

The leaves of standing plants were measured twelve weeks after *R. solanacearum* Rs5 infection. Control: uninoculated seedlings (neither *Bacillus* sp. nor *R. solanacearum* Rs5), Rs5: seedlings infected with *R. solanacearum* Rs5 alone, Cp_+Rs5: seedlings primed by streptomycin and infected with *R. solanacearum* Rs5, BFL2_+RA37_+Rs5: seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37, and infected with *R. solanacearum* Rs5. Mean values (bar charts) bearing different letter are significantly different ($p \leq 0.05$) as determined by the Tukey's HSD post hoc test.

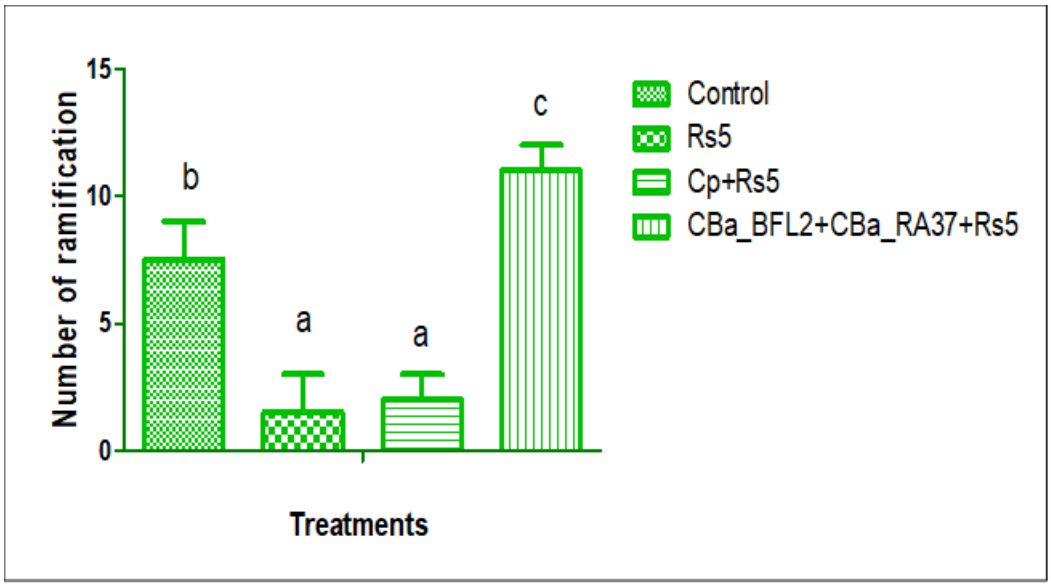

Fig. 41: Number of ramifications of plants as a function of treatment.

Ramification of standing plants was measured twelve weeks after R. solanacearum Rs5 infection. Control: uninoculated seedlings (neither *Bacillus* sp. nor *R. solanacearum* Rs5), Rs5: seedlings infected with *R. solanacearum* Rs5 alone, Cp_+Rs5: seedlings primed by streptomycin and infected with *R. solanacearum* Rs5, BFL2_+RA37_+Rs5: seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37, and infected with *R. solanacearum* Rs5. Mean values (bar charts) bearing different letter are significantly different ($p \leq 0.05$) as determined by the Tukey's HSD post hoc test.

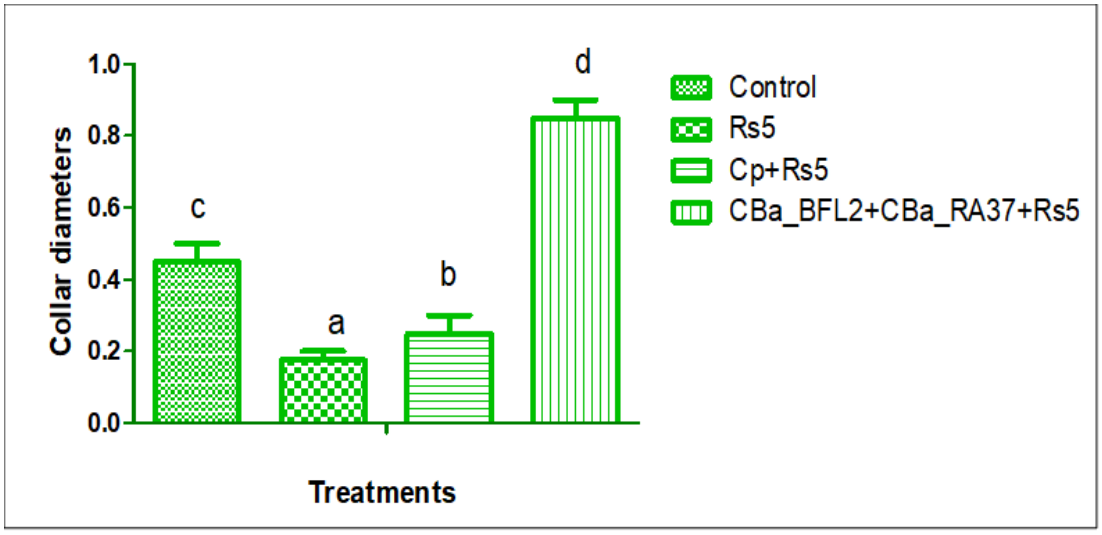

Fig. 42. Variation in plant collar diameters as a function of treatment.

Standing plants were measured twelve weeks after R. solanacearum Rs5 infection and expressed in cm. Control: uninoculated seedlings (neither *Bacillus* sp. nor *R. solanacearum* Rs5), Rs5: seedlings infected with *R. solanacearum* Rs5 alone, Cp_+Rs5: seedlings primed by streptomycin and infected with *R. solanacearum* Rs5, BFL2_+RA37_+Rs5: seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37, and infected with *R. solanacearum* Rs5. Mean values (bar charts) bearing different letter are significantly different ($p \leq 0.05$) as determined by the Tukey's HSD post hoc test.

Fig. 43: Biological control of bacterial wilt and enhanced growth performance in tomato seedlings under pot conditions by the use of the best consortia CBa_RA37+CBa_BFL2+Rs5 and challenged with *R. solanacearum* Rs5 after 45 days.

(A) plantlets primed with streptomycin and grown on *R. solanacearum* Rs5 infected soil. (B) plantlets primed by *R. solanacearum* Rs5 alone. (C) uninoculated seedlings (control) neither by *Bacillus* spp nor *R. solanacearum* Rs5. (D) plantlets coinoculated with *B. amyloliquefaciens* CBa_RA37 and CBa_BFL2 and infected with *R. solanacearum* Rs5.

3.13.6 Effect of treatments on field yield under field conditions

Ninety days after transplantation, the results indicated that the application of CBa_BFL2/CBa_RA37 to the plants greatly promoted the growth parameters and yield of the

plants. On the other hand, plants infected with *R. solanacearum* alone showed a remarkable decrease in the number of branchels/plant. Similarly, treatment with biocontrol agents has showed the best yield in terms of flowers and fruit in comparison with the negative control and positive control

Table 11: Flowering and fruit attributes of tomato under field conditions.

Treatments	Number of days for floworing	Number of days for 50% floworing	Number of flowers per plant	Weight of the fruit (g)	Length of the fruit (cm)	Diameter of the fruit (cm)	Yield (t/ha)
Control	47	50	11	42.01	7	13.38	20.2
Rs5	50	51	5	17	3.1	9.78	3.6
Cp + Rs5	51	51	7	20.33	3.3	10.5	7.3
CBa_BFL2+CBa_RA37+Rs5	40	48	19.67	81.15	13	16.40	30.74
S.Em	2.1	2.5	2.25	1.87	1.76	1.54	2.9
CD (P=0.05)	5.2	3.3	6.89	4.6	6.7	4.3	6.5

Control: uninoculated seedlings neither by *Bacillus* spp nor *R. solanacearum* Rs5. Rs5: plantlets primed by *R. solanacearum* Rs5 alone. C+Rs5: plantlets primed with streptomycin and grown on *R. solanacearum* Rs5 infected soil. CBa_BFL2+CBa_RA37+Rs5: plantlets coinoculated with *B. amyloliquefaciens* CBa_RA37 and CBa_BFL2 and infected with *R. solanacearum* Rs5. S.Em: Standard error of the mean. CD: critical difference.

