THE UNIVERSITY OF YAOUNDE I

CENTRE FOR RESEARCH AND TRAINING IN GRADUATE STUDIES IN LIFE, HEALTH AND ENVIRONMENTAL SCIENCES
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RESEARCH AND DOCTORATE TRAINING UNIT IN LIFE SCIENCES

DEPARTMENT OF BIOCHEMISTRY

UNIVERSITE DE YAOUNDE I

CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE, SANTE ET ENVIRONNEMENT
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UNITE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE

DEPATEMENT DE BIOCHIMIE

DEPARTMENT OF BIOCHEMISTRY
DEPARTEMENT DE BIOCHIMIE

LABORATORY FOR PHYTOBIOCHEMISTRY AND MEDICINAL PLANTS STUDIES
LABORATOIRE DE PHYTOBIOCHIMIE ET D'ETUDE DES PLANTES MEDICINALES

ANTIMICROBIAL AND BIOCONTROL AGENTS UNIT
UNITE DES AGENTS ANTIMICROBIEN ET DE BIOCONTROLE

DESERT SPURGE-DERIVED ENDOPHYTIC BACTERIA INDUCE SYSTEMIC RESISTANCE AGAINST FUSARIUM ROT IN MAIZE (*Zea mays* L.) UNDER SEVERE DROUGHT CONDITIONS

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

BY

NYA DINANGO VANESSA
Registration N° 11R0721
M.sc in Biochemistry

Co-supervised by:

Pr. NANA WAKAM Louise
Associate Professor,
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Academic year : 2023-2024

THE UNIVERSITY OF YAOUNDE I

CENTRE FOR RESEARCH AND TRAINING IN GRADUATE STUDIES IN LIFE, HEALTH AND ENVIRONMENTAL SCIENCES
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ANTIMICROBIAL AND BIOCONTROL AGENTS UNIT
UNITE DES AGENTS ANTIMICROBIEN ET DE CONTRÔLE BIOLOGIQUE

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DEPARTMENT OF BIOCHEMISTRY

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 : « **Desert spurge-derived endophytic bacteria induce systemic resistance against *Fusarium* rot in maize (*Zea mays* L.) under severe drought conditions**» defended on friday, 21th of June 2024 at 9 :00 a.m in the Multimedia room of the faculty of Sciences by **Mrs. NYA DINANGO Vanessa (registration number 11R721)**, 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 .....

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FEKAM BOYOM Fabrice	NGAKOU Albert	MOUNDIPA FEWOU Paul	MOUNDIPA FEWOU Paul
NANA WAKAM Louise	EWANE Cécile	Professeur Enzymologie - Toxicologie	
	NGUEFACK Julienne		

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ACADEMIC YEAR 2023/2024

(by Department and by Grade)

LAST UPDATE 04 June 2024

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22.	MEGNEKOU Rosette	Associate Professor	On duty
23.	MOUNGANG Luciane Marlyse	Associate Professor	On duty
24.	MONY Ruth épse NTONE	Associate Professor	On duty
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28.	NOAH EWOTI Olive Vivien	Associate Professor	On duty
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36.	GONWOUO NONO Legrand	Senior Lecturer	On duty
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39.	MAPON NSANGOU Indou	Senior Lecturer	On duty
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44.	TADU Zephyrin	Senior Lecturer	On duty
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46.	YOUNOUSSA LAME	Senior Lecturer	On duty

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48	NDENGUE Jean De Matha	Assistant	On duty
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10	MALA Armand William	Associate Professor	On duty
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13	NGONKEU MAGAPTCHE Eddy L.	Associate Professor	***CT / MINRESI***
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16	ONANA JEAN MICHEL	Associate Professor	On duty

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24.	GODSWILL NTSOMBOH NTSEFONG	Senior Lecturer	On duty
25.	KABELONG BANAHO Louis-Paul-Roger	Senior Lecturer	On duty
26.	KONO Léon Dieudonné	Senior Lecturer	On duty
27.	LIBALAH Moses BAKONCK	Senior Lecturer	On duty
28.	LIKENG-LI-NGUE Benoit C	Senior Lecturer	On duty

29.	TAEDOUNG Evariste Hermann	Senior Lecturer	On duty
30.	TEMEGNE NONO Carine	Senior Lecturer	On duty
31.	DIDA LONTSI Sylvere Landry	Assistant	On duty
32.	METSEBING Blondo-Pascal	Assistant	On duty

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5.	NGOMO Horace MANGA	Professor	*Vice Chancelor/UB*
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7.	NJOYA Dayirou	Professor	On duty

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10.	KAMGANG YOUBI Georges	Associate Professor	On duty
11.	KEMMEGNE MBOUGUEM Jean C.	Associate Professor	On duty
12.	KENNE DEDZO GUSTAVE	Associate Professor	On duty
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3.	NGOUELA Silvère Augustin	Professor	***Head of Department UDS***
4.	PEGNYEMB Dieudonné Emmanuel	Professor	***Rector UBe/ Head of Department***
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8.	EYONG Kenneth OBEN	Associate Professor	On duty
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19.	TAGATSING FOTSING Maurice	Associate Professor	On duty
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25.	NGOMO Orléans	Senior Lecturer	On duty
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30.	TSAFFACK Maurice	Senior Lecturer	On duty
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22.	NKONDOCK. MI. BAHANACK.N.	Assistant	On duty

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15.	DOUANLA YONTA Herman	Senior Lecturer	On duty
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17.	LOUMNGAM KAMGA Victor	Senior Lecturer	On duty
18.	MBAKOP Guy Merlin	Senior Lecturer	On duty
19.	MBATAKOU Salomon Joseph	Senior Lecturer	On duty
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30.	GUIDZAVAI KOUCHERE Albert	Assistant	On duty
31.	MANN MANYOMBE Martin Luther	Assistant	On duty
32.	MEFENZA NOUNTU Thiery	Assistant	On duty
33.	NYOUMBI DLEUNA Christelle	Assistant	On duty

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22.	MAYI Marie Paule Audrey	Assistant	On duty
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7.	HONA Jacques	Professor	On duty
8.	NANA ENGO Serge Guy	Professor	On duty
9.	NANA NBENDJO Blaise	Professor	On duty
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12.	NOUAYOU Robert	Professor	On duty
13.	SAIDOU	Professor	***Chief of Center IRGM/MINRESI***
14.	SIMO Elie	Professor	On duty
15.	TABOD Charles TABOD	Professor	***Dean FS UBa***
16.	TCHAWOUA Clément	Professor	On duty
17.	WOAFO Paul	Professor	On duty
18.	ZEKENG Serge Sylvain	Professor	On duty
19.	ENYEGUE A NYAM épse BELINGA	Associate Professor	***Head of continuing and distance learning Division /ENSPY***
20.	FEWO Serge Ibraïd	Associate Professor	On duty
21.	FOUEJIO David	Associate Professor	***Head Unit MINADER***
22.	MBINACK Clément	Associate Professor	On duty
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24.	MELI'I Joelle Larissa	Associate Professor	On duty
25.	MVOGO ALAIN	Associate Professor	On duty
26.	NDOP Joseph	Associate Professor	On duty
27.	SIEWE SIEWE Martin	Associate Professor	On duty
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17.	MBESSE Cécile Olive	Associate Professor	On duty
18.	METANG Victor	Associate Professor	On duty
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21.	NGUEUTCHOUA Gabriel	Associate Professor	***CEA/MINRESI***
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23.	NYECK Bruno	Associate Professor	On duty
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30.	ESSONO Jean	Senior Lecturer	On duty

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32.	MINYEM Dieudonné	Senior Lecturer	***Chief division/UMa***
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34.	NGO'O ZE ARNAUD	Senior Lecturer	On duty
35.	NOMO NEGUE Emmanuel	Senior Lecturer	On duty
36.	NTSAMA ATANGANA Jacqueline	Senior Lecturer	On duty
37.	TCHAPTCHET TCHATO De P.	Senior Lecturer	On duty
38.	TEHNA Nathanaël	Senior Lecturer	On duty
39.	FEUMBA Roger	Senior Lecturer	On duty
40.	MBANGA NYOBE Jules	Senior Lecturer	On duty

41.	KOAH NA LEBOGO Serge Parfait	Assistant	On duty
42.	TENE DJOUKAM Joëlle Flore, épouse KOUANKAP NONO	Assistant	On duty

Distribution of permanent lecturers in the faculty of science according to departments

NUMBER OF LECTURERS

Department	**Professors**	**Associate Professors**	**Senior Lecturers**	**Assist. Lecturers**	**Total**
BCH	8 (01)	20 (12)	9 (04)	6 (05)	**43 (22)**
BPA	11 (01)	19 (09)	16 (05)	3 (02)	**49 (17)**
BPV	6 (01)	10 (02)	14 (08)	2 (00)	**32 (11)**
CI	7 (01)	14 (04)	5 (01)	1 (00)	**27 (06)**
CO	7 (01)	15 (05)	11 (05)	1 (00)	**33 (11)**
RE	1(00)	/	/	/	**1(0)**
IN	2 (00)	2 (00)	14 (01)	4 (00)	**22 (01)**
MAT	1 (00)	8 (00)	19 (02)	5 (01)	**33 (03)**
MIB	3 (01)	7 (03)	9 (05)	5 (02)	**24 (11)**
PHY	18 (01)	12 (04)	11 (01)	1 (00)	**42 (06)**
ST	10 (00)	17 (03)	13 (03)	3 (01)	**43 (07)**
Total	**74 (07)**	**124 (42)**	**121 (35)**	**31 (11)**	**350 (95)**

A total of……**349 (95)**, with :

Professors……**73 (07)**

Associate Professors……..**124 (42)**

Senior Lecturers……..**121 (35)**

Assistant Lecturers……..**31 (11)**

( ) = Number of women…….**95**

DEDICATION

I dedicate this thesis to my family particularly to my lovely mother: NYABEYE CECILE and my late father NYA GERAF

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ACC deaminase	1-aminocyclopropane-1-carboxylic acid deaminase
APX	Ascorbate peroxidase
BCAs	Biocontrol agents
BLAST	Basic local alignment search tool
CAT	Catalase
CLA	Carnation leaves agar
CMC	Carboxymethyl cellulose
DAS	Days after sowing
DNA	Desoxyribonucleic acid
DON	Deoxynivalenol
DW	Dry weight
EA	Enzyme activity
EL	Electrolyte leakage
EPS	Exopolysaccharide
FAOSTAT	Food and Agriculture Organization of the United Nations
FFSC	Fujikuroi species complex
FW	Fresh weight
FV	*Fusarium verticillioides*
GPX	Guaiacol peroxidase
H2O2	Hydrogen peroxide
IAA	Indole acetic acid
IRAD	Agricultural research institute for development
ISR	Induce systemic resistance
ITS	Internal transcribed spacer
JA	Jasmonic acid
LA	Leaf area
LB	Lurea Bertani
MPa	Mega pascal
NCBI	National center for biotechnology information
OD	Optical density
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
PGP	Plant growth promotion
POD	Peroxidase
RWC	Relative water content
ROS	Reactive oxygen species
RDS	Reduction of the disease severity

ABSTRACT

Fungal-plant diseases pose a significant challenge to global agriculture, with *Fusarium verticillioides* being particularly harmful to maize cultivation. The increased frequency of drought events worldwide further exacerbates maize vulnerability to these harmful fungi, leading to substantial yield losses. Current strategies often address pathogenic infections or drought stress individually but are frequently ineffective. The aim of this study was to assess the ability of endophytic bacteria from the desert plant (*Euphorbia antiquorum*) to enhance maize tolerance against *Fusarium* rot, drought stress, and their combined effects. *Fusarium verticillioides* was isolated from symptomatic maize tissues and identified through molecular characterization using internal transcribed spacer (ITS) and translation elongation factor (TEF-1α) sequences. Subsequently, the antagonistic potential of 25 endophytic bacteria isolated from *Euphorbia antiquorum* L. was assessed against the pathogenic fungus through various *in vitro* and *in planta* assays. Notable reductions in *Fusarium verticillioides* mycelial growth were observed, with a decrease of 68.59% and 71.7% in direct and indirect confrontations, respectively. *In vitro* assays showed that all tested bacteria were able to solubilize phosphate and produce extracellular enzymes and plant growth factors such as indole acetic acid (IAA), ammonia, and siderophores. These activities enabled the identification of ten potent bacterial strains that significantly reduced the severity of *Fusarium* ear and root rot disease in maize plants. Furthermore, the ten bacteria as biological agents demonstrated resilience to drought conditions induced by varying concentrations of polyethylene glycol (PEG). Four bacterial strains showed tolerance to high-stress levels (-1.76 MPa), which was linked to their ability to produce biofilms under these conditions. Additionally, the strains exhibited resistance to salt stress, while four of them (*C. indologenes* LPR17, *B. velezensis* BE1, *B. amyloliquefaciens* BFL1, and *S. maltophilia* LPR6+) were shown to carry the ACC deaminase gene, which plays a crucial role in stress mitigation (AcdS gene). Significant increases in shoot and root lengths, as well as biomass, were observed in drought-stressed maize after bacterial inoculation, particularly with two strains, unidentified LPR1+ and *B. megaterium* RR13. These strains were the most promising for inducing drought tolerance, showing significant improvements in various physiological and biochemical parameters. They notably increased relative water content (RWC) by up to 63.3%, leaf area by 127.2%, chlorophyll a and total chlorophyll contents by 116.7% and 121.1%, respectively. Furthermore, they reduced the antioxidant guaiacol peroxidase (27.8%) and catalase (54.3%) activities, while total phenols and flavonoids increased by 106.9% and 74.2% respectively. The two highly effective bacterial agents (unidentified LPR1+ and *B. megaterium* RR13), showed remarkable efficacy when plants were subjected to combined *Fusarium* rot and drought conditions. The performance of the unidentified bacterial strain LPR1+ was particularly noteworthy, with an increase in root length by up to 64.7%, leaf area by 84.4%, relative water content by 9.6%, and a significant decrease in proline content. These findings highlight the need for further investigations to develop a potential biopesticide based on this unidentified bacterial agent to address the urgent challenge of combined stresses impacting maize cultivation.

Keywords: Maize, *Fusarium verticillioides*, drought stress, combined stress, endophytic bacteria.

RESUME

Les maladies fongiques des plantes posent un défi important à l'agriculture mondiale, avec *Fusarium verticillioides* particulièrement nuisible à la culture du maïs. La fréquence accrue des événements de sécheresse dans le monde exacerbe encore la vulnérabilité du maïs à ces champignons nuisibles, entraînant des pertes de rendement substantielles. Les stratégies actuelles traitent souvent les infections pathogènes ou le stress dû à la sécheresse individuellement, mais sont fréquemment inefficaces. L'objectif de cette étude était d'évaluer la capacité des bactéries endophytes provenant de la plante désertique *Euphorbia antiquorum*, à améliorer la tolérance des plants de maïs contre la pourriture fusarienne, la sècheresse ainsi que ces stress combinés. *Fusarium verticillioides* a été isolé à partir de tissus de maïs symptomatiques et identifié par caractérisation moléculaire utilisant les séquences des espaces transcrits internes (internal transcribed spacer- ITS) et du facteur d'élongation de traduction (TEF-1α). Par la suite, le potentiel antagoniste de 25 bactéries endophytes obtenues de *Euphorbia antiquorum* L. a été évalué contre le champignon pathogène à travers diverses analyses *in vitro* et *in planta*. Des réductions notables de la croissance mycélienne de *Fusarium verticillioides* ont été observées, avec des diminutions de 68,59% et 71,7% lors des confrontations directes et indirectes, respectivement. Les analyses *in vitro* ont montré que toutes les bactéries testées produisaient des enzymes extracellulaires et des facteurs de croissance des plantes tels que l'acide indole acétique (AIA), l'ammoniac, les sidérophores et le phosphate solubilisé. Ces activités ont conduit à l'identification de dix souches bactériennes puissantes qui ont considérablement réduit la gravité de la pourriture des épis et des racines du maïs causée par *Fusarium* . En outre, les dix agents biologiques ont démontré une résilience aux conditions de sécheresse induites par des concentrations variables de polyéthylène glycol (PEG). Quatre souches bactériennes ont montré une tolérance à des niveaux de stress élevés (-1,76 MPa), liée à leur capacité à produire des biofilms dans ces conditions. De plus, les souches ont montré une résistance au stress salin, et quatre d'entre elles (*C. indologenes* LPR17, *B. velezensis* BE1, *B. amyloliquefaciens* BFL1 et *S. maltophilia* LPR6+) portaient le gène de l'ACC déaminase, jouant un rôle crucial dans l'atténuation du stress (gène AcdS). Des augmentations significatives de la longueur des pousses et des racines, ainsi que de la biomasse, ont été observées chez le maïs soumis au stress hydrique après inoculation bactérienne, en particulier avec les souches Unidentified LPR1+ et *B. megaterium* RR13. Ces souches étaient les plus prometteuses pour induire la tolérance à la sécheresse, montrant des améliorations significatives de divers paramètres physiologiques et biochimiques. Notamment, elles ont augmenté la teneur relative en eau (RWC) jusqu'à 63,3%, la surface foliaire de 127,2%, les contenus en chlorophylle a et en chlorophylle totale de 116,7% et 121,1%, respectivement. De plus, elles ont réduit l'activité des enzymes antioxydantes, y compris la gaïacol peroxydase de 27,8% et la catalase de 54,3%, tout en augmentant les phénols totaux de 106,9% et les flavonoïdes de 74,2%. Les agents bactériens très efficaces, unidentified LPR1+ et *B. megaterium* RR13, ont montré une efficacité remarquable lorsque les plantes étaient soumises aux conditions combinées de pourriture par *Fusarium* et de sécheresse. Ces souches ont considérablement amélioré la croissance du maïs et divers paramètres physiologiques et biochimiques. La performance de la souche bactérienne unidentified LPR1+ était particulièrement remarquable, avec des augmentations de la longueur des racines jusqu'à 64,7%, de la surface foliaire de 84,4%, de la teneur relatice en eau de 9,6% et une diminution significative du contenu en proline. Ces résultats soulignent la nécessité de poursuivre les recherches pour développer un biopesticide potentiel utilisant cet agent bactérien afin de relever le défi urgent des stress combinés affectant la culture du maïs.

Mots clés: Maïs, *Fusarium verticillioides*, sècheresse, stress combinés, bactéries endophytes.

INTRODUCTION

INTRODUCTION

Food insecurity is a large human burden and is particularly exacerbated by the onset of climate change and its associated extreme weather events. Hence, forecasts predict a significant decline in the production of vital agricultural commodities such as rice, wheat, soybeans, and maize by 2050, potentially leading to a 20% increase in malnourished children around the globe. However, cereal crops including maize, are vital for human nutrition owing to their widespread distribution and high content of essential macro- and micronutrients. This significance is especially pronounced in developing nations and areas experiencing crises worldwide **(Ignaciuk and Mason-D'Croz, 2014; FAOStat, 2021)**.

Maize (*Zea mays* L.) is the most extensively cultivated and consumed grain globally **(Shahbandeh, 2022)**. Maize cultivation spans approximately 33 million ha, accounting for approximately 17% of the anticipated 200 million ha of cultivated land in Sub-Saharan Africa **(Shi and Tao, 2014)**. Maize is a versatile crop with a wide array of uses involving over 600 derived products used in human diets, animal nutrition for livestock and poultry, and even the energy sector **(Ntsama and Kamgnia, 2019)**. Although major producers such as the United States, China, Brazil, and Argentina dominate global maize production, numerous countries achieve significant yields annually **(Erenstein *et al.*, 2022)**. Cameroon, for instance, ranks 44th in the world and is Africa's 13th largest maize producer, contributing 0.18% to global production **(FAO *et al.*, 2022)**. Maize is the most affordable crop in Cameroon in terms of market price and seed cost **(Epule and Bryant, 2014)**. The annual sale of grain generates approximately 25 billion XAF, serving as a key source of income and employment for more than three million small rural farmers across the world **(Ntsama and Kamgnia, 2008; Mbah *et al.*, 2023)**.

According to the Food and Agricultural Organization (FAO), Cameroon produced 2.2 million tons of dry corn in 2022 and 2023, decreasing to 600,000 tons to meet the country's annual demand of 2.8 million tons **(USDA, 2023)**. In fact, between 2018 and 2020, a decline in yield of 5.27% was recorded, leading to an import value of 3.305 thousand USD (1.65 billion FCFA) in 2019 **(World Data Atlas, 2021)**. Furthermore, despite the global goal of achieving 2.4% yield improvement each year, estimations predict a 7% yield reduction per year owing to biotic and abiotic stressors, representing challenges in meeting production targets **(Ray *et al.*, 2013; Admin, 2022)**.

With the advent of climate change, the prevalence of compounded abiotic and biotic stressors is increasing, thereby amplifying the individual anticipated effects of each stressor. This situation tends to enhance the lone expected impact of each stressor **(Pandey *et al.*, 2017b)**. Indeed, high salinity levels, low or high temperatures, and drought are the key abiotic stressors currently impeding maize productivity as a result of climate change **(NkamLeu, 2004; Takam, 2017)**. Drought, in particular, emerges as the leading cause of crop failure and associated yield loss in all climate change scenarios. Moreover, drought damage to agricultural systems has been increasing as global temperatures continue to rise **(Li *et al.*, 2020)**.

Climate change models estimate that average maize yields are expected to decline between 5% and 33% by 2050, depending on the magnitude of the change, with the most severe repercussions anticipated in the least developed nations **(Maize, 2016)**. Farmers who opt not to utilize drought-tolerant (DT) maize cultivars could face a reduction in maize production yield of approximately 13.3%, resulting in a 12.9% increase in impoverished farmers and an 84.0% food shortage **(Wossen *et al.*, 2017)**. For instance, considerable greater damage was recorded between 2012 and 2015 in Cameroon, when maize seedlings experienced extremely high mortality **(Epule *et al.*, 2021)**. In addition to its direct effects on yield, drought stress has been shown to enhance plant susceptibility to phytopathogenic microbes. Such an imbalance in the disease triangle is expected to produce unforeseen losses, notably when pathogens such as *Pythium*, *Verticillium*, *Macrophomina*, and *Fusarium* spp. are involved **(Pandey *et al.*, 2017b)**.

Indeed, *Fusarium* is a cosmopolitan fungal genus that is renowned for inducing seedling failure and ear, root, and stalk rot, impeding development and grain output at harvest **(Munkvold and Desjardins, 1997)**. *Fusarium* species, including *F. graminearum*, *F. subglutinans*, *F. proliferatum*, and *F. verticillioides*, have been pinpointed as major causative agents of these diseases **(Munkvold, 2003)**. Among the former, *F. verticillioides* has strong specificity for maize **(Bankole and Mabekoje, 2004; Schjøth *et al.*, 2008; Ncube *et al.*, 2018)**. The fungus thrives in hot and dry seasons, exploiting the crop's weakened defense system **(Hernández-Rodríguez *et al.*, 2008; Pfordt *et al.*, 2020)**. *F. verticillioides* gains entry into maize via wounds caused by insects or through seeds and silks **(Pfordt *et al.*, 2020)**. Disease propagules are then transmitted from seeds to kernels in four major steps which are: (i) from seed to seedling, (ii) colonizing the stalk, (iii) moving into the ear, and (iv) by spreading within the ear, resulting in high economic costs of up to 50% grain yield and contaminated seeds **(Munkvold and**

Carlton, 1997; Munkvold, 2003; Horst, 2013; Tagne *et al.*, 2021). In the main maize production hotspots of Cameroon, *F. verticillioides* causes up to 70% yield losses in severe cases **(Ngoko *et al.*, 2001; Tagne *et al.*, 2021)**. Furthermore, the disease is frequently found wherever the crop is grown and it is often linked to the production of fumonisin, a highly toxic mycotoxin known for its multiple hazardous effects on human health, including carcinogenicity and multiple organ failure. Hence, fumonisin has been categorized by the International Agency for Research on Cancer as a "Group 2B carcinogen" **(Eskola *et al.*, 2020)**.

Generally, several methods have been used to address drought and *Fusarium* stressors separately. Drought-resilient breeding programs and irrigation are two strategies employed to mitigate drought, although they encounter various technical and economic challenges **(Blum, 2011; Nuccio *et al.*, 2018)**. Furthermore, conventional chemical fungicides and fumigants are commonly used to mitigate the lethal effects of *F. verticillioides* on maize crops **(Capo *et al.*, 2020)**. Chemical compounds such as Apron Star, Benlate/benomyl, and carbofuran have consistently been provided to Cameroonian farmers to manage outbreaks of *Fusarium* rot disease **(Tagne *et al.*, 2013)**. Unfortunately, the adverse effects of these chemicals have been extensively documented (**Latimer and Close, 2019)**. Moreover, the drawbacks associated with agrochemicals are the need for a steady reorientation toward innovative ecological approaches **(Eke *et al.*, 2016 and 2019)**. In this regard, biocontrol agents (BCAs) are gaining acceptance as eco-friendly and cost-effective biochemical fungicides against a wide array of agriculturally destructive phytopathogens **(Medeiros *et al.*, 2012; Panth *et al.*, 2020)**. *Bacillus* sp., *Enterobacter* sp., and *Pseudomonas* sp., for instance, have all demonstrated biocontrol effects on *F. verticillioides* **(de Fátima *et al.*, 2021; Kara and Soylu, 2022)**. However, in the climate change era, these measures tend to fail due to the concurrent occurrence of diseases and drought stress, compelling plants to develop unique physiological responses that require unique control strategies **(Eke *et al.*, 2023a)**. Endophytic organisms sourced from specific environments, such as deserts, have recently been investigated to help solve this issue **(Alsharif *et al.*, 2020)**. Studies have demonstrated that natural occurring endophytes from deserts can support plant survival in harsh ecosystems, due to their ability to improve host nutrient uptake and increase resilience to biotic and abiotic stresses **(Lundberg *et al.*, 2012)**. Although little is known about desert plant endophytes, research suggests that, given the environmental difficulties facing plant production, they may provide a more ecologically friendly option than the existing traditional methods **(Zhang and James, 2021)**. **Eke *et al.*, (2019)** and **Zahra *et al.*, (2020)** demonstrated

the ability of desert endophytes to trigger drought tolerance in tomatoes and sunflowers, respectively. Moreover, substantial outcomes depict the biocontrol effects of desert endophytes on bacterial wilt **(Youmbi *et al.*, 2022)**, root rot in common beans **(Eke *et al.*, 2023b)**, and postharvest decay of groundnuts (Wandji, 2022, personal communication). Nevertheless, there is still a lack of understanding regarding the potential of these endophytes to induce cross-tolerance against *Fusarium* rot, and drought stress.