Fig. 44: Biological control of bacterial wilt and improvement of growth performance in tomato seedlings under field conditions by use of selected treatment (consortia CBa_BFL2+CBaRA37).

Differences in the level of flowering between the treatments. Control: uninoculated seedlings (neither *Bacillus* sp. nor *R. solanacearum* Rs5), Rs5: seedlings infected with *R. solanacearum* Rs5 alone, Cp_+Rs5: seedlings primed by streptomycin and infected with *R. solanacearum* Rs5, BFL2_+RA37_+Rs5: seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37, and infected with *R. solanacearum* Rs5.

Fig. 45: Biological control of bacterial wilt and improvement ofgrowth performance in tomato seedlings under field conditions by use of selected treatment (consortia CBa_BFL2+CBaRA37).

Differences on the level of fruits between the treatments. Control: Uninoculated seedlings (neither *Bacillus* sp. nor *R. solanacearum* Rs5), Rs5: seedlings infected with *R. solanacearum* Rs5 alone, Cp_+Rs5: seedlings primed by streptomycin and infected with *R. solanacearum* Rs5, BFL2_+RA37_+Rs5: seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37, and infected with *R. solanacearum* Rs5.

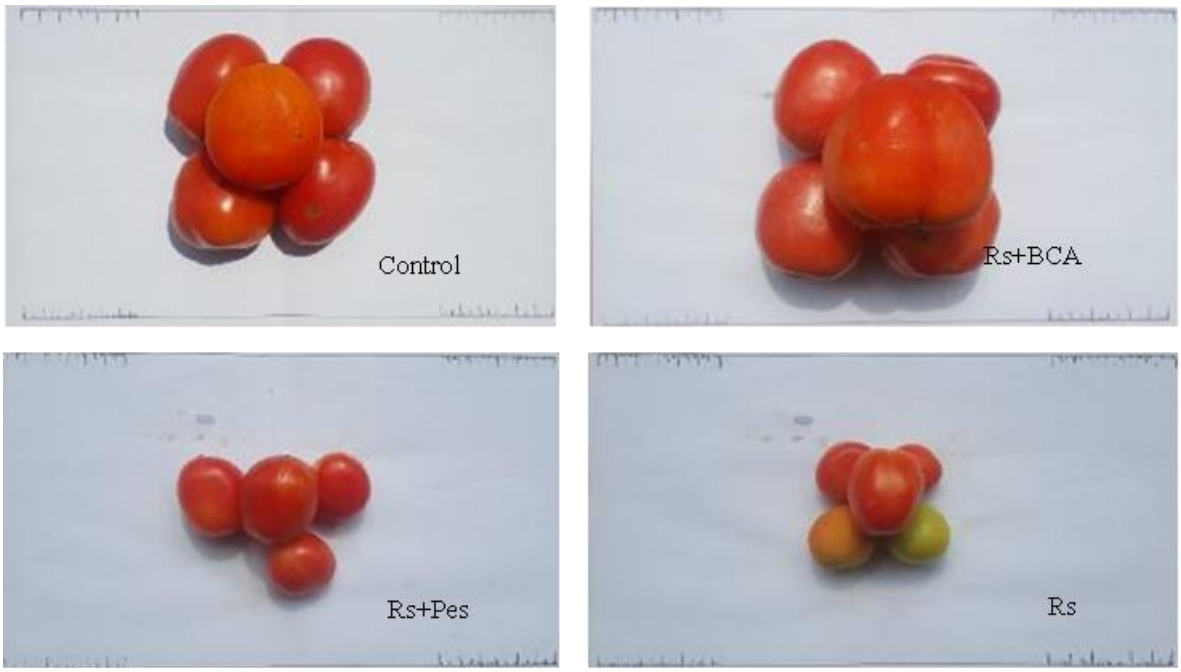

Fig. 46: Biological control of bacterial wilt and improvement of growth performance in tomato seedlings under field conditions by use of selected treatment (consortia CBa_BFL2+CBaRA37). Differences on the level of fruit haversted between the treatments. Control: uninoculated seedlings (neither *Bacillus* sp. nor *R. solanacearum* Rs5), Rs5: seedlings infected with *R. solanacearum* Rs5 alone, Cp_+Rs5: seedlings primed by streptomycin and infected with *R. solanacearum* Rs5, BFL2_+RA37_+Rs5: seedlings dually primed with *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37, and infected with *R. solanacearum* Rs5.

DISCUSSION

Chapter 4: Discussion

The interplay between the components at the three vertices of the "disease triangle" encompassing virulent pathogens, susceptible hosts and environmental conditions, has since been established as a determining factor underlying the destructive effects of phytopathogens. Disrupting this equilibrium by acting directly or indirectly on one or multiple elements is the backbone of plant disease control strategies (Wang *et al*., 2018). Agriculture has long been heavily still been dependent on chemical inputs to serve this role but their deleterious effects have led to a quest for novel and reliable technologies for higher quantity and quality food provision without jeopardizing human health (Karimi *et al*., 2016; Alori *et al*., 2017; Alori and Babalola, 2018). For this purpose, microbial inoculants are being steadily advocated because their inherent traits including site colonization and competition for nutrients, plant growth promotion (PGP), antibiotic production, cell lysis effects, and induced systemic resistance (ISR) can influence the plant-environment-pathogen system to support a plant's health and productivity. Herein, we substantially demonstrated that ten *Bacillus* strains originating from desert triangular spurge displayed promising biocontrol potential both *in vitro* and *in planta*. Out of ten endophytic bacteria, *B. velezensis* CBv_BE1, *B. amyloliquefaciens* CBa_BFL2 and CBa_RA37 showed outstanding antibacterial activities either through dual culture or their respective culture filtrate and ethyl acetate-based extracts, which totally suppressed (100% inhibition) the growth of *R. solanacearum* at low concentrations (CMI = 0.625 mg/ml). Such inhibitory actions of *Bacillus* species have widely been reported against a vast array of agriculturally impactful pathogenic fungi (Alvarez *et al*., 2012; Karimi *et al*., 2016) and bacteria, including *R. solanacearum,* causing bacterial wilt in peanut (Karimi et al., 2016), tobacco (Tahir *et al*., 2017) and tomato (Almoneafy *et al*., 2014; Cao *et al*., 2018). Our finding contradicts the evidence that antibiosis plays a role in biocontrol mechanisms produced by biocontrol bacilli. While the antimicrobial potential has commonly been ascribed to lipopeptides, Chen *et al*. (2019) identified 32 putative gene clusters intervening in the synthesis of antimicrobial compounds in the genome of *Bacillus velezensis* LDO2 when sensing the plant pathogenic *Aspergillus flavus*, of which 7 well known antimicrobials consisted of dipeptides (bacilysin), cyclic lipopeptides (surfactin and fengycin) and polyketides (bacillaene, butirosin, macrolactin and difficidin). The observed activity of culture filtrate and ethyl acetate extract would be do to the presence of such compounds (cyclo(L-Phe-D-pro), cyclo (L-Val-L-pro), musicacid) present in extract as demonstrated by HPLC analysis. Indeed, the biological activities of somes of these compounds have already been demonstrated. Muhanna *et al*. (2017) have demonstrated the

antimicrobial activity of cyclo (L-phe-Dpro) and cyclo (L-val-L-pro). Similarly, Ae et al., 2020 have shown the ability of cyclo (L-val-Lpro) derived from *Bacillus thuringiensis* JCK_1233 to control pine wilt disease by elicitation of moderate hypersensitive reaction. The present study was able to improve the germination of tomato seeds *in vitro* and enhance the growth of the plants under pot conditions. Plants treated with *Bacillus* species were more vigorous, and their growth was significantly increased regardless of inoculation with the *R. solanacearum* pathogen. Similarly, Abo-Elyousr et al. (2019) reported growth improvement in tomato plants inoculated with a species of *B. amyloliquefaciens.* The induced increase in root growth may be attributed to the ability of bacteria to improve plant phosphate solubilization and the synthesis of plant growth-regulating substances such as IAA and siderophores (Antunes *et al*., 2017). Indeed, phosphorous nutrition enhances the overall growth of plants and helps in root development (Jones and Darrah, 1994), and increasing IAA production may positively influence the development of the root system and allow plants to improve nutrient uptake which is critical for their growth. Nutrient acquisition could also be enhanced through siderophore production, which is known to increase the bioavailability of nutrients such as iron (Glick, 2003).