Hypothesis

Given the aforementioned, we hypothesized the following:

Endophytic bacteria from the desert spurge *Euphorbia antiquorum* protect maize seedlings from both *Fusarium* root rot and drought stress.

Objectives

General objective

This study aimed at investigating the mitigating effects of *Euphorbia antiquorum*-derived endophytic bacteria on the individual and combined effects of *F. verticillioides* root rot and severe drought in maize (*Zea mays* L.) seedlings.

Specific objectives

Specifically, we intended to:

1. Evaluate the antifungal potential of some desert-spurge (*E. antiquorum*)-derived endophytic bacteria against *F. verticillioides*.

2. Determine the ability of the antagonistic candidates to induce drought tolerance in maize seedlings under greenhouse conditions.

3. Assess the mitigating effects of drought-resilient and antagonistic candidates on the co-occurrence of *F. verticillioides* root rot and severe drought.

CHAPTER I:

LITTERATURE REVIEW

CHAPTER I: LITTERATURE REVIEW

I.1. Host plant: maize (*Zea mays* L.)

I.1.1. Origin and diffusion of maize

Many speculations have been proposed about the origin of maize, and three hypotheses have been proposed. First, (1) maize comes from pol corn, which differs from normal maize in that the seeds are enclosed in glumes. Second (2), maize originated from teosinte, its closest relative, by direct selection, large-scale mutations, or hybridization of teosinte with another grass, and third (3), maize, teosinte and Tripsacum, the more distantly related genus descended along independent lines from a remote common ancestor **(Montgomery, 1906; Collins, 1912; Weatherwax, 1935)**. Therefore, the second hypothesis was the most accepted, and since teosinte was found in Mexico and Guatemala, it has been assumed that maize must have had its origins there. Maize was first domesticated 7000 to 10,000 years ago in south-central or southwestern Mexico and spread fairly quickly throughout the Americas, reaching northeastern U.S./southeastern Canada just before European colonization (Figure 1). Teosinte is constantly hybridized with maize, and the hybrids are backcrossed to both maize and teosinte, leading to germplasm exchange and the appearance of new cultivars **(Goodman and Galinat, 1988)**.

Maize was the food base that allowed the development of several important civilizations over the centuries, such as the Incas in Peru and a wide region located in the Andes, the Aztecs in Mexico, and the Maya in Central America and southern Mexico. Navigators are responsible for maize seed transfer to Europe, Asia, and Africa, and today, on the African continent, cereal is the most cultivated crop with the highest total grain production **(Prasanna, 2012; FAOSTAT, 2018)**.

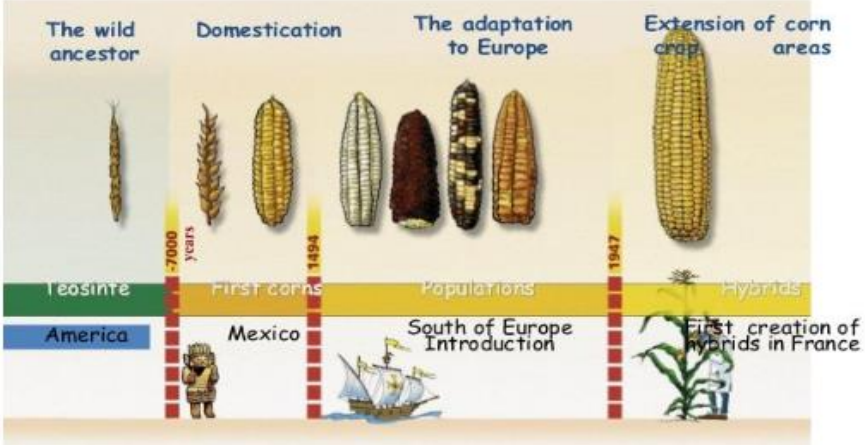

Figure 1: Illustration of the evolution of maize **(Madec, 2019)**.

I.1.2. Taxonomy

Maize or corn is a robust monoecious annual plant that belongs to the subkingdom Tracheobionta, division Magnoliophyta, class Liliopsida, and is a member of the Maydeae tribe of the grass family Poaceae. The tribe Andropogoneae includes seven genera, which are grouped into old and new taxa. The old world is composed of Coix (2*n* = 10/20), Chionachne (2*n* = 20), Sclerachne (2*n* = 20), Trilobachne (2*n* = 20), and Polytoca (2*n* = 20), and the new world comprises Zea (2*n* = 20) and Tripsacum **(USDA-ARS, 2018)**. The genus Zea was separated into two sections: Luxuriantes and Zea. The luxuriantes section contains three species, namely, *Z. luxurians*, *Z. diploperennis*, and *Z. perennis*, and very recently, it has included *Z. nicaraguensis* **(Ilt is and Benz, 2000)**. The Zea section comprises only one species, *Z. mays*, the most economically important, which is subdivided into three subspecies: ssp. mays, for maize, ssp. mexicana and ssp. Parviglumis **(Wilkes, 1967; Wllkes, 1977)**.

I.1.3. Plant description

Maize plants are composed of a simple stem of nodes and internodes, and a pair of large leaves extend off each internode (a total of 8 to 21 per plant). The leaves are linear to lanceolate, arranged alternately, and can reach 30 to 100 cm in length. The flower-bearing regions of the plant constitute the male and female inflorescences, which are separately positioned along the plant. The male inflorescence located at the top of the plant, known as the tassel, contains anthers in which a large number of pollen grains are born. The female inflorescence also called the ear, can be found in 1-3 parts of the plant covered by silks, which serve to catch and anchor pollen grains. A total of 30 to 1000 maize grains or kernels are encased in husks per ear, and under field conditions, 97% of these kernels are pollinated by other plants. The annual crop can only grow during the rainy season and can reach 2-3 m in length (Figure 2A) **(Rakshit *et al.*, 2023)**.

Approximately 50 species exist and exhibit different colors, textures, grain shapes, and sizes (Figure 2B). White, yellow, and red are the most common types. White and yellow varieties are preferred by most people depending on the region **(Singh *et al.*, 2019)**. The crop is processed and prepared in various forms depending on the country. Ground maize is prepared in porridge in Eastern, Southern, and West Africa. Ground maize is also fried or baked in many countries. In all parts of Africa, green (fresh) maize is boiled or roasted on its cob and serves as a snack **(Ekpa *et al.*, 2018)**. Popcorn is also a popular snack. In Cameroon, for instance, maize is used for different dishes in many cultures, including sanga, fufu corn, koki corn, and pap (paste).

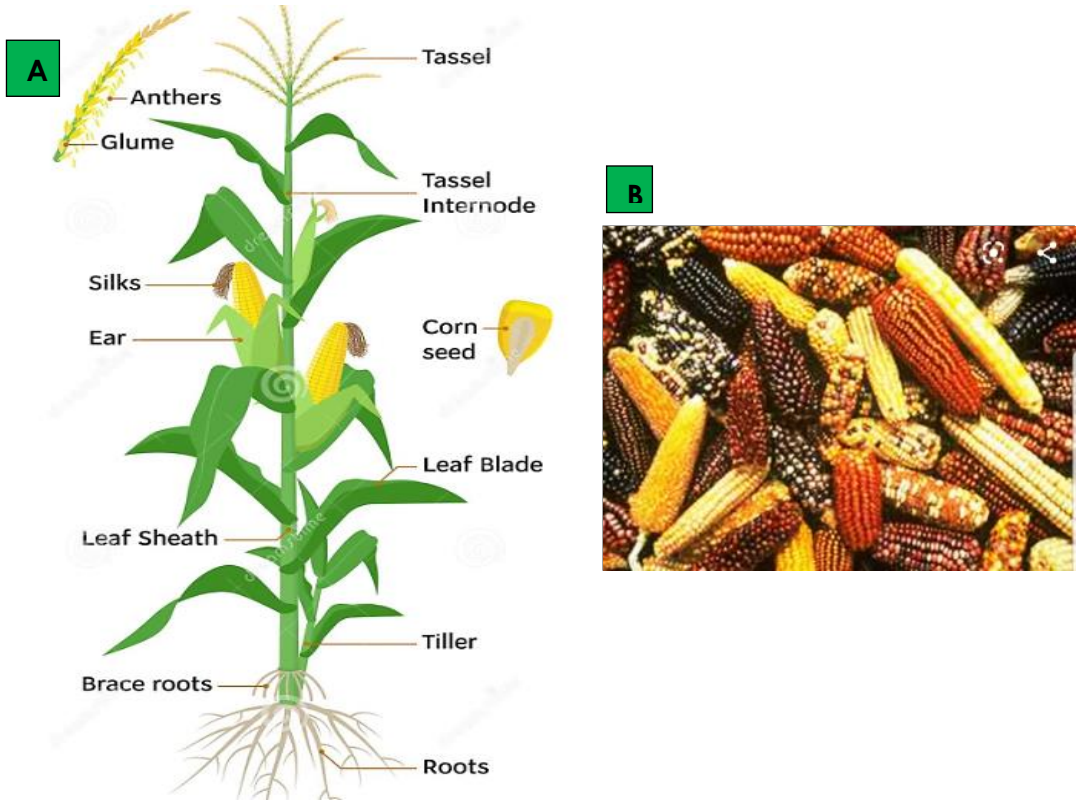

Figure 2: Illustration of maize structure and varieties. The entire maize plant and the names of the different parts (A) and different colors, shapes, sizes and textures of some maize kernels (B) **(Bezvershenko, 2019)**.

I.1.4. Ecology of *Zea mays*

Maize is a warm weather crop but can be cultivated in all types of soil and different climates. Although minimal conditions are required for production, as soil temperatures range from 10-38°C with an optimal temperature between 25-30°C, temperatures of approximately 32°C critically affect the yield. Rain between 350 and 450 mm is required to produce a yield of 3,152 kg/ha per annum, whereas the optimum rainfall range is 750-1500 mm. Every millimeter of water absorbed leads to approximately 10 to 16 kg of grain produced. At maturity, 250 L of water was added to each plant in the absence of moisture stress. The most suitable soil for maize culture should be characterized by good internal drainage, an optimal moisture regime, a good effective depth, favorable morphological properties, sufficient and balanced quantities of plant nutrients (approximately 200 kg/ha N; 50 to 80 kg/ha P and 60 to 100 kg/ha K) and chemical properties that are favorable for maize production **(Du Plessis, 2003; Cofas, 2018)**.

I.1.5. Cultivation practices

Recent research has led to the development of suitable soil and crop management practices for increasing resource use efficiency while reducing greenhouse gas emissions and maintaining

soil health. To reduce climate change impacts and for successful maize production, we need to enhance the use of conservation agriculture practices **(Kamara *et al.*, 2020)**. This includes the application of 2-3 shallow tillage operations, and seeds must be dibble 2 to 4 cm deep (should not be more than 5 cm deep). For most of the areas, a spacing of 25 cm between plants in rows 60 cm apart was found to be optimal. The crop can be cultured alone or intercropped with soybean or bean. The optimal yield is achieved by growing one row of soybean plants between 2 rows of maize (60 cm spacing) and one row of maize plants alternating every 4 rows of urd bean or black gram (30 cm spacing). The seed germination percentage must be more than 85%, and planting rates of 20-25 kg/ha for pure crops, 40-50 kg/ha for fodder maize and 10 kg/ha (1:3) or 15 kg/ha (1:2) for intercropping with soybean are generally recommended **(Karki *et al.*, 2014)**. For the control of diseases and pests, crop rotation should be adopted every 3 years, and the same family crop should not be continuously cultivated.

I.1.6. Importance of *Zea mays*

Maize or corn is an important cereal worldwide and is consumed as a vegetable, although it is a grain crop **(Mulyati *et al.*, 2021)**. Maize is the most important cereal in Africa and an important staple food for more than 1.2 billion people in Africa. It is a multiverse crop with more than 600 derived products with various applications **(Ntsama and Kamgnia, 2019)**. All parts of the crop can be used for large-scale nutritional, health, and industrial benefits related to food and nonfood products, and grain is the most important part **(Badu-Apraku and Fakorede, 2017)**.