The triggered metabolic shift and reprogramming of the host transcriptome, resulting in elicitation of the innate immune system are other pathways by which biocontrol agents confer protection and enhance crop growth. Prior to this, host inner tissue colonization and establishment are required to efficiently deliver any desired trait *in planta*, and are indispensable in imparting tolerance against soilborne diseases (Bais *et al*., 2004; Liu *et al*., 2017). Trending omics analyses have indeed defined key traits enabling endophytes to invade, colonize and translocate in the host inner tissue including but not limited to chemotaxis, motility, plant cell-wall degradation, and biofilm formation abilities (Sheibani-Tezerji *et al*., 2015; Liu *et al*., 2017). Our results are in agreement with ~~these~~ the above findings, as our strains could synthesize and release lipases, proteases, and cellulases and form biofilms under *in vitro* conditions. In fact, biofilms are essential tools that help bacterial cells adhere to the plant cell surface and initiate colonization. Furthermore, Straub *et al*. (2013) detected a large number of copies of genes encoding cell-wall degrading enzymes in the metagenome of rice endophytic bacteria, probably to break plant cell walls and translocate in the apoplast.

Once in plant tissues, researchers have agreed on the involvement of a finely balanced molecular regulatory network mediated by hormone-controlled signaling pathways. For instance, the jasmonic and salicylic acid regulation pathways, including their cross-talk restrict

the endogenous endophyte's population level to an extent that it benefits not only the latter but also the survival of the bacteria. Accordingly, Hein *et al*. (2008) and Sheoran *et al*. (2016) argued that *Pseudomonas putida* PpBP25 could partially repress and induce 74 and 131 genes, among which were components of defense and salicylic acid (SA) signaling such as ATEXO70B2, CBP60G, PLA2, CRK18 and ATFBS1 allowing initial colonization and establishment in *Arabidopsis thaliana*. Meanwhile, the transcription factor WRKY, which regulates the cross-talk between the JA and SA pathways, was found to be upregulated upon endophyte colonization, inferring the probable reactivation of the previously repressed genes and the activation of the jasmonate signaling pathway, leading to restricted endophyte colonization and subsequently induced systemic resistance to future pathogenic attacks (Sheoran *et al*., 2016). Herein, we recorded a relatively constant endophyte population density (approximately log10 CFU = 4.29 per gram fresh weight) in tomato seedlings despite the increase in inoculum concentration and irrespective of the bacterial strain. Subsequent to the strengthened defense response, all the tested bacteria significantly suppressed bacterial wilt compared to pathogen-infected and unprimed seedlings (Rs5). *B. amyloliquefaciens* CBa_BFL2 and *B. amyloliquefaciens* CBa_RA37 the consortium among being the most (CBa_BFL2+CBa_RA37) effective by protecting the plants from infection at as 90% (PWP = 10) and slowing the symptom progress in infected tomato plantlets by up to 89% (Fig. 16 and 17). The achieved data are in agreement with previous findings advocating the application of microbial inoculant-based consortia for sustainable disease control rather than individual microbes (Sarma et al., 2014; Eke *et al*., 2016). This technology is being touted for its reliability and improved efficacy and consistency under diverse soils and environmental conditions (Sarma *et al*., 2014). In this study, we further dissected the ISR at the subcellular level through some biochemical indicators, such as oxidative burst, and the accentuation of the phenylpropanoid pathway. The Pearson correlation model showed positive and significant correlations between the specific activities of GPX ($r = 0.85$; $p = 0.006$), PAL ($r = 0.77$; $p = 0.02$) and SOD ($r = 0.72$; $p = 0.05$) and bacterial wilt severity, indicating enhanced expression of these genes and their role in disease suppression. In fact, it is noteworthy that polyphenol biosynthesis is a signal in response to induced systemic resistance. Notably, when the pathogen's epitope conserved pathogen associated molecular patterns (PAMPs), is detected by plant pattern recognition receptors (PRRs), a series of adaptative measures are taken, including increasing antioxidant activities, tickening the host cell membrane by lignification as a means of blocking pathogen entry, and the synthesis of protective agents against pathogen ingress (Jetiyanon, 2007; Al-askar and Rashad, 2010; Abdel Fattah *et al*., 2011).

Overall, although substantial applied and basic work is still needed, these findings motivate further vulgarization of the use of microbes in assisted crop production. However, biocontrol candidates are usually poorly effective when tested in field conditions. A 3-month field study from sowing to harvest was carried out using the best treatment that was the consortium (CBa_BFL2+CBa_RA37). We demonstrated that, in addition to being nontoxic toward tobacco leaflets and perfectly compatible, the consortium *B. amyloliquefaciens* CBa_RA37 and *B. amyloliquefaciens* CBa_BFL2 could substantially promote tomato growth and suppression of bacterial wilt induced by *R. solanacearum* more than any other treatment under our field conditions. We noted a 87.5% reduction in the severity of the disease in the treatment having received the bacterium consortium. On the other hand, the batch that received *Ralstonia solanacearum* alone presented a severity of 100%. Similarly, we obtained a significant difference between the agronomic parameters of the treatment by the consortium and the treatment constituting the normal control with tomato fruit diameters ranging from 11 to 18 mm unlike the normal control whose fruit diameters ranged from 11 to 18 to 14.5mm.

The resulting data provided additional evidence of the superiority of dual bioagent application over field application in uncontrolling plant diseases. However, deep knowledge regarding the modes of action is paramount as they may perhaps shed light on the observed outputs derived from the addition of individual performances or from microbe-microbe or microbial-plant interactions.

CONCLUSION AND PERSPECTIVES

4. Conclusion and perspectives

4.1 Conclusion

This work aimed to survey the ability of endophytic bacteria isolated from *E. antiquorum* to protect tomato crops against the pathogen *R. solanacearum* and promote the growth of plant. From the 10 initial endophytic bacteria chosen for this study, 3 of the genus Bacillus, namely, *B. amyloliquefaciens* CBa_RA37, *B. amyloliquefaciens* CBa_BFL2 and *B. velezensis* CBv_BE1, showed promising inhibitory potentials in vitro and in vivo against the *R. solanacearum* pathogen. In pot and field experiment the combine application of *B. amyloliquefaciens* CBa_BFL2 and *B. amyloliquefaciens* CBa_RA37 (CBa_BFL2/CBa_RA37) significantly promoted shoot and root growth. Additionally, the consortium CBa_BFL2/CBa_RA37 stood out by suppressing wilt incidence and severity by 90% and 87.5%, respectively in field experiement.

According our specifics objectives the following conclusions were drawn.

- Out of the ten Bacillus strains used in this work, *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37 and *Bacillus velezensis* CBv_BE1 were selected for *in planta* testing because of their ability to inhibit 100% of *R. solanacearum* growth. Additionally, they have been the best strains in terms of growth-promoting parameters (ammonia, siderophores, phosphate solubilization, IAA).
- *Bacillus amyloliquefaciens* CBa_BFL2, *Bacillus amyloliquefaciens* CBa_RA37 and *Bacillus velezensis* CBv_BE1 successsfully colonized the tissues of young tomato plants and the results revealed a positive correlation between colonization and the amount of salicylic acid produced.
- All the BCA treatments led to a significant reduction in the disease in tomato plants, and a significant growth of agro-morphological parameters. The consortium *Bacillus amyloliquefaciens* CBa_BFL2 and *Bacillus amyloliquefaciens* CBa_RA37 was the best treatment in the pot and in the open field with reductions of 89% and 87.5% in severity, respectively. In addition, they acted on the metabolism of phenolic compounds and some oxidative stress enzymes. This resulted in reduced activities of guaicol peroxidase

(GPX), phenylalanine ammonia lyase (PAL) and superoxide dismutase (SOD) compared to the *R. solanacearum* treatment alone.