The chemical composition of maize depends on the type, and starch is the major nutrient, with up to 72 to 73% of the kernel weight being made up of amylose (25 to 30% of the starch) and amylopectin (70 to 75%) **(Cortez and Wild-Altamirano, 1972a)**. Starch is directly followed by protein, which varies from approximately 8 to 11% of the kernel weight (Table I). Maize grains are rich in vitamins A, C, K, and E, carbohydrates, and essential minerals. Yellow maize contains many important vitamins, predominantly vitamin A, such as carotenoids, and vitamin E, such as tocopherols. White maize contains small amounts of total carotenoids or does not contain carotenoids **(Galani *et al.*, 2022)**. The grains are also rich in dietary fiber and calories, which are good sources of energy and B-complex vitamins that are good for the skin, hair, heart, brain, and digestion. A heavy reliance on maize in the diet can lead to malnutrition and nutrient deficiency diseases such as night blindness and kwashiorkor. The presence of essential fatty acids, especially linoleic acid, in maize oil plays an important role in the diet by maintaining

blood pressure, regulating blood cholesterol levels, and preventing cardiovascular diseases **(Dupont *et al.*, 1990)**. Corn provides a large part of the daily folate requirement and constitutes a significant source of zinc and potassium **(Galani *et al.*, 2022)**.

For medicinal purposes, decoctions of silk, roots, and leaves are used for bladder problems, nausea, and vomiting, while decoctions of cobs are used for stomach complaints **(Kumar and Jhariya, 2013**). Maize is highly beneficial to human beings and is believed to have potential anti-HIV activity due to the presence of Galanthus nivalis agglutinin (GNA) lectin, also referred to as GNA-maize **(Rouf Shah *et al.*, 2016)**.

Starch from maize can also be made into plastics, fabrics, adhesives, and many other chemical products. Corn steep liquor, a plentiful watery byproduct of the maize wet-milling process, is widely used in the biochemical industry and in research as a culture medium to grow many kinds of microorganisms. Maize is increasingly used as a feedstock for the production of ethanol fuel and can be used to replace carcinogenic petroleum products that are major components of cosmetic preparations **(Mohanty and Swain, 2019)**. Maize is an important crop that provides an avenue for making various types of foods.

Table I: Major chemical components and contents in grams (g) for 100 g of maize kernels of different maize types **(Cortez and Wild-Altamirano, 1972b)**.

Maize type	Moisture	Ash	Protein	Crude fiber	Ether extract	Carbohydrate
Salpor	12.2	1.2	5.8	0.8	4.1	75.9
Crystalline	10.5	1.7	10.3	2.2	5.0	70.3
Floury	9.6	1.7	10.7	2.2	5.4	70.4
Starchy	11.2	2.9	9.1	1.8	2 2	72 8
Sweet	9 5	1 5	12.9	2.9	3.9	69.3
Pop	10.4	1.7	13.7	2.5	5.7	66.0
Black	12.3	1.2	5.2	1.0	4.4	75.9

I.1.7. Maize production

Due to its high yield potential among cereals, maize is globally known as the queen of cereals. The crop is produced on nearly 100 million hectares in developing countries, with almost 70% of the total maize production in the developing world coming from low- and lower middle-income countries **(FAOSTAT, 2010)**. The largest producer of maize is the United States of America (USA), which contributes approximately 35% of the total world maize production,

with 392.45 million tons per year, followed by China, with an estimated production of 257.34 million tons per year. South Africa is the first producer in Africa, with 12.51 million tons produced per year **(FAO, 2020)**. In large parts of Africa, maize is the principal staple crop, accounting for up to 51% of consumed calories. It is the number one crop in terms of the area planted, occupying more than 33 million ha each year, covering nearly 17% of the estimated 200 million ha of cultivated land in sub-Saharan Africa and accounting for 50-60% of total cereal production **(Shi and Tao, 2014)**. In Cameroon, maize is the most affordable crop in terms of market price and cost of seeds, and the country occupies 44th place worldwide and 13th place in Africa, with an estimated production of 2.34 million tons **(FAO *et al.*, 2022)**. The maize market represents approximately 25 billion XAF each year and constitutes an important source of income and employment for more than three million small rural farmers in the country **(Ntsama and Kamgnia, 2008; Mbah *et al.*, 2023)**.

I.1.8. Maize production constraints

Maize yields in many of the sub-Saharan African countries, where it is the most important staple food, are often extremely low, averaging approximately 1.8 tons/ha yearly compared to a significant yield of up to 4.9 tons/ha obtained in developed countries **(Rezende *et al.*, 2020)**. Elsewhere, a 7% yield decline is forecasted per annum due to multiple biotic and abiotic factors, therefore biasing the global goal to record a 2.4% yield improvement per year to feed the overgrowing world population by 2050 **(Ray *et al.*, 2013)**. In Cameroon, for instance, maize production decreased from 2.316 to 2.200 thousand tons between 2018 and 2020, even though it was in a hurry to increase production to reach the 2.800,000 tons/year expected by the Ministry of Agriculture to meet local demand **(FAO, 2020)**. Consequently, an import value estimated in 2019 of 3.305 thousand US dollars, or approximately 1.65 billion FCFA, was registered **(World Data Atlas, 2021)**. This situation is directly attributed to several constraints, including an array of abiotic and biotic stresses affecting the crop.

I.1.8.1. Biotic constraints

Diseases are one of the major biotic constraints reducing crop yield and deteriorating the quality of products which ultimately reduces the market price. Maize plants are affected by a large number of diseases caused by bacteria, viruses, many other mycoplasma-like organisms, nematodes, parasites, and fungal pathogens in various fields **(Gong *et al.*, 2014)**. Approximately 112 diseases have been reported in maize crops **(USDA, 1996)**. In Cameroon, for instance, biotic constraints are responsible for 15-50% of maize yield losses **(Ngonkeu *et***

***al.*, 2017)**. Therefore, several fungal diseases have been reported to be devastating in maize crops **(Cobo-Díaz *et al.*, 2019)**. More dramatically, biotic stress is often coupled with abiotic stress caused by present climate change conditions. This increases host plant sensitivity to harmful organisms and insects while decreasing weed competition, resulting in uncountable yield losses **(Pandey *et al.*, 2017b)**.

I.1.8.2. Abiotic constraints

Climate change intensifies the variability in crop yield, and maize is the most negatively affected **(Tebaldi and Lobell, 2018)**. Drought, extreme temperatures, salinity, and nutrient deficiency are especially known to be prime environmental perturbations that negatively affect global maize production. The impact is associated with a long-term trend toward higher temperatures, greater evapotranspiration, and an increase in the frequency of extreme weather events such as heat spells and temporary droughts, at least in some major maize production regions **(Campos *et al.*, 2004)**.

Under the extreme climate change scenario, maize yields are expected to decline by 10-20% by the end of the twenty-first century, even if maize is provided with all of the necessary water **(Xu *et al.*, 2016)**. In this context, maize cultivars with improved abiotic stress resistance, especially drought tolerance, are critical for overcoming current climatic circumstances as well as addressing food security concerns of growing human populations **(Fedoroff *et al.*, 2010)**.

I.2. Drought stress

Water deficit is the most severe cause of yield reduction in global agricultural production, and climate change tends to aggravate this scenario **(Ribaut *et al.*, 2009; Nadeem *et al.*, 2019)**. The drought tolerance of maize is generally considered poor due to its large transpiration surface area and poorly developed root system **(Camacho and Caraballo, 1994)**. Therefore, drought is undoubtedly a major cause of yield loss in maize worldwide **(Bänziger and Araus, 2007),** and projections of decreasing precipitation and increasing evaporative demand within rainfed maize areas will further exacerbate losses **(IPCC, 2007)**. Many factors influence the severity of drought, such as the occurrence and distribution of rainfall, evaporative demand, and the moisture storage capacity of soils. Drought can be classified as moderate (-1.2 b SPI b -0.8), severe (-1.5 b SPI b -1.3), extreme (-1.9 b SPI b -1.6), or exceptional (SPI b -2.0) based on the specific standardized precipitation index (SPI), resulting in up to 50% yield losses concerning the severity level **(Wery *et al.*, 1994; Leng and Hall, 2019)**. Reported studies from 1980 to 2015 revealed up to 21% yield reductions in wheat and 40% in maize due to drought stress

worldwide **(Daryanto *et al.*, 2016)**. The highest water requirements for maize are concentrated in the pre-flowering and flowering stages and can compromise the entire production process **(Fancelli and Dourado-Neto, 2000; Bergamaschi and Matzenauer, 2014)**.

In Cameroon, for instance, two climatic regions are distinguished: the humid equatorial region in the south (temperature = 25°C, annual rainfall often averages 1500 mm) and the semiarid northern portion (temperature = 25 - 34°C, rainfall averages 500 mm) (Figure 3). The Far North, Adamaoua, and North Regions, located closer to the Sahel regions, constitute the first maize consumers in the country. Unfortunately, the prevailing climatic conditions in these regions (with rainfall less than the required range of 750-1500 mm) reduce the production capacity; consequently, 64 to 77% of the total consumption is purchased despite representing 37% of the national production **(World Data atlas, 2021)**.

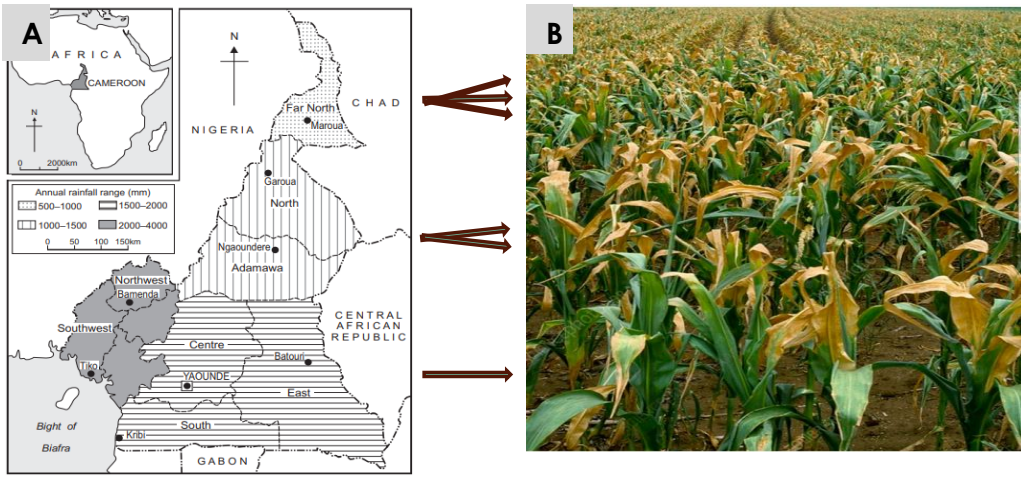

Figure 3: Impact of rainfall distribution on maize growth. Annual rainfall distribution in the different regions of Cameroon (A) and direct impact of drought stress on young maize plants (B)

I.2.1. Impact of drought on plant development

Under water stress, plants can respond by developing morpho-anatomical, biochemical, physiological, and molecular adaptation mechanisms (Figure 4) **(Taiz and Zeiger, 2013)**.

I.2.1.1. Impact of drought on plant physiological parameters

Physiological alterations, such as changes in leaf water potential, stomatal conductance, and chlorophyll content, correspond to changes that occur at the cellular level. In fact, a lack of water reduces the turgor of cells and consequently their growth, enhances the synthesis of abscisic acid (ABA), which induces stomatal closure, reduces CO_2 assimilation and the

transpiration rate, and accelerates senescence and leaf abscission **(Kapoor *et al.*, 2020)**. Therefore, physiological alterations that ameliorate production yield under stress conditions include a lower reduction in water potential, prolonged stomatal closure, a reduction in CO_2 assimilation rates, a greater chlorophyll concentration, and deep roots with less lateral branching. Additionally, the capacity to control stomatal opening, through which plants limit water loss, is considered an important mechanism that allows plants to continue CO_2 assimilation and perform photosynthesis **(Oliveira *et al.*, 2002)**. Although the biosynthesis and level of free proline, such as amino acids, are highly regulated, their levels increase many-fold in living systems under stress due to a short decrease in feedback inhibition **(Zhang *et al.*, 1995)**. Proline is a compatible solute that plays a pivotal role in protecting cellular metabolism under stress by (i) regulating the NAD(P)H/NAD(P)+ ratio **(Alia and Saradhi, 1993)**, (ii) regulating the carboxylase/oxygenase activity of Rubisco **(Sivakumar *et al.*, 2001)**, (iii) scavenging reactive oxygen species **(Shabnam *et al.*, 2014)**, (iv) protecting macromolecular complexes such as photosystem PS II **(Alia and Saradhi, 1991)**, and (v) osmoregulating **(Sanchita *et al.*, 2015)**.

I.2.1.2. Impact of drought on plant morphological parameters

Morphoanatomical alterations correspond to an increase in the interval, in days, between female and male flowering; accelerated senescence of culms and leaves; and a reduction in plant height, prolificacy, the number of branches of the tassel and total dry matter **(Balbaa *et al.*, 2022)**. However, recent studies revealed that an increase in root length and reduced lateral roots enhance drought tolerance in maize **(Zhan *et al.*, 2015; Ali *et al.*, 2016)**.

I.2.1.3. Impact of drought on plant biochemical parameters

Drought induces the overproduction of reactive oxygen species (ROS), such as O2−, H2O2, and •OH radicals, responsible for oxidative stress. These ROS increase lipid peroxidation by increasing the content of malondialdehyde (MDA), a suitable marker for membrane lipid peroxidation and thus oxidative stress **(Møller *et al.*, 2007)**. Therefore, the biochemical response consists of a complex of enzymatic and nonenzymatic antioxidant systems developed by the plant to avoid injuries caused by active oxygen species and guarantee normal cellular function **(Shakeel *et al.*, 2011)**. The nonenzymatic system consists of the production of low-molecular-weight antioxidants such as glutathione, ascorbate, carotenoids, total phenols, and flavonoids, which cooperate to maintain the integrity of photosynthetic membranes. The enzymatic system refers to the synthesis of antioxidant enzymes such as superoxide dismutase

(SOD), peroxidase (POD), catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase (APX), which directly scavenge ROS or may produce nonenzymatic antioxidants **(Kholova *et al.*, 2010; Ramazan *et al.*, 2021)**.

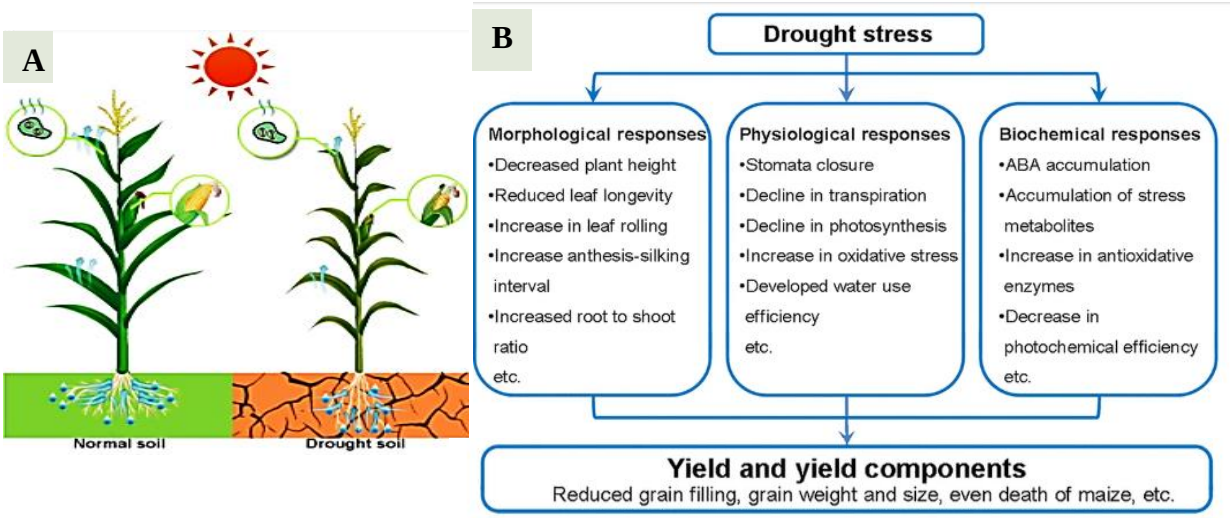

Figure 4: Drought stress development and different plant defense mechanisms. Drought effects on maize plants and soil (A); morphological, physiological, and biochemical responses of plants to drought stress (B) **(Nadeem *et al.*, 2019)**.

I.2.2. Mechanism of action of bacterial endophytes for drought stress management

To manage drought stress, plants mediate various morphophysiological, biochemical, and molecular responses; therefore, endophytic bacteria alter this process to improve drought tolerance. A wide range of bacterial strains have been shown to induce drought tolerance in some plants. This is the case for *Enterobacter* sp., which enhances drought tolerance in maize **(Naveed *et al.*, 2014b)**, *Bacillus amyloliquefaciens* in grapevine **(Jiao *et al.*, 2016)**, and *Pantoea alhagi* in wheat **(Chen *et al.*, 2017),** among many others. Endophytic bacteria are known to release various plant growth-regulating and drought-tolerant substances, such as auxins, gibberellins, ABA, cytokinins, and ACC deaminase. They are also known to enhance root growth, antioxidant activity, and relative water content (Figure 5). In fact, a deeper root system with an increased number of roots is associated with improved yield under drought stress. This is related to the elasticity of the root structure, which enables better adaptation to physical and chemical soil properties **(Chapman *et al.*, 2012)**. Additionally, ABA increases root length and density for better contact with high moisture to enhance optimal water and nutrient acquisition **(Vysotskaya *et al.*, 2017)**. Endophytic bacteria are also known to increase the levels of compatible solutes such as sugars and proline, responsible for osmotic adjustment, one of the most important cellular responses that help plants tolerate drought damage **(Ullah *et***

***al.*, 2019)**. Moreover, ROS produced under drought conditions are responsible for oxidative damage to lipids, proteins, and other macromolecules; therefore, the application of bacterial endophytes during stress reduces ROS through the production of antioxidants such as peroxidase (POD), glutathione reductase (GR), catalase (CAT) and malondialdehyde (MDA) **(Hussain *et al.*, 2019)**. To survive under drought conditions, bacteria form a matrix called biofilm which constitute a self-organized and cooperating community in which bacterial cells adhere to each other on living or non-living surfaces **(Singh and Chauhan, 2017)**. Biofilm formation allows bacteria to be synergistically more stable and perform with consistency for optimum plant protection under drought stress conditions **(Wang *et al.*, 2019)**.

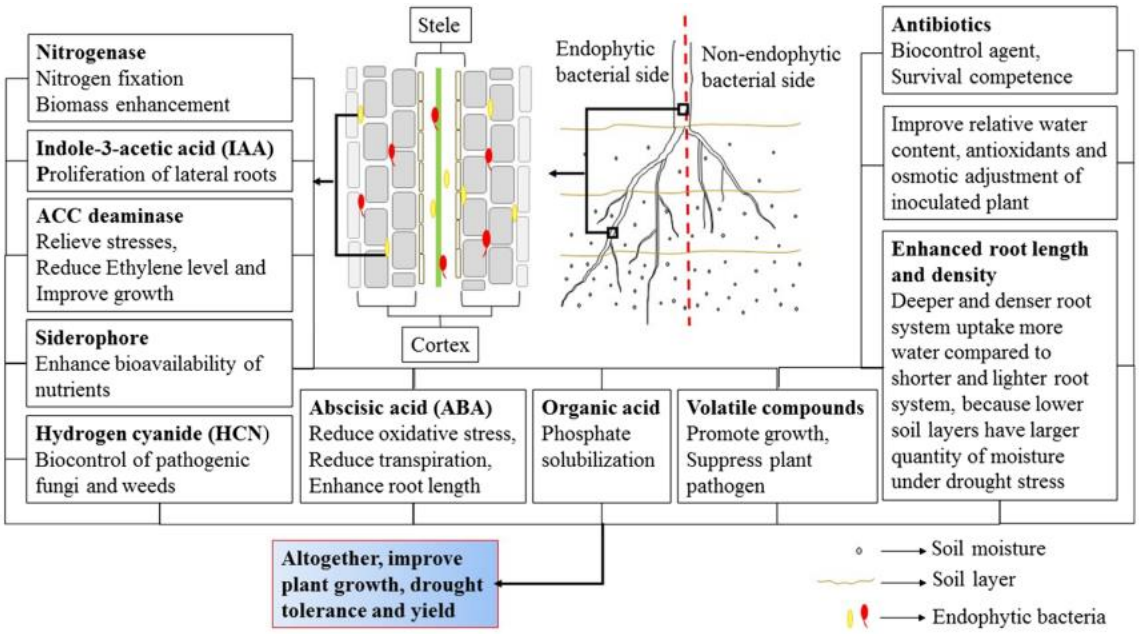

Figure 5: Enzymes and molecules released by bacterial endophytes in the cortex and stele of plants induce tolerance to drought and enhance plant growth **(Ullah *et al.*, 2019)**.

I.3. Maize diseases

Maize is affected by more than 60 diseases **(Hooda *et al.*, 2018),** which are mainly caused by bacteria, fungi, and viruses. All these organisms can spread extremely quickly under good conditions, and the infection of a single plant can easily cause severe disease outbreaks in the field **(Singh *et al.*, 2020)**.

In most cases, bacterial pathogens penetrate plants through wounds created by insects, wind, hail, or blowing soil. The major bacterial diseases affecting maize are bacterial stalk rot (caused by *Erwinia chrysanthemi*), Stewart's wilt (*Erwinia stewartii*), and bacterial leaf stripe (caused by *Pseudomonas rubrilineans*) **(The CIMMYT Maize Program, 2004; Hooda *et al.*, 2018)**.

Moreover, viral diseases are common in maize-growing locations around the world and can cause major losses for farmers. Viral infections are usually detected by the appearance of symptoms such as streaks, mosaics, and chlorosis. The leaves of older plants may turn reddish or purple, and dwarfing or stunting is prevalent in early affected plants **(Redinbaugh and Zambrano, 2014)**. The most widely prevalent maize viruses are maize chlorotic dwarf virus (MCDV), maize chlorotic mottle virus (MCMV), maize dwarf mosaic virus (MDMV), sugarcane mosaic virus (SCMV), maize lethal necrosis (MLN), maize streak virus (MSV), maize rough dwarf virus (MRDV) and maize bushy stunt (MBS) **(Zambrano *et al.*, 2014)**.

Among the disease-causing pathogens in maize, fungal diseases are the most dangerous because of their ability to sporulate prolifically and because spores provide inoculum, which may infect other plants. Additionally, they need only a few days between infection and the production of further infectious propagules (spores) **(Richard, 2006)**. The major and devastating diseases of maize caused by fungi are grouped into two categories: foliar diseases and stalk and ear rot. The most damaging foliar diseases are gray leaf spot (the major foliar disease problem in the United States and sub-Saharan Africa), and the causal agent is *Cercospora zeae-maydis* **(Ward *et al.*, 1999; Gordon *et al.*, 2006)**; northern corn leaf blight (also known as turcicum leaf blight), which is caused by *Exserohilum turcicum* **(Welz and Geiger, 2000);** and southern rust, which is caused by *Puccinia polysora* **(Jines *et al.*, 2007)**. Stalk and ear rots are the most economically damaging corn diseases and occur wherever corn is grown. They are generally associated with mycotoxin contamination, which is most dangerous for humans because it has been associated with a variety of diseases, including cancer **(Bhatnagar *et al.*, 2002)**. The most expended and devastating fungus is Aspergillus ear rot, which is very damaging to maize, and the fungus *Aspergillus flavus*, which produces a mycotoxin called aflatoxin, is the first incriminated strain. Gibberella stalk and ear rot is added to the list and is caused by the fungus *Gibberella zeae* (synonym *Fusarium graminearum*), which also produces several mycotoxins, including DON (deoxynivalenol, also known as vomitoxin). *Fusarium* ear rot is caused by the fungi *Fusarium verticillioides* (synonym *F. moniliforme*) and *F. proliferatum* (*T. Matsushima*), which are responsible for kernel fumonisin accumulation **(Clements *et al.*, 2003; Clements *et al.*, 2004)**. **(Balint-Kurti and Johal, 2009)**. Therefore, among these pathogens, *Fusarium verticillioides* is the most commonly and devastatively reported fungal species infecting maize, causing up to 30 to 50% yield losses **(Leslie and Summerell, 2006; Bacon *et al.*, 2008; Tom and Patel, 2021)**.

I.3.1. *Fusarium verticillioides*

I.3.1.1. Taxonomy

The name of this taxon is subject to many controversies, with some taxonomists calling it *F. moniliforme* and others calling it *F. verticillioides*. The name *F. verticillioides* has priority and is now generally accepted for its use **(Seifert *et al.*, 2003)**. *F. verticillioides* is a member of the Fujikuroi species complex (FFSC), which encompasses several morphologically diverse species supported by multilocus analyses involving the genes encoding translation elongation factor 1-α, β tubulin and several other housekeeping and metabolic loci **(O'Donnell *et al.*, 2000)**. The species complex is divided into American, Asian, and African clades, with *F. verticillioides* belonging to the African trade **(De Vos *et al.*, 2014)**. However, *Fusarium verticillioides* is widely distributed throughout the world and is particularly associated with maize, where it can cause stalk rot, cob rot, and seed rot that results in significant yield losses and reductions in grain quality **(Castro del Ángel *et al.*, 2021)**. Kingdom: Fungi

Division: Ascomycota

Class: Sordariomycetes

Order: Hypocreales

Family: Nectriaceae

Genus: *Fusarium*

Species: ***F. verticillioides***

I.3.1.2. Morphology

The cultures initially present a white mycelium that can develop a violet pigment with age. On PDA media, pigmentation can vary from white or grayish orange to violet gray, dark violet, or dark magenta (almost black). Blue–black sclerotia may develop in some isolates but are not diagnostic, but they could indicate a high level of fertility (Figure 6A and B). The growth rate varies from 21 to 35 mm after three days in total darkness and depends on the temperature (25-30°C) **(Leslie and Summerell, 2006).**

Microscopic observations of a 7- to 10-day culture revealed a long and slender macroconidium that was slightly falcate or straight with 3 to 5 septate and thin walls (Figure 6C). Apical cells are curved and often tapered to a point, and basal cells present a foot-shaped morphology.