4.2 Perspectives

As this work is only the beginning of research on this subject, we will consider the following perspectives:

- Assess the qualitative analysis of tomato fruit obtained after the treatment with our biopesticide (consortium CBa_BFL2/CBa_RA37).
- Evaluate the impact of our biopesticide on soil quality during cultivation and after harvest.
- Assess the biochemical molecules stimulated by BCAs during resistance to *Ralstonia solanacearum* in a pot.
- In the field, assess the formulation's effectiveness against bacterial wilt caused by *R. solanacearum*

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Appendices

Appendices

Appendice 1: Preparation of in vitro culture media

❖ Muller Hinton Agar (MHA)

✓ **Composition**

Beef extract : 2.00 g
Acid hydrolysate of Casein: 17.50 g
Starch : 1.50 g
Agar : 17.00g
Eau distillée : 1L

✓ Preparation

The Muller Hinton Agar medium (MHA) was prepared according to the manufacturer's recommendations by dissolving 38 g of powder of the medium in 1000 ml of distilled water. Sterililization was carried out in an autoclave at 121°C for **15 minutes**

❖ Tetrazolium chloride (TZC)

Composition
Beef extract : 2.00 g
Acid hydrolysate of Casein : 17.50 g
Starch : 1.50 g
Eau distillée : 1L

✓ Preparation

The Tetrazolium chloride medium (TZC)) was prepared according to the manufacturer's recommendations by dissolving 10 g of 2,3,5-triphenyl tetrazolim chloride in 1000 ml of distilled water and placed in a light-proof capped bottle. Sterilization was carried out in an autoclave at 121°C for 8 minutes

❖ **Luria Bertani Agar**

✓ **Composition**

Trpton : 10g
Fungal extract : 5g
Nacl : 5g
Agar : 10g
Eau distilée : 1L

✓ Preparation

The Luria Bertani Agar medium (LBA) was prepared according to the manufacturer's recommendations by dissolving 38 g of powder of the medium in 1000 ml of distilled water. Sterilization was carried out in an autoclave at 121°C for 15 minutes

❖ Pikovskaya

✓ Composition

Glucose : 10g
Sulfate d'ammonium : 0.2g
Sulfate de manganese : 0.002g
Yeast extract : 0.5g
Sulfate de magnesium : 0.1g
Chlorure de potassium : 0.2g
Sulfate de fer : 0.002g
CaHPO4 : 5g

✓ Préparation

Pikovskaya medium (PVK) was prepared according to the manufacturer's recommendations by dissolving each of the medium powder ingredients in 1000 ml of distilled water. Sterililization was carried out in an autoclave at 121°C for 15 minutes

Appendice 2: Antibacterial activity of culture filtrate of BCA at different time

	LPR19	BFL1	RA37	BFL2	RR10	BE1
0h	0	0	0	0	0	0
24h	0	0	0	0	0	0
48h	0	0	0	0	0	0
72h	0	0	0	0	0	1000

96h	125	125	1000	62.5	0	1000
120h	125	125	62.5	31.25	62.5	15.625
144h	0	0	0	1000	0	250

Appendice 2: OD of ammonia production

	LPR19	BFL1	LPR11	RA37	LPR2	BFL2	RR10	RA14	LPR8	BE1	Cont
	0.896	1.0932	1.0665	1.227	0.986	1.169	1.037	0.999	0.942	1.167	0.398
	0.927	1.124	1.094	1.238	1.118	1.166	1.023	1.049	0.989	1.147	0.432
	0.928	1.113	1.064	1.211	1.054	1.141	0.965	1.135	0.962	1.219	0.39
	0.964	1.0971	1.059	1.229	0.986	1.117	0.991	0.994	0.967	1.098	0.367
Means	0.92875	1.106825	1.070875	1.22625	1.036	1.14825	1.004	1.04425	0.965	1.15775	0.39675
ODr	0.92875	1.106825	1.070875	1.22625	1.036	1.14825	1.004	0.6475	0.56825	0.761	0

Appendice 3 : Indol acetic acid production

	LPR19	BFL1	LPR11	RA37	LPR2	BFL2	RR10	RA14	LPR8	BE1	Cont
	0.3019	0.2331	0.2686	0.3082	0.3201	0.3938	0.2957	0.3293	0.3569	0.3545	0.1003
	0.3558	0.2037	0.3069	0.3058	0.2749	0.3622	0.2789	0.3366	0.3697	0.4051	0.1009
	0.3931	0.2557	0.3194	0.3266	0.3235	0.3826	0.2804	0.3508	0.363	0.4092	0.1006
	0.3161	0.2412	0.2922	0.2936	0.334	0.378	0.2692	0.2942	0.3336	0.3678	0.1008
Means	0.3558	0.233425	0.296775	0.30855	0.30855	0.37915	0.28105	0.327725	0.3558	0.38415	0.10065
ODr	0.241075	0.132775	0.1961	0.2079	0.227075	0.2785	0.1804	0.227075	0.25515	0.2835	0.00143
Qtity (ug/ml)	1.45	3.62	3.86	4.28	3.3	1.34	11.32	12.14	25.32	15.31	19.35

Appendice 4 : Salysilic acid production

	LPR19	BFL1	LPR11	RA37	LPR2	BFL2	RR10	RA14	LPR8	BE1	Cont
	0.1288	0.2566	0.2967	1.6312	0.1267	1.9946	0.2748	0.1757	0.7769	0.7797	0.1357
	0.1286	0.2753	0.2901	1.6336	0.1184	1.9669	0.2603	0.1799	0.7683	0.8067	0.1389
	0.1289	0.2753	0.3053	1.731	0.1239	1.9403	0.2801	0.1797	0.7561	0.8009	0.1345
	0.1344	0.2702	0.2618	1.6675	0.1144	1.9763	0.2638	0.1852	0.7496	0.7777	0.1332
Means	0.130175	0.26935	0.288475	1.53025	0.12085	1.969525	0.26975	0.1852	0.762725	0.7777	0.135575
ODr	-0.0054	0.133775	0.1529	1.53025	-0.014725	1.83395	0.2638	0.1852	0.62715	0.655675	0
Qt (µg/mL)	0	1.031	1.386	26.986	0	10.201	1.038	0	1.038	29.539	0

Appendice 5 : Carboxilic siderophores

	LPR19	BFL1	LPR11	RA37	LPR2	BFL2	RR10	RA14	LPR8	BE1
	0.2987	0.2998	0.2897	0.3948	0.2012	0.3698	0.3458	0.2967	0.2967	0.4387
	0.3001	0.2986	0.2987	0.3375	0.2134	0.3267	0.3867	0.2899	0.3005	0.4475
	0.3012	0.2899	0.2865	0.2997	0.2212	0.3768	0.2987	0.2945	0.2988	0.5101
	0.2976	0.2985	0.2898	0.3111	0.2098	0.3564	0.3013	0.2898	0.3026	0.4934
DO Means	0.2994	0.2967	0.291175	0.335775	0.2114	0.357425	0.333125	0.292725	0.29965	0.472425
% product	7.18	6.22	4.24	20.2	0.2	27.96	16.26	4.79	7.27	69.13

Appendice 6 : Hydroxamate production

	LPR19	BFL1	LPR11	RA37	LPR2	BFL2	RR10	RA14	LPR8	BE1
	0.1201	0.0663	0.0631	1.5445	0.1108	0.0964	0.1043	0.4317	0.491	1.8319
	0.0872	0.0664	0.0797	1.4867	1.8319	1.1646	0.4526	0.4708	0.4267	1.7739
	0.6875	0.4547	0.4907	1.5577	1.7739	1.2924	0.6045	0.4837	0.4391	1.1432
	0.9958	0.6979	0.7602	1.7507	1.1432	1.4557	0.459	0.4873	0.4382	1.0234
	1.0106	0.4698	0.4705	0.075	1.2428	1.3614	0.4774	0.0745	0.0878	1.2865
Means	0.58024	0.35102	0.37284	1.28292	1.22052	1.0741	0.41956	0.3896	0.37656	1.41178
% product	59	35	37	100	100	100	41	38	37	100