Macroconidia are difficult to find, whereas microconidia are abundant (Figure 6D). The microconidia are predominantly long chains or false heads and are occasionally found in small aggregates (Figure 6E). Chlamydospores are not produced, although some isolates may produce swollen cells in hyphae that are easily confused with chlamydospores or pseudochlamydospores **(Leslie and Summerell, 2006)**.

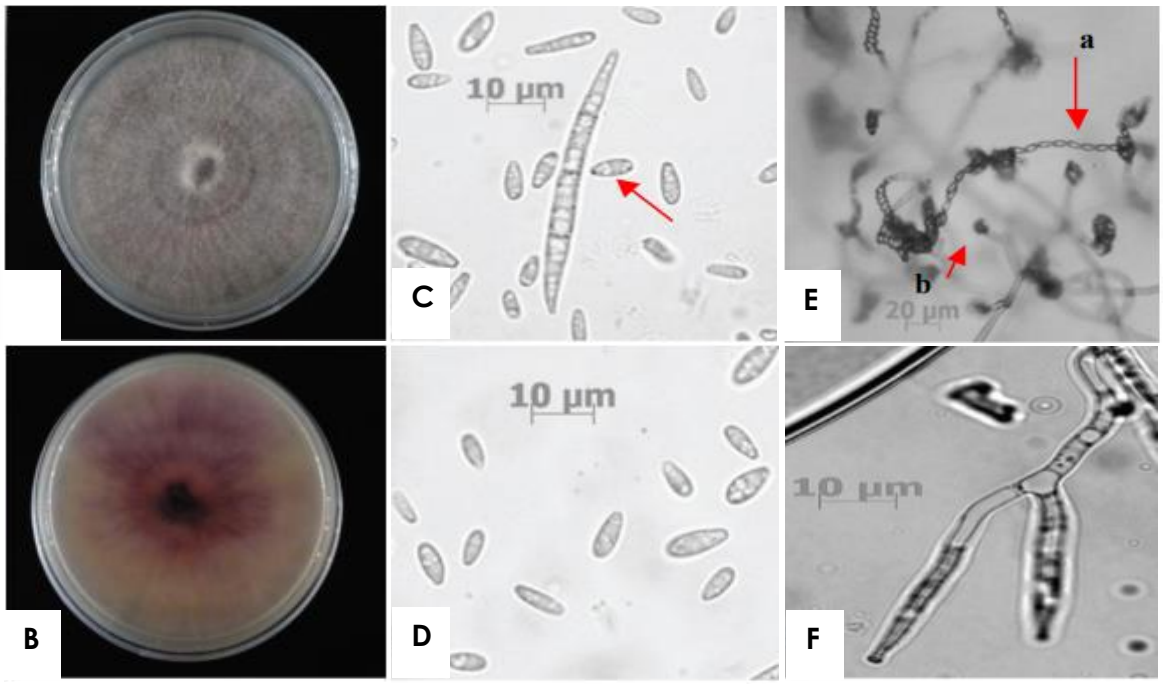

Figure 6: Macroscopic and microscopic characteristics of *Fusarium verticillioides*. A-B: Colony features on PDA upper and lower surfaces, respectively; C.: macroconidia (arrow); D: microconidia; E: microconidia that form long chains (a) and false heads (b); F: branched monophialides **(Leslie and Summerell, 2006)**.

I.3.1.3. Infection cycle

The soil serves as a reservoir and primary source of *Fusarium verticillioides* inoculum, although it can survive through resistant structures or as mycelium and conidia on residues from remnant harvest **(Galindo-Castañeda *et al.*, 2019)**. *F. verticillioides* infection of maize can occur via several routes. First, the fungus can invade the root system, grow endophytically or asymptomatically, and then reach the ears and grains of the plant, causing further systemic infection. The second most commonly reported infection route is through airborne conidia that infect silks **(Headrick and Pataky, 1991; Munkvold and Carlton, 1997)**. After invading the silk, the fungus infects the kernels, but usually, only a small percentage of the infected kernels become symptomatic (Figure 7) **(Munkvold *et al.*, 1997)**. Another reported infection pathway is systemic infection through seeds. In this case, fungal conidia or mycelia carried inside the

seeds or on the seed surface may develop inside the young plant, moving from the roots to the stalk and finally to the cob and kernels **(Murillo-Williams and Munkvold, 2008a)**. Systemic infection may also arise from conidia or mycelia that survive in crop residues in the soil. Additionally, the fungus can colonize maize stalks systemically without causing visible disease symptoms **(Schoeman *et al.*, 2018)**. When a genetically uniform host is planted, diseased and asymptomatic plants can be observed in the same field. Globally, symptoms vary widely and range from asymptomatic infection to severe rotting of all plant parts. Therefore, the reasons why asymptomatic infections or rotting and wilting occur in diseased tissues are still not known. However, environmental conditions, the genetic background of the plant and the pathogen, and water availability may all be important factors in disease development **(Pfordt *et al.*, 2020)**.

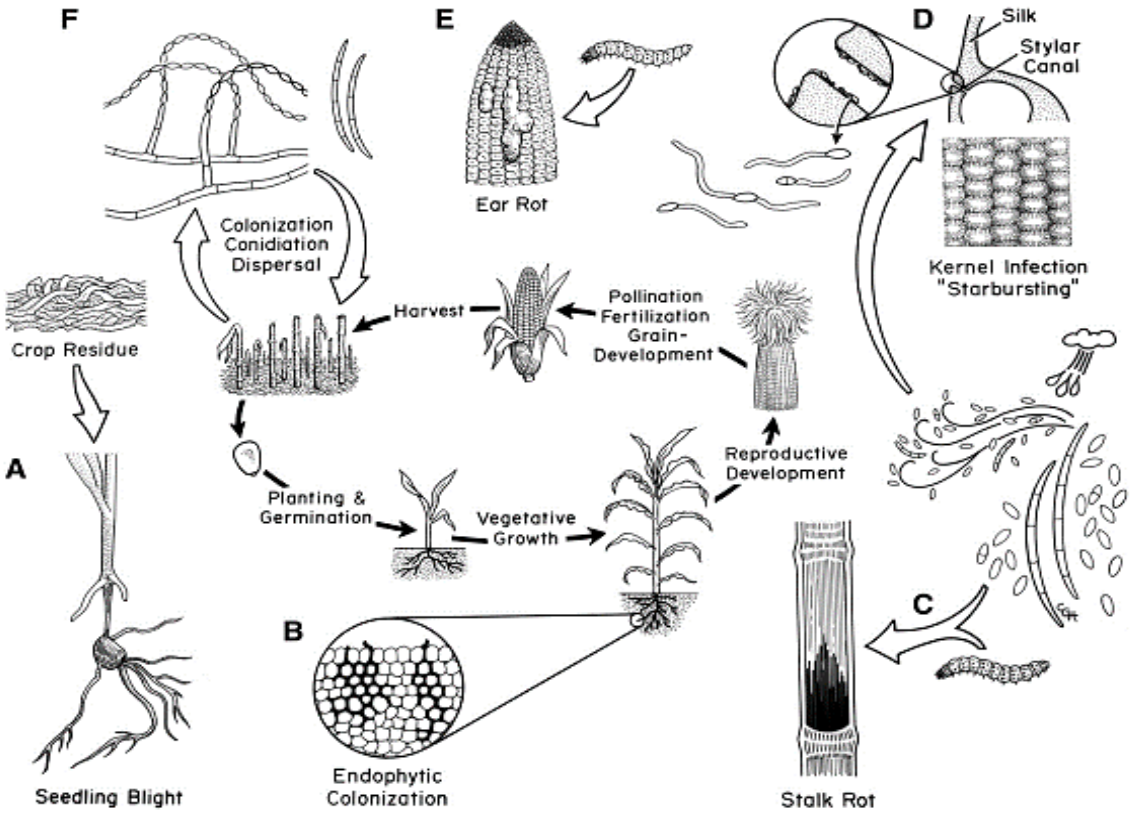

Figure 7: *Fusarium verticillioides* maize disease cycle **(Blacutt *et al.*, 2018)**

A, Seed germination in *Fusarium* -infested soils may result in aggressive rot and seedling blight; B, under inappropriate disease conditions, endophytic colonization may occur; C, stalk wounds from insect or mechanical damage may cause *F. verticillioides* infection and result in stalk rot; D, the fungus can colonize maize kernels via the stylar canal, giving rise to the starburst pattern on kernels; E, larvae of the European corn borer moth feed on leaves, stalks, ears and collar tissues, providing infection courts for *F. verticillioides* stalk and ear rot; F, after harvest, the fungus can survive and sporulate on crop residue, providing inoculum for further infections.

I.4. Management of maize rot caused by *F. verticillioides*

I.4.1. Cultural practices

The control strategies for *F. verticillioides* include soil tillage and crop rotation to break the disease cycle by removing the primary source of inoculum that survives on the previous crop residues **(Cotten and Munkvold, 1998; Mabuza *et al.*, 2018)**. Ensuring good soil drainage and soil fertilization are added to these strategies. Additionally, plant susceptibility to *F. verticillioides* increases during stress and can be managed using proper irrigation, as well as nutrient supplementation using soil amendments **(Alakonya *et al.*, 2008)**. Maize harvest and transport should be refined to reduce the risk of physical damage that creates wounds allowing the pathogen to enter and insect damage or hailstorms that predispose maize plants to stalk rot. The collected kernels were well-dried to reduce fungal infection **(Munkvold, 2003)**. These methods are sometimes difficult to implement and are not sufficient to protect the culture and ensure good productivity **(Tran *et al.*, 2021)**.

4.2. Resistant cultivars

The purpose of producing resistant maize cultivars was to obtain hybrids with high yield potential, good resistance to leaf, ear and stalk rot, and good emergence and seedling vigor traits that are adaptable to the area and that exhibit proper heat and drought tolerance for growing conditions. Therefore, the use of hybrid germplasms in maize is difficult because 95% of the grains are fertilized by the pollen of other plants **(Cooper *et al.*, 2014)**. On the other hand, the success of plant breeding is not guaranteed on the market due to consumer responses to genetically modified plants varying among countries **(Fedoroff *et al.*, 2010)**. Although genetic resistance is one of the main strategies for disease management in maize, there are still no resistant or tolerant hybrids against *Fusarium* ear and stalk rot disease, and chemical control is now one of the most widely used measures by farmers **(Lanza *et al.*, 2016)**.

4.3. Chemical control

The success of chemical control of diseases in maize is well known **(Degani *et al.*, 2014)**. However, the results for *Fusarium* ear rot are not as efficient as those for foliar diseases. Better results against *F. verticillioides* were obtained with the combined use of pyraclostrobin + epoxiconazole in two applications **(Junqueira *et al.*, 2017)**. Additionally, a significant reduction in the incidence of this disease was observed with the combined application of azoxystrobin and cyproconazole **(Silva *et al.*, 2021)**. However, a mixture of fludioxonil and

metalaxyl-M induces an increase in the production of FB1-type fumonisins **(Miguel *et al.*, 2015)**. Moreover, these fungicides have experienced low success rates over the years due to their exhaustive use and lack of long-term efficacy, resulting in the development of resistance and nontarget effects **(Lucas *et al.*, 2015)**. In addition, the worldwide trend toward environmentally safe methods for plant disease control in sustainable agriculture calls for reducing the use of synthetic chemicals and opens the opportunity for another management technique **(Chandra *et al.*, 2010)**. Thus, an attempt has been made to develop environmentally friendly fungicidal compounds known as biological control agents for sustainable agriculture **(Corrêa *et al.*, 2014)**.

I.4.4. Biological control

Biological control refers to the reduction or suppression of the activities and populations of one or more plant pathogens through the management of common components of ecosystems or the use of antagonistic agents for plant protection. For this purpose, multiple living organisms called biocontrol agents (BCAs) are used for the control of several plant pathogens and diseases. **(Tchameni *et al.*, 2017; Lahlali *et al.*, 2022)**. Yeasts, viruses, actinomycetes, fungi, and bacteria are generally used as BCAs and have a range of effects on plant pathogens **(Lahlali *et al.*, 2022)**. The agents act in a friendly way to the plant; they have no toxic residual effect and act as nonchemical agents, although they have been reported in many cases to be as effective as chemical control agents **(Deepa *et al.*, 2021)**. BCAs are recommended for the integrated management of plant diseases because of their ability to avoid diseases of high severity levels, reducing quantitative and qualitative damage **(He *et al.*, 2021)**. In fact, according to **Berg, (2009),** there is a growing market for microbial inoculants across the world that has an annual rate of approximately 10%. In addition, microbial inoculants, compared with chemical pesticides and fertilizers, are a) safer, b) reduce environmental damage, c) have more targeted activity, d) are effective in smaller quantities, e) can multiply but are controlled by plants and indigenous microbes, f) have quicker decomposition procedures, g) are less likely to induce resistance by pathogens and pests, and finally, g) can be used either in organic or conventional agriculture **(Berg, 2009; Berg *et al.*, 2020)**.