Appendice 7 : Catecholate production

	LPR19	BFL1	LPR11	RA37	LPR2	BFL2	RR10	RA14	LPR8	BE1
	0.4317	0.8145	0.3631	0.7708	0.6663	1.6317	0.6211	0.3263	0.5201	1.2645
	0.4408	0.9011	0.3707	0.8319	0.6664	1.87395	0.5066	0.2364	0.5172	1.3924
	0.4437	0.8576	0.4907	0.7739	0.5547	1.1333	0.5575	0.3147	0.5275	1.5558
	0.4073	0.9507	0.3602	1.1432	0.6979	1.0034	0.5523	0.319	0.6003	1.3617
	0.4445	0.8205	0.3705	0.9428	0.6698	1.3365	0.5499	0.3698	0.5106	1.2021
Means	0.4468	0.8975	0.3715	0.9433	0.645401	1.39577	0.5543	0.3273	0.52001	1.3553
% product	44.68	89.75	37.15	94.33	64.54	100	55.43	32.73	52	100

Appendice:

	EA Cell (a+b)/a	**EA Pro (a+b)/a**	**EA Amy (a+b)/a**
BE1	1.5483871	2.33333333	2.5
BFL2	1.72413793	1.89655172	2
BFL1	1.33333333	1.92	1.55555556
RA37	1.41935484	2.16	2.11111111
RA14	1.72727273	1.5483871	1.13333333
RR10	1	1.2972973	1.06666667
LPR11	1.4	2.51724138	1.15384615
LPR19	1.15	1.43333333	1.15384615
LPR8	1	1.42105263	1

LPR2	1.6	1.9	1

Infection index (%)

Appendice 8:

Treatments

	Days 0	Day 7	Day 14	Day 21	Day 28
Control	0	0	0	0	0
RS_5	0	0	4	30	100
$BFL2RS_5$	0	0	0	0	16
$RA37RS_5$	0	0	0	14	22
$BE1RS_5$	0	0	0	16	18
$BFL2BE1RS_5$	0	0	0	0	4
$RA37BE1RS_5$	0	0	0	0	18
$RA37BFL2RS_5$	0	0	0	0	16

Appendice 9: In vitro capacity of BCA on disease severity

Number of wilted plants daily during the 28 days of pot test

Treatments	**Days**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Cont		0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0		0		0		0	0	0	0	0
RS_5		0	0	0		0	1	1	1	1	1	2	3	4	6	6	6	6	7	7	7	7	8	8	8	8	9	10	10

$BFL2RS_5$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	2	2	3	3	3	3	3
$RA37RS_5$	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	3	3	3	4	4	5	5	6	6	6	6	6
BE1+RAS	0	0	0	0	0	0	0	0	0	0	1	1	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
$BFL2BE1RS_5$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1
$RA37BE1RS_5$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	2	2	2	2	3
$BFL2RA37RS_5$	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1

Appendice 10: Root part length (cm)

Treatments	C	RAS	BFR3	BFR4	BE1	BFR3BFR4	BFR3BE1	BFR4BE1
Means ±ET	16.73± 1.85	4.07±0.90	17.29 ±2.12	14.71 ±2.11	17.86 ±2.23	20.20± 1.82	14.89± 1.93	14.99± 1.51

Appendice 11: length of the aerial part (cm)

Treatments	C	RAS	BFR3	BFR4	BE1	BFR3BFR4	BFR3BE1	BFR4BE1
Mea,s±ET	11.57±1.52	4.77±0.49	14.32±1.51	11.91±1.034	12.40±1.0	15.88±1.32	13.00±1.205	12.85±1.57

Appendice 12: Aerial dry biomass (g)

Treatments	C	RAS	BFR3	BFR4	BE1	BFR3BFR4	BFR3BE1	BFR4BE1
	0.18	0.02	0.446	0.254	0.212	0.199	0.065	0.069
	0.171	0.013	0.33	0.272	0.235	0.135	0.102	0.073
	0.107	0.017	0.291	0.14	0.141	0.088	0.08	0.071
	0.175	0.014	0.361	0.207	0.191	0.079	0.096	0.057
	0.14	0.017	0.331	0.238	0.298	0.151	0.063	0.057
	0.133	0.029	0.334	0.49	0.192	0.061	0.082	0.063
Means	0.151	0.018	0.349	0.267	0.212	0.119	0.081	0.065
Means±ET	0.151± 0.02	0.018 ±0.01	0.349 ±0.0	0.267± 0.11	0.212 ±0.05	0.119± 0.05	0.081± 0.01	0.065± 0.006

Appendice 12: Dry root biomass (g)

Treatments	C	RAS	BFR3	BFR4	BE1	BFR3BFR4	BFR3BE1	BFR4BE1
Means±ET	0.046± 0.01	0.00632± 0.03	0.126 0.00	0.102± 0.0	0.087 ±0.014	0.116±0.04	0.077 ±0.01	0.068± 0.006

Appendice 13: Enzymatic assay

- Calibration BSA (Bovine Serum Albumine)

Quantities BSA (µg)	0	0.01	0.03	0.04	0.05	0.07
OD 595nm	0	0.024	0.034	0.062	0.057	0.078

- OD total protein content

Treatments	C	RAS	BFL2	RA37	BE1	BFL2BE1	RA37BE1	BFL2RA37
OD1	0.085	0.01	0.111	0.029	0.125	0.094	0.148	0.181
OD2	0.101	0.012	0.119	0.053	0.155	0.123	0.174	0.159

- Total protein content (eq BSA/g of fresh material)

m 1	0.656	0.077	0.857	0.224	0.965	0.725	1.142	1.397
m 2	0.780	0.093	0.918	0.409	1.196	0.949	1.343	1.227
Means	0.718	0.085	0.888	0.316	1.080	0.837	1.243	1.312
Means±ET	0.718±0.06	0.085±0.01	0.088±0.03	0.316±.0.09	1.080±0.12	0.837±0.11	1.243±0.10	1.312±0.08

- OD SOD activity (OD 80s- OD 20s)

Treatments	C	RS_5	BFL2	RA37	BE1	BFL2BE1	RA37BE1	BFL2RA37

OD1	0.233	0.167	0.032	0.11	0.125	0.125	0.178	0.019
OD2	0.238	0.183	0.018	0.123	0.058	0.224	0.199	0.129

- Activité SOD (nombre d'unités SOD/mg protéines)

Treatments	C	RS_5	BFL2	RA37	BE1	BFL2BE1	RA37BE1	BFL2RA37
Means±ET	1.223±0.11	15.568±1.67	17.904±1.45	18.905±1.22	12.788±0.44	12.066±1.61	10.342±0.89	4.559±1.33

- OD specific activities GPX
- Specific activity GPX (µmol/min/mg de protéines)

Treatments	C	RS_5	BFL2	RA37	BE1	BFL2BE1	RA37BE1	BFL2RA37
Means±ET	7.717±000	0.912±000	9.543±000	3.402±000	11.61±001	9.004±001	13.361±001	14.108±000

- Activité spécifique de la PAL (DO/min/g de protéines)

Treatments	C	RS_5	BFL2	RA37	BE1	BFL2BE1	RA37BE1	BFL2RA37
Means±ET	9.126±0.00	141.078±0.00	116.18±0.00	133.6±0.01	90.041±0.01	77.174±0.01	95.435±0.01	34.024±0.01