The use of BCAs has been mainly documented in the literature as a potential alternative to control *Fusarium* spp. **(Pellan *et al.*, 2021; Abdellatif *et al.*, 2022)**. Recent studies have identified bacterial agents, predominantly *Bacillus* and *Pseudomonas*, as the most commonly investigated microorganisms for the control of *Fusarium* spp. **(Müller and Behrendt, 2021;**

Balthazar *et al.*, 2022; Palazzini, 2023). In this regard, bacteria naturally occurring within plants without causing any damage to their host, called endophytic bacteria, can be good candidates for use as BCAs **(Muthukumar *et al.*, 2017)**. In fact, endophytic bacteria are more directly related to plants than to the rhizospheric bacterial community and can produce more interesting effects in biological control **(Ullah *et al.*, 2019)**.

I.5. Bacterial endophytes in biological control

Exploitation of the plant microbiome is often touted for its enormous potential to substitute environmentally deleterious agrochemicals such as fertilizers and pesticides that are crucial for current agricultural productivity **(Ghosh and Jha, 2023)**. Given that the failure of biocontrol agents is generally attributed to the poor competence of microorganisms facing UV radiation and fluctuations in temperature and moisture encountered in the phyllosphere of plants, endophytes may have a greater survival rate and may have better abilities than non endophytes to enter and colonize plants when inoculated on the plant surface **(Schulz *et al.*, 2002; Comby *et al.*, 2017)**. In fact, endophytic bacteria can penetrate and disseminate in host plants, colonizing the same ecological niche as plant pathogens, and therefore can easily act as biocontrol agents against pathogens **(Afzal *et al.*, 2019; Rabiey *et al.*, 2019)**. The benefits associated with the use of endophytic bacteria for crop protection have been well-documented in recent years **(Ali *et al.*, 2022b; Mushtaq *et al.*, 2023; Tariq *et al.*, 2023)**. Several mechanisms are responsible for this antagonistic activity (Figure 8), such as the synthesis of hydrolytic enzymes that can hydrolyze cell components of pathogenic microorganisms (such as glucanases, chitinases, proteases, and lipases) **(Carro and Menéndez, 2020; Mishra *et al.*, 2020)**; competition for nutrients and proper colonization of niches at the surface of plant roots **(Liu *et al.*, 2017)**; the production of siderophores and antibiotics directed against the growth of pathogens; and the regulation of plant stress hormones in response to stress imposed by infection **(Parray *et al.*, 2016)**. In addition, endophytic bacteria can degrade harmful compounds, reduce the effects of drought and saline stress, and improve seed germination and plant growth, among other benefits.

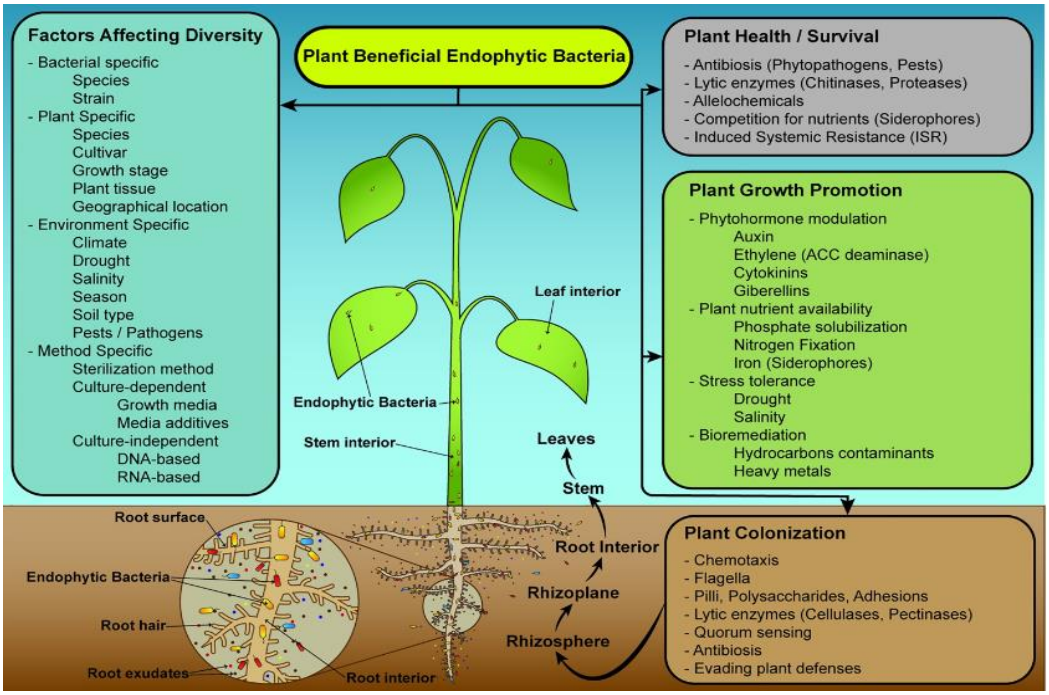

Figure 8: Endophytic bacteria colonizing plant tissues, factors affecting their diversity in plants and consequent impacts on plant growth and protection **(Afzal *et al.*, 2019)**.

I.6. Biomarkers for plant defense against biotic and abiotic stresses

Biomarkers are indicators of the cellular state of an organism in response to external factors. Plants induce or synthesize them in response to abiotic and biotic stresses **(Michel and Soto, 2016)**. Biological agents can help plants under stress conditions by acting on these biomarkers. Drought and pathogenic fungi may induce changes in the plant's morphology, physiology, and biochemistry **(Ghanbary *et al.*, 2021)**. Morphological changes can be detected in the roots and leaves and can be the proliferation of lateral roots in drought cases, the reductions in shoot and root growth, and leaf size in other stresses **(Bano *et al.*, 2022)**.

Phenotypic parameters that can be directly evaluated in drought-stressed plants are not limited to (1) Leaf area adjustment, which reduces the photosynthetic rate **(Silva *et al.*, 2019)**. (2) Leaf chlorosis can be monitored by observing the leaf area in which chlorophyll is lost **(Niu *et al.*, 2020)**. (3) Decrease in chlorophyll a and b contents, directly measured by spectrophotometric quantification **(Simova-Stoilova *et al.*, 2010)**. (4) Changes in the lipid profile of the plasma membrane, mainly caused by temperature fluctuations and ROS-mediated lipid peroxidation **(Morgan *et al.*, 2007)**. (5) Decrease in carotenoid content due to oxidation. Carotenoids are excellent antioxidants and their abundance is associated with greater tolerance to the deleterious effects of ROS accumulation **(Sharma *et al.*, 2012)**. (6) Decrease in stomatal conductance to

minimize water loss through transpiration **(Verma *et al.*, 2020)**. (7) Decrease in relative water content. Relative water content refers to the water content of cells compared at two different points. Higher relative water content indicates less transpiration, with a consequent improvement in the oxidative performance of cells and less leaf wilt **(Semida *et al.*, 2020)**. (8) Increased electrolyte leakage under stress. Electrolyte leakage is a parameter that infers membrane integrity, with the accumulation of ROS and the deleterious effects of stresses on the plasma membrane, there may be electrolyte leakage caused by membrane rupture **(Bajji *et al.*, 2002)**.

At the biochemical level, abiotic and biotic stresses trigger specific plant defense activating secondary metabolites **(Edreva *et al.*, 2007; Sachdev *et al.*, 2021)**. More than 100,000 secondary metabolites are produced by plants subjected to environmental challenges, and this plethora of organic compounds display many biological functions, such as osmoprotectants, thermos-protectants, signaling molecules, co-enzymes, antioxidants, bio repellents, and others **(Radušienė *et al.*, 2012)**. Under these stress conditions, free radicals and reactive oxygen species such as singlet oxygen, superoxide ion, and hydrogen peroxide accumulate in plant tissues, leading to oxidative damage and cell death **(Dumanović *et al.*, 2021)**. In response to increased ROS production, plants upregulate the synthesis of antioxidant enzymes to scavenge and maintain cellular ROS homeostasis **(Huang *et al.*, 2019)**. Catalase (CAT), peroxidase (POX), guaiacol peroxidase (GPX), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GuPx), and glutathione reductase (GR) are the most common antioxidants enzymes activated in response to abiotic and biotic stresses in plants **(Rajput *et al.*, 2021)**. SOD catalyzes the conversion of superoxide radicals to O_2 and H_2O_2, POX scavenges H_2O_2 within extracellular spaces and CAT converts two molecules of H_2O_2 into water and O_2. In addition, APX utilizes ascorbic acid to reduce H_2O_2 to water, GPX catalyzes the breakdown of H_2O_2 and GR catalyzes the conversion of oxidized glutathione (dimeric GSSG) to reduced glutathione (monomeric GSH) **(Chakrabarty *et al.*, 2016; Rajput *et al.*, 2021)**. Flavonoids have also been associated with ROS scavenging and antioxidant activities in plants **(Agati *et al.*, 2020)**.

Phenolic compounds are the most abundant secondary metabolites in plants with antimicrobial and antioxidant properties against a broad range of biotic and abiotic stresses **(Desai *et al.*, 2023; Dumanović *et al.*, 2021)**. It is estimated that about 2% of all carbon photosynthesized by plants is converted into flavonoids or closely related compounds **(Pretorius, 2003)**. Flavonoids are a type of phenylpropanoids that act as pigments; they are soluble in water and are stored in

the vacuoles of plant cells offering a broad range of colors to plants **(Dong and Lin, 2021)**. Flavonoids also have antioxidant properties and function as phytoalexins, which protect plants against damage from biotic and abiotic stresses **(Cavaiuolo *et al*., 2013)**.

Osmoprotectants are a special class of low-molecular-weight organic compounds produced by plants that maintain cell homeostasis under salinity and drought stresses **(Zulfiqar *et al.*, 2020)**. Osmolytes can be ammonium compounds (polyamines, glycinebetaine, *b*-alanine betaine, dimethyl-sulfonio propionate, and choline-*O*-sulfate), sugars and sugar alcohols (fructan, trehalose, mannitol, D-ononitol and sorbitol) and amino acids (proline and ectoine) **(Singh *et al*., 2015)**. They are thought to protect cells by stabilizing proteins, maintaining membrane integrity, and scavenging reactive oxygen species. Proline is a primary osmoprotectant produced in the cytosol, chloroplast, or both **(Verslues and Sharma, 2010)**. In addition to serving as an osmoprotectant, proline is involved in preventing cytoplasmic acidosis and maintaining proper NADP/NADPH ratios for metabolism **(Mansour and Salama, 2020)**.

I.7. Maize infection by *F. verticillioides* under drought stress

Plant stress, particularly drought, is one of the factors that increases the incidence and severity of *Fusarium* disease **(Liu and Liu, 2016).** *F. verticillioides* grows well at temperatures above 26°C; the optimal and maximum temperatures for *F. verticillioides* are 31 and 35°C, respectively, while the minimum growth temperatures range between 22 and 24°C **(Murillo-Williams and Munkvold, 2008b)**. The pathogen can function at temperatures under which maize plants may experience stress and therefore is more common in regions with hot and dry growing conditions **(Rossi *et al.*, 2009)**. Therefore, fungi take advantage of plant susceptibility due to stress. A study by **Murillo-Williams and Munkvold, (2008)** confirm that high temperatures favor the development of systemic infection by *F. verticillioides* within maize stalks. In fact, high temperatures increase the extent of colonization of internodes infected with seeds inoculated with *F. verticillioides* and subsequently increase the infection rate.

I.8. Endophytic bacteria against combined drought and fungal stress

In nature, plants are generally exposed to various abiotic and biotic stresses or their combinations. In the case of maize, combinations of drought and soilborne *Fusarium-induced* diseases are the most common combinations of stresses, significantly reducing crop yields worldwide. In this regard, bacterial endophytes have become more interesting because they colonize the internal tissues of their host plants, triggering plant tolerance to harmful conditions and protecting plants against various pathogenic microbes **(Afzal *et al.*, 2019). Lastochkina *et***

***al.*, (2020)** demonstrated the potential of *Bacillus subtilis* to improve wheat growth and tolerance under the influence of drought, *Fusarium* rot, and combinations of these stresses. In fact, endophytic bacteria act through various mechanisms, including the synthesis of plant growth regulators **(Beneduzi *et al.*, 2012)**, exopolysaccharides, osmoprotectants **(Mohammadipanah and Zamanzadeh, 2019)**, the modulation of plant physio-biochemical constituents **(Hashem *et al.*, 2016)** and antifungal metabolites **(Gond *et al.*, 2015)**. However, knowing that plant responses to simultaneous stresses result in a much more complex scenario, knowledge on the interactions between maize, endophytes, and pathogens under adverse drought stress conditions is still rather limited.