Table : Table of dereplication of extract BE1

	Compounds name (LibraryID)	Molecular formular	RTMean_min	number of spectra	parent mass	precursor mass	sum(precursor intensity)
1	N/A	C5H11NO	8,742	2	102	102	42662
2	N/A	C5H11NO	8,745	2	102	102	16226
3	N/A	C5H11NO2	0,447	6	119	119	1299580
4	N/A	C3H3ClO3	5,789	217	123	123	8015400
5	N/A	C8H10O	5,62	178	123	123	3567220
6	N/A	C6H9N3	17,083	4	124	124	58268
7	N/A	C5ClNO2	4,039	5	142	142	100000
8	L-Glutamine		4,363	8	147	147	600000
9	N/A	C10H15N	16,268	15	150	150	200000
10	N/A	C9H16N2	10,779	26	153	153	600000
11	N/A	C9H16N2	14,215	103	153	153	1373330
12	N/A	C9H16N2	12,762	19	153	153	500000
13	N/A	C9H16N2	10,853	2	153	153	46109
14	N/A	C6H4Cl2O3	6,035	267	159	159	4708540
15	N/A	C6H4Cl2O3	6,149	180	159	159	1967940
16	N/A	C6H4Cl2O3	7,307	7	159	159	100000
17	N/A	C9H11NO2	0,526	5	166	166	500000
18	N/A	C9H11NO2	0,518	2	166	166	100000
19	Acamprosate| 3-acetamido-1-propanesulfonic acid	C5H11NO4S	1,968	8	180	180	200000

20	N/A	C12H17NO	8,553	2	192	192	29164
21	cyclo(L-Val-L-Pro)	C10H16N2O2	1,56	13	197	197	300000
22	1 Cyclo(proline-leucine)		4,889	47	211	211	700000
23	N/A	C11H18N2O2	5,143	3	211	211	53209
24	N/A	C11H18N2O2	4,914	2	211	211	21720
25	N/A	C12H12N2O2	1,638	2	217	217	20715
26	N/A	C12H6N4O	11,994	4	223	223	100000
27	N/A	C13H24N2O	8,829	2	225	225	16376
28	N/A	C13H24N2O	10,356	2	225	225	22856
29	N/A	C13H24N2O	11,999	30	225	225	600000
30	N/A	C13H24N2O	13,601	57	225	225	900000
31	N/A	C13H24N2O	9,375	2	225	225	25745
32	N/A	C13H24N2O	11,369	2	225	225	31352
33	N/A	C13H24N2O	12,388	2	225	225	35134
34	N/A	C13H24N2O	14,309	2	225	225	28887
35	6,11-dioxododecanoic acid	C12H20O4	12,256	2	225	225	35761
36	N/A	C13H24N2O	15,519	2	225	225	48120
37	N/A	C13H24N2O	16,91	2	225	225	38862
38	MUCIC ACID	C6H10O8	8,867	9	228	228	200000

39	AZELAIC ACID	C9H16O4	8,891	6	228	228	65146
40	N/A	C18H120	0,575	9	245	245	200000
41	cyclo(L-Phe-D-Pro)		6,735	14	245	245	800000
42	N/A	C10H23NO6/C7H15N11	0,42	2	254	254	57410
43	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-[(4-hydroxyphenyl)methyl]-	C10H12N8O2	1,57	20	261	261	700000
44	N/A	C18H30O2/C14H26N6	10,417	3	279	279	62409
45	(2S,8R)-8-hydroxy-2-[(1S)-1-hydroxyheptyl]-2,3,4,6,7,8-hexahydrochromen-5-one	C16H26O4	9,873	49	281	281	600000
46	N/A	C14H28N6	10,071	54	281	281	1383870
47	N/A	C14H28N6	9,773	6	281	281	100000
48	Linoleic acid		9,3	4	281	281	92604
49	N/A	C14H28N6	10,031	2	281	281	49452

50	N/A	C14H28N6	10,325	2	281	281	47620
51	N/A	C14H28N6	10,392	2	281	281	53830
52	N/A	C14H28N6	10,412	2	281	281	50107
53	N/A	C18H34O2	11,346	10	283	283	200000
54	N/A	C18H30O3	8,677	12	295	295	100000
55	N/A	C18H30O3	8,646	4	295	295	55737
56	N/A	C18H30O3	8,758	2	295	295	30000

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57	N/A	C18H30O3	8,783	2	295	295	28666
58	Phytol,mixture of isomers|3,7,11,15-Tetramethyl-2-hexadecen-1-ol|(E,7R,11R)-3,7,11,15-tetramethylhexadec-2-en-1-ol		12,026	3	297	297	53290
59	N/A	C14H28N6O	10,299	9	297	297	82040
60	N/A	C14H28N6O	10,12	2	297	297	30897
61	N/A	C14H28N6O	10,49	9	297	297	200000
62	N/A	C16H28N6O	10,166	9	321	321	200000
63	N/A	C16H28N6O	10,181	3	321	321	81651

64	N/A	C22H32O2	11,325	7	329	329	200000
65	N/A	C22H43NO	14,027	10	338	338	600000
66	N/A	C22H43NO	14,04	2	338	338	100000
67	Cyclopentasiloxane, decamethyl-		13,243	16	371	371	1152460
68	Cyclopentasiloxane, decamethyl-		13,382	12	371	371	800000
69	1-O-b-D-glucopyranosyl sinapate|1-O-Sinapoyl-beta-D-glucose|1-O-Sinapoyl beta-D-glucoside|[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] (E)-3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-eno		9,24	6	387	387	100000

m

70	Austinoneol		9,788	11	415	415	500000
71	7b,9-Dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-5-oxo-1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-9aH-cyclopropa[3,4]benzo[1,2-e]azulen-9a-yl acetate		9,78	9	432	432	400000
72	N/A	C24H14N8O3	12	10	463	463	900000
73	N/A	C24H14N8O3	12,005	5	463	463	400000
74	N/A	C16H45N17O2	12,691	75	508	508	7000230
75	N/A	C28H44N6O3	12,761	84	513	513	9241810
76	N/A	C28H44N6O3	12,742	43	513	513	2647700

77	N/A	C32H56ClN3	13,24	15	518	518	1583670
78	N/A	C27H43N11	11,466	30	522	522	1348730
79	N/A	C23H43ClN10O2	11,597	21	527	527	1109570

80	N/A	C27H50N2O8	12,649	3	531	531	200000
81	N/A	C27H58ClN5O3	12,818	45	536	536	4091140
82	N/A	C28H44N6O5	11,668	17	545	545	700000
83	N/A	C33H28ClNO5	12,846	12	554	554	3073560
84	N/A	C33H28ClNO5	12,851	6	554	554	1585130
85	N/A	C24H43N7O8	6,972	2	558	558	13983
86	N/A	C24H43N7O8	6,977	3	558	558	21279

87	N/A	C24H43N7O8	6,981	3	558	558	22983
88	N/A	C35H25N2O5	13,903	3	568	568	200000
89	N/A	C36H27N3O5	12,884	9	582	582	500000
90	N/A	C40H26ClN5O	13,568	21	628	628	8882360
91	N/A	C40H32ClNO5	14,517	2	642	642	200000
92	N/A	C42H50ClN5O	13,554	17	656	656	1480180
93	N/A	C34H33Cl2N9O4	14,174	16	702	702	7140600
94	N/A	C34H33Cl2N9O4	14,179	8	702	702	4653830
95	N/A	C41H65N5O5	16,947	3	709	709	100000
96	N/A	C36H30ClN11O4	15,261	2	716	716	100000

Footnote: Derplication here consist of the use of chromatographic and spectroscopic analysis to recognize previously isolated substances present in the BE1 extract. N/A means not available.

Table : Table of dereplication of extract BFL2

	Compounds name (LibraryID)	**Molecular formular**	**RTMean_min**	**number of spectra**	**parent mass**	**precursor mass**	**sum(precursor intensity)**
1	N/A	C5H11NO	8,7329	4	102,1	102,1	59323,2
2	N/A	C5H9NO2	0,4204	4	116,1	116,1	527668,0

3	N/A	C5H11NO2	6,8515	6	118,1	118,1	221907,0
4	N/A	C3H3ClO3	5,4984	226	123,0	123,0	8145860,0
5	N/A	C4ClN3O	5,1304	129	142,0	142,0	3187280,0
6	N/A	C4ClN3O	4,8551	141	142,0	142,0	1858160,0
7	N/A	C4ClN3O	4,4556	40	142,0	142,0	900638,0
8	N/A	C9H14N2O2	0,337	4	147,1	147,1	387425,0
9	N/A	C5H8O5	11,056	2	149,0	149,0	25482,4
10	N/A	C10H15N	15,8983	3	150,1	150,1	49991,7
11	N/A	C9H16N2	9,8478	20	153,1	153,1	245044,0
12	N/A	C9H16N2	9,8689	29	153,1	153,1	659479,0
13	N/A	C9H16N2	12,6039	7	153,1	153,1	330552,0
14	N/A	C9H16N2	14,8537	78	153,1	153,1	1018290,0
15	N/A	C9H16N2	16,3739	2	153,1	153,1	51556,9
16	N/A	C6H3ClO3	5,9148	242	159,0	159,0	4338890,0
17	N/A	C6H3ClO3	5,8982	181	159,0	159,0	2011760,0
18	N/A	C6H3ClO3	6,0597	4	159,0	159,0	79139,9
19	N/A	C6H3ClO3	7,1809	8	159,0	159,0	152817,0
20	N/A	C6H3ClO3	8,1684	3	159,0	159,0	55632,3
21	N/A	C7H5N3O2	6,6496	4	164,1	164,1	59154,6
22	Acamprosate| 3-acetamido-1-propanesulfonic acid		1,9731	4	180,1	180,1	65907,1
23	cyclo(L-Val-L-Pro)		1,577	16	197,1	197,1	393950,0

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24	Cyclo(proline-leucine)		4,6267	18	211,1	211,1	290352,0
25	Cyclo(proline-leucine)		5,0505	29	211,1	211,1	332599,0
26	N/A	C7H14N8	5,0603	2	211,1	211,1	37978,9
27	N/A	C12H6N4O	11,5334	2	223,1	223,1	57328,1
28	N/A	C12H6N4O	11,8602	6	223,1	223,1	165140,0
29	N/A	C12H6N4O	12,046	2	223,1	223,1	57984,5
30	N/A	C13H24N2O	12,0095	51	225,2	225,2	656878,0
31	N/A	C13H24N2O	8,453	2	225,2	225,2	20491,5
32	phenazine-1-carboxylic acid		11,9771	35	225,2	225,2	593360,0
33	N/A	C13H24N2O	13,6246	3	225,2	225,2	63207,7
34	N/A	C13H24N2O	11,7719	2	225,2	225,2	33013,9
35	N/A	C13H24N2O	13,278	2	225,2	225,2	42440,3
36	MUCIC ACID		8,864	10	228,2	228,2	196877,0
37	N/A	C13H25NO2	8,8584	8	228,2	228,2	97075,3
38	N/A	C13H25NO2	8,9574	2	228,2	228,2	40160,7
39	cyclo(L-Phe-D-Pro)		6,7064	19	245,1	245,1	858040,0

40	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-[(4-hydroxyphenyl)methyl]-		1,5891	22	261,1	261,1	734263,0
41	N/A	C16H35NO2	8,5031	2	274,3	274,3	25606,8
42	N/A	C18H32O2	10,1302	24	281,2	281,2	595548,0
43	(2S,8R)-8-hydroxy-2-[(1S)-1-hydroxyheptyl]-2,3,4,6,7,8-hexahydrochromen-5-one		9,9758	48	281,2	281,2	655721,0
44	N/A	C18H32O2	9,9729	27	281,2	281,2	730960,0
45	N/A	C18H32O2	9,9954	2	281,2	281,2	53137,9
46	N/A	C18H32O2	10,5829	2	281,2	281,2	48711,5
47	Glycerol 1-myristate		11,5986	11	283,3	283,3	215773,0
48	N/A	C18H34O2	11,5644	9	283,3	283,3	109154,0
49	N/A	C14H26N6O	8,8443	9	295,2	295,2	97342,7
50	N/A	C14H26N6O	8,7624	5	295,2	295,2	73551,6

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51	Phytol,mixture of isomers|3,7,11,15-Tetramethyl-2-hexadecen-1-ol|(E,7R,11R)-3,7,11,15-tetramethylhexadec-2-en-1-ol		11,8706	6	297,1	297,1	134129,0
52	N/A	C14H28N6O	10,4322	9	297,2	297,2	158959,0
53	N/A	C14H28N6O	10,1891	2	297,2	297,2	33250,8
54	N/A	C14H28N6O	10,3118	2	297,2	297,2	33735,0
55	N/A	C9H22N12	7,7715	7	299,2	299,2	153017,0
56	N/A	C15H32N2O5	10,0816	3	321,2	321,2	74946,9
57	N/A	C15H32N2O5	10,0002	4	321,2	321,2	102545,0

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58	N/A	C15H32N2O5	10,082	6	321,2	321,2	79662,5
59	N/A	C15H32N2O5	10,1142	2	321,2	321,2	55274,4
60	(E)-10-(3,5-dihydroxy-6-methyloxan-2-yl)oxyundec-2-enoic acid		11,3623	8	329,3	329,3	180370,0
61	N/A	C22H41NO	11,8169	5	336,3	336,3	139271,0
62	N/A	C22H43NO	14,027	11	338,3	338,3	671954,0
63	Cyclopentasiloxane, decamethyl		13,2759	30	371,1	371,1	2460340,0
64	Cyclopentasiloxane, decamethyl		13,0537	4	371,1	371,1	348621,0

65	beta-D-Glucopyranoside, 4-hydroxy-2-(hydroxymethyl)phenyl, 6-benzoate		9,2519	8	387,2	387,2	351465,0
66	N/A	C18H25N7O4	9,2455	6	404,2	404,2	273368,0
67	2-(4-hydroxyphenyl)-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2,3-dihydrochromen-4-one		9,7949	5	415,2	415,2	98680,8
68	N/A	C20H29N7O4	9,783	2	432,2	432,2	40640,8
69	N/A	C20H29N7O4	9,1367	2	432,3	432,3	28683,1
70	N/A	C22H23ClN2O7	11,9946	10	463,1	463,1	1355130,0

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71	N/A	C22H23ClN2O7	11,9999	5	463,1	463,1	723653,0
72	N/A	C27H54N6O	11,7614	3	479,4	479,4	99675,2
73	N/A	C30H53NO5	12,5195	57	508,4	508,4	4860390,0
74	N/A	C27H48N2O7	12,6779	43	513,4	513,4	4551770,0
75	N/A	C27H48N2O7	12,6877	37	513,4	513,4	2258910,0
76	N/A	C27H48N2O7	12,7192	29	513,4	513,4	3232930,0

77	N/A	C32H55NO4	13,1894	18	518,4	518,4	2160610,0
78	N/A	C32H55NO4	13,1946	9	518,4	518,4	567768,0
79	N/A	C18H55N5O5	12,3145	23	521,3	521,3	1968690,0

y

80	N/A	C27H47ClN4O4	11,5812	12	527,3	527,3	644160,0
81	N/A	C34H21N3O4	15,5624	32	536,2	536,2	3346060,0
82	N/A	C27H48N2O9	11,6456	18	545,3	545,3	730493,0
83	N/A	C33H28ClNO5	12,8367	12	554,2	554,2	4408040,0
84	N/A	C33H28ClNO5	12,842	6	554,2	554,2	2345500,0
85	N/A	C36H27N3O5	12,8594	12	582,2	582,2	758902,0
86	N/A	C24H18ClN17O2	14,4465	16	612,2	612,2	2890160,0

z

87	N/A	C40H26ClN5O	13,6005	18	628,2	628,2	8031160,0
88	N/A	C40H26ClN5O	13,6057	9	628,2	628,2	4533750,0
89	N/A	C47H29NO3	13,5735	21	656,2	656,2	2295830,0
90	N/A	C39H32ClN5O6	14,1684	16	702,2	702,2	9244740,0
91	N/A	C39H32ClN5O6	14,1736	8	702,2	702,2	5897410,0
92	N/A	C41H65N5O5	15,0721	20	708,5	708,5	1582480,0
93	N/A	C41H30ClN9O2	15,1376	9	716,2	716,2	677128,0

Footnote: Derplication here consist of the use of chromatographic and spectroscopic analysis to recognize previously isolated substances present in the BFL2 extract. N/A means not available.

Table : Table of dereplication of extract BA37

	Compunds names (LibraryID)	**Molecular formular**	**RTMean_min**	**number of spectra**	**parent mass**	**precursor mass**	**sum(precursor intensity)**
1	N/A	C5H11NO	8,72946	3	102	102	63189
2	N/A	C4ClNO	3,34765	7	114	114	176202
3	N/A	C3H3ClO3	5,44266	318	123	123	8415950
4	N/A	C3H3ClO3	6,63278	71	123	123	2710020
5	N/A	C6H9N3	16,97958	2	124	124	17769

aa

6	N/A	C4ClN3O	4,81129	126	142	142	2480800
7	N/A	C4ClN3O	5,21353	129	142	142	1659940
8	N/A	C4ClN3O	4,47632	29	142	142	653770
9	N/A	C4ClN3O	3,62221	4	142	142	86485
10	N/A	C4ClN3O	3,40841	3	142	142	59084
11	N/A	C4ClN3O	6,74662	2	142	142	44033
12	N/A	C4ClN3O	10,11062	4	142	142	103379
13	N/A	C6H2N4O	9,58463	11	147	147	519171
14	N/A	C10H15N	16,39935	18	150	150	294950
15	N/A	C10H15N	16,26068	2	150	150	57710
16	N/A	C9H16N2	9,96945	32	153	153	758337
17	N/A	C9H16N2	10,87027	36	153	153	434158
18	N/A	C9H16N2	9,95658	2	153	153	57832
19	N/A	C9H16N2	10,8524	7	153	153	183879
20	N/A	C9H16N2	15,20162	3	153	153	85607
21	N/A	C9H16N2	15,39359	80	153	153	1149800
22	N/A	C9H16N2	14,97713	13	153	153	396091
23	N/A	C9H16N2	15,38644	3	153	153	85028
24	N/A	C6H3ClO3	5,97657	270	159	159	4569710
25	N/A	C6H3ClO3	5,68069	201	159	159	2087790

bb

26	N/A	C6H3ClO3	7,31462	3	159	159	56380
27	N/A	C6H3ClO3	5,46413	3	159	159	77266
28	N/A	C8H12N2O2	0,54755	2	166	166	84303
29	N/A	C8H12N2O2	0,55103	2	166	166	55021
30	N/A	C9H12N2O2	0,57584	2	181	181	31219
31	cyclo(L-Val-L-Pro)		1,61896	11	197	197	179274
32	Cyclo(leucylprolyl)		5,13053	10	211	211	103361
33	N/A	C7H14N8O	5,09946	12	211	211	85057
34	N/A	C10H18N2O3	0,48882	3	215	215	122237
35	N/A	C12H6N4O	12,01113	2	223	223	48479
36	N/A	C12H6N4O	12,0181	3	223	223	67061
37	N/A	C13H24N2O	12,99785	69	225	225	929089
38	N/A	C13H24N2O	12,23538	3	225	225	58329
39	N/A	C13H24N2O	12,93642	47	225	225	876911
40	N/A	C13H24N2O	10,63112	2	225	225	31607
41	N/A	C13H24N2O	13,8572	2	225	225	32707
42	N/A	C13H24N2O	11,5037	2	225	225	32612

cc

43	N/A	C13H24N2O	13,85372	2	225	225	41393
44	N/A	C13H24N2O	11,39143	2	225	225	36645
45	N/A	C13H24N2O	11,63448	4	225	225	68011
46	N/A	C13H24N2O	13,82689	5	225	225	105260
47	N/A	C13H24N2O	16,52053	2	225	225	27218
48	MUCIC ACID	C13H25NO2	8,88259	10	228	228	219142
49	N/A	C13H25NO2	8,88851	5	228	228	57550
50	N/A	C13H12N2O3	0,53842	3	245	245	78416
51	cyclo(L-Phe-D-Pro)		6,72764	11	245	245	298451
52	N/A	C10H23NO6	0,41918	2	254	254	56486

dd

53	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-[(4-hydroxyphenyl)methyl]	C10H12N8O	1,582	17	261	261	299744
54	N/A	C24H26N6	10,51901	9	279	279	171945
55	(2S,8R)-8-hydroxy-2-[(1S)-1-hydroxyheptyl]-2,3,4,6,7,8-hexahydrochromen-5-one	C14H28N6	9,9855	47	281	281	651166
56	N/A	C14H28N6	9,78947	2	281	281	42628
57	N/A	C14H28N6	10,00614	41	281	281	1082240
58	N/A	C14H28N6	9,94742	3	281	281	70818
59	N/A	C14H28N6	10,2895	5	281	281	122365

ee

60	N/A	C14H28N6	10,2954	14	281	281	351409
61	3-Hydroxyoctadecanoic Acid	C14H30N6	11,12283	7	283	283	144037
62	N/A	C14H30N6	11,2457	12	283	283	175098
63	14-(hydroxymethyl)-5,9-dimethyltetracyclo[11.2.hexadecan-5-ol	C14H26N6O	8,67591	8	295	295	73986
64	N/A	C14H26N6O	8,68712	2	295	295	27455
65	N/A	C14H26N6O	8,64969	2	295	295	27695
66	N/A	C14H26N6O	8,95953	2	295	295	33953

ff

67	N/A	C14H28N6O	10,86808	7	297	297	125964
68	N/A	C14H28N6O	10,48709	2	297	297	34138
69	N/A	C14H28N6O	10,29039	9	297	297	82616
70	N/A	C15H32N2O5	10,06058	13	321	321	339762
71	N/A	C15H32N2O5	10,07339	7	321	321	96625
72	(E)-10-(3,5-dihydroxy-6-methyloxan-2-yl)oxyundec-2-enoic acid	C29H32O2	11,1129	4	329	329	93269

gg

73	N/A	C29H32O2	10,92317	2	329	329	34408
74	N/A	C23H43NO	14,03289	11	338	338	655702
75	N/A	C16H19ClN2O6	13,30826	3	371	371	151990
76	Cyclopentasiloxane, decamethyl	C16H19ClN2O6	13,32347	21	371	371	1236990
77	beta-D-Glucopyranoside, 4-hydroxy-2-(hydroxymethyl)phenyl, 6-benzoate		9,22326	9	387	387	377863
78	N/A	C19H30N2O8	9,79466	2	415	415	35206
79	2-(4-hydroxyphenyl)-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2,3-dihydrochromen-4-one	C19H30N2O8	9,79988	4	415	415	76399
80	7b,9-Dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-5-oxo-1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-9aH-cyclopropa[3,4]benzo[1,2-e]azulen-9a-yl acetate	C19H33N3O8	9,78902	3	432	432	53920
81	N/A	C23H19ClN6O3	11,97934	8	463	463	631160
82	N/A	C23H19ClN6O3	11,98457	4	463	463	313825

hh

83	N/A	C30H53NO5	12,62331	72	508	508	6685850
84	N/A	C28H44N6O3	12,69798	62	513	513	6812620
85	N/A	C28H44N6O3	12,78194	50	513	513	3174730
86	N/A	C27H48N2O7	13,00498	27	513	513	3302090
87	N/A	C27H48N2O7	13,79972	3	513	513	324173
88	N/A	C27H48N2O7	13,20635	24	518	518	2416530
89	N/A	C27H43N11	11,54995	39	522	522	1898760
90	N/A	C23H43ClN10O2	11,71344	15	527	527	884988
91	N/A	C32H57NO5	12,96055	32	536	536	3281740
92	N/A	C32H57NO5	12,91136	6	536	536	538291
93	N/A	C28H44N6O5	11,52856	19	545	545	834030
94	N/A	C28H44N6O5	11,49017	11	545	545	303845
95	N/A	C27H48N2O9	12,8235	10	554	554	2171690
96	N/A	C27H48N2O9	12,82873	5	554	554	1123640
97	N/A	C40H26ClN5O	13,5703	21	628	628	6082980
98	N/A	C40H26ClN5O	13,55818	12	656	656	903184
99	N/A	C35H28ClN11O4	14,17701	24	702	702	7938700

Footnote: Derplication here consist of the use of chromatographic and spectroscopic analysis to recognize previously isolated substances present in the RA37 extract. N/A means not available.