CHAPTER II:

MATERIALS AND METHODS

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II.1. Materials

II.1.1. Bacterial strains

The twenty-five (25) bacterial strains (Table II) used in this study were obtained from the core collection of the Biocontrol Agents Sub-Unit, Antimicrobial and Biocontrol Agents Unit, Faculty of Science, University of Yaounde I, Cameroon. They were isolated from healthy roots, seeds and spines of *Euphorbia antiquorum*, which is naturally grown in the hot Far North Region of Cameroon (Latitude: 12º 5' 13.4988'' N; longitude 15º 0' 53.3952''E), bordering the Sahara Desert. The data pertaining to their identity (16S-rDNA gene sequencing) are given in **Eke *et al*., (2019)**. These bacteria were selected for their abilities to improve tomato resistance to drought stress **(Eke *et al*., 2019b)**, as well as to boost tomato growth and induce systemic resistance against the bacterium wilt pathogen *R. solanacearum*.

Table II: List of bacterial strains used: codes, names and GenBank accession numbers

	Strains codes	Strains identities	Accession number
1	BFL1	*Bacillus Amyloliquefaciens*	MH788971
2	RA37	*Bacillus Amyloliquefaciens*	MH788970
3	BFR2	*Bacillus Amyloliquefaciens*	MH788971
4	RR13	*Bacillus megaterium*	MH788974
5	BE1	*Bacillus velezensis*	MH788975
6	RA4	*Bacillus xiamenensis*	MH788976
7	LPR17	*Chryseobacterium indologenes*	MH788978
8	LPR11+	*Chryseobacterium indologenes*	MH788978
9	LPR4+	*Citrobacter fruendii*	MH788979
10	LGR26	*Enterobacter cloacae*	MH788981
11	RR19	*Klebsiella pneumoneae*	MH788983
12	PA2+	*Lelliottia minipressuralis*	MH788984
13	LGR12+	*Lelliottia minipressuralis*	MH788984
14	LGR12	*Lelliottia minipressuralis*	MH788984
15	LPR19	*Lynsinibacillus fusiformis*	MH788987
16	LPR20	*Paracoccus* sp.	MH788988
17	RA24	*Proteus peneri*	MH788991
18	PA3+	*Pseudomonas putida*	MH788990
19	RR20	*Pseudomonas putida*	MH788990
20	RR4	*Pseudomonas aeruginosas*	MH788989
21	RA33	*Stenotrophomonas maltophila*	MH788993
22	LPR6+	*Stenotrophomonas maltophilia*	MH788995
23	LPR1+	*Unidentified*	/
24	LPR3	*Unidentified*	/
25	LPR3+	*Unidentified*	/

II.1.2. Pathogenic fungi

The pathogenic fungus used in the present study was isolated from symptomatic maize plants exhibiting stalk rot, root rot, chlorosis, or stunting in commercial maize parcels in Cameroon's Centre Region (latitude 3° 42' N and longitude 11° 20' E). The isolate was grown for 7 days at

25°C and then kept at 4°C in a solution containing glycerol and PDB at a 1:1 ratio to avoid genetic changes.

II.1.3. Planting material and culture substrates

- **Maize seeds**

The maize seeds used in this study were from the variety BENEDICT CMS (Cameroon Maize Series) 8704 (Figure 9) provided by the Institute of Agricultural Research for Development (IRAD, Yaoundé) and are the most consumed variety of maize in Cameroon but are more sensitive to dry conditions.

The characteristics of the seeds were as follows: variety, CMS 8704; life cycle, 105-110 days; color, yellow; average yield, 6-8 t/ha; and ecological zone, humid forest **(Ekobo, 2006)**.

Figure 9: Maize seeds used during the greenhouse experiments, variety CMS 8704.

- **Sowing substrate**

The seeds were sown in garden soil substrate provided by the Department of Plant Biology of the University of Yaoundé 1 which was previously air-dried, passed through a 4-mm sieve, and then autoclaved two times at 121°C for 1 h.

Soil characteristics:

The soil pH was 5.89, and it was composed of CH_3COONa: 0.52 mm; Ca: 3.97 Cmol/kg; Mg: 0.92 Cmol/kg; K: 0.28 Cmol/kg; Na: 0.023 Cmol/kg; P: 1.54 ppm; Al: 0.43 Cmol/kg; Mn: 0.02 Cmol/kg; clay: 51.9%; and C/N: 11.17 **(Tchameni *et al.*, 2012)**.

II.2. Methods

II.2.1. Isolation and identification of the pathogen

II.2.1.1. Isolation of the pathogenic fungi

The pathogenic fungus of interest was isolated from young maize stems and roots exhibiting rot symptoms, collected from a commercial farm in Cameroon's Centre Region (4° 45 0 N, 12 0 0 E), as reported by **Gai *et al.*, (2017)** with minor changes. Diseased sections were cut into pieces (0.5-1 cm long), sequentially cleaned with tap water, disinfected with 70% ethanol (2 min) and 2% sodium hypochlorite (5 min), and then rinsed three times with sterile distilled water. Sterilized fragments were dried on blotting paper in a laminar flow cabinet before being seeded onto potato dextrose agar (PDA) plates supplemented with 500 mg/L chloramphenicol. The plates were sealed with parafilm tape and incubated (25 ± 2°C). After three days of incubation, mycelial tips emerging from the explants were subcultured on new PDA media until pure cultures were obtained. Strains that revealed morphological traits associated with the *Fusarium* genus were selected for the pathogenicity test.

II.2.1.2. Evaluation of the virulence of the selected isolates

The virulence of the selected isolates was evaluated on young maize plants using cornmeal sand for conidia proliferation according to **Grisham and Anderson, (1983)**. In brief, 200 g of autoclaved (121°C/30 min) cornmeal sand was added to flasks and inoculated with 4 cm diameter mycelial plugs from a seven-day-old *F. verticillioides* culture on PDA media. The flasks were shaken every two days to homogenize fungal growth, and the slurry was air-dried and ground to powder after 28 days of incubation at 25 ± 2°C. The resulting inoculum (colonized cornmeal) was carefully mixed with sterilized garden soil in a proportion of 5% and 10% (w/w), as described by **Sumner and Minton, (1989)**, with slight modifications. Next, the potting mix was placed in 1 kg plastic pots alongside the control set, which consisted of noninfested autoclaved cornmeal sand and garden soil. Maize seeds (CMS-8704 composite) were surface disinfected with 5% sodium hypochlorite for 5 min, washed three times with sterile distilled water, and seeded at 4 cm depth (four seeds per pot, 10 pots per treatment). The pots were then arranged in the greenhouse following a randomized block design and watered every two days. Emergence percentages were determined at pre- and postemergence stages from 3 to 21 days after sowing (das). The number of infected plants was counted, and the disease incidence was determined using formula (1) below.

Incidence = (Number of infected plants / Total number of plants) ×100 (1)

The degree of virulence of the isolates was evaluated based on the percentage of pre- and postemergence damping-off (which is an outcome of seeds that never germinated and germinated seeds with completely rotted shoots), as well as severity using a 0 to 5 disease rating scale described by **Mańka, (1989)** (Table III).

Table III: Scale for estimation of the virulence of *Fusarium* isolates

Scale numbers	Rating scale	Degree of virulence
0	0 - 10%	Not Virulent
1	11 - 20%	Very lowly Virulent
2	21 - 40%	Lowly Virulent
3	41- 60%	Moderately Virulent
4	61 - 80%	Highly Virulent
5	81 - 100%	Very Highly Virulent

II.2.1.3. Morphological identification of the virulent isolate

The most virulent fungus was identified using macroscopic and microscopic characteristics. A 5 mm agar plug from a seven-day-old fungus culture was deposited at the center of a PDA plate and macroscopic characteristics such as growth rate, mycelium aspect, and color were observed after 5 days. The strain was cultivated on carnation leaves agar (CLA) for microscopic examination, and the number of macroconidia and microconidia, the basal and apical morphologies, the number of macroconidia septations, the presence or absence of chlamydospores, and the disposition of microconidia were recorded and compared to those of the *Fusarium* identification keys **(Leslie and Summerell, 2006)** and **(Navi *et al.*, 1999)**.

II.2.1.4. Genomic characterization of the virulent strain

The most virulent isolate was subjected to genomic characterization based on the nucleotide sequence of the ITS1-5.8S rRNA-ITS2 region specific to every fungal species.

♣ ***DNA extraction***

DNA was extracted from mycelial pieces of a fungal culture cultivated on PDA following the method described by **Sánchez Márquez *et al.*, (2012)**. The pathogenic fungus was grown in potato dextrose broth (PDB) medium for three days under constant shaking (160 rpm), and the mycelia mats were recovered by filtering. Ten milligrams of mycelium were placed in 2 mL Eppendorf tubes and incubated with RNase and buffer to facilitate RNA breakdown. DNA was extracted using the commercial Red

Extract-N-Amp Plant PCR kit (Sigma Aldrich) according to the manufacturer's instructions. After incubating the sample at 95°C for 10 min, 200 µL of chloroform was added, and the mixture was centrifuged at 15000 rpm for 15 min. The supernatant was transferred to fresh 2 mL Eppendorf tubes, and 5 µL of potassium acetate and 100 µL of isopropanol were added before centrifuging the mixture. The supernatant was then discarded, and 100 µL of ethanol (80%) was added to wash the salts out before centrifuging at 10000 rpm for 10 min after which the supernatant was discarded. After drying for 10 min, the DNA was suspended in 100 µL of Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8,0) and refrigerated at -20°C.

♣ ***DNA amplification***

The internal transcribed spacer (ITS) of nuclear ribosomal DNA was used for PCR amplification of the ITS1-5.8S rRNA-ITS2 region using primers ITS4 (5′ -TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′ -GGAAGTAAAAGTCGTAACAAGG-3′) **(White, 2008)**. The two-time PCR Extract-N-Amp PCR ReadyMix, consisting of 10 µL of Taq polymerase master mix, 3 µL of primer mix (5 µmol/L each), 2 µL of genomic DNA, and 2 µL of double distilled water, was utilized. The thermal cycling parameters for amplification were as follows: initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s of denaturation, 58°C for 1 min of annealing, 72°C for 1 min of extension, and 72°C for 3 min of final extension. The resulting PCR amplicons were separated via agarose gel electrophoresis and subsequently purified.

♣ ***Agarose gel electrophoresis***

To ensure that the PCR was successful, the amplified DNA was separated by electrophoresis (Electrophoresis Power Supply-EPS 600) on a 1% agarose gel. For migration, a TEA (TrisHCl 1.6 mM; sodium acetate 1.6 mM; EDTA 0.04 mM; pH 8) buffer was used with a constant voltage of 70 volts applied for 80 min. A DNA ladder ranging from 250 to 10000 base pairs

was used as a marker. After migration, 2 µL of Midori green direct DNA stain was added to the gel, and the gel was exposed to a UV-transluminator (Fluor-S Multimager) to visualize the DNA as yellow bands.

- ***Sequences analysis***

The resulting PCR products were sequenced at the DNA sequencing service of the University of Salamanca, Spain. To assess the percentage of similarity, the sequences obtained were compared with reference strains from the National Centre for Biotechnology Information (NCBI) GenBank using the Basic Local Alignment Search Tool (BLAST) program. Sequences with more than 97% similarity were considered to belong to the same species.

II.2.1.5. Sequencing of the TEF 1-α gene

Given that TEF-1 is the most suitable gene for distinguishing closely related *Fusarium* species, we sequenced the translation-elongation factor-1 alpha gene (TEF-1α) to determine the identity of the so-called pathogen **(Mirhendi *et al.*, 2015)**. The primer pairs EF1 (50-ATGGGTAAGGARGACAAGAC-30) and EF2 (50-GGARGTACCAGTSATCATGTT-30) were used, and the PCR conditions were similar to those indicated previously (ITS), with the exception that the annealing step was conducted at 55°C instead of 58°C. Theresulting PCR amplicons were separated on a 1% agarose gel and purified according to the instructions provided by the manufacturer.

- ***Phylogenetic analysis***

The sequences generated were matched with reference sequences in the GenBank nucleotide database to determine the closest relatives for the phylogenetic investigations. The DNA sequences retrieved in this work, along with those obtained from GenBank, were aligned for each of the two loci (ITS and TEF1) using the ClustalW technique of the MEGA X software package. For the maximum likelihood phylogenetic analysis, the best evolutionary model for each partition was determined using MEGA X and added to the analyses. The maximum likelihood phylogenetic tree searches were performed in MEGA X with the tree bisection and reconnection (TBR) algorithm, with gaps considered missing data. The robustness of the topology was evaluated by 1000 bootstrap replications. Thus, the consensus sequences were searched in the NCBI nucleotide databases using BLAST and *FUSARIUM* -ID databases. The most plausible identity of the strain was recorded.

II.2.2. Evaluation of the antagonistic potential of the BCAs against *F. verticillioides* mycelial growth

II.2.2.1. Direct antagonism assay

The ability of BCAs to suppress *F. verticillioides* development in dual culture was tested *in vitro* **(Khalili and Kamyab, 2016)**. In brief, a 5 mm agar plug from a 3-day-old *F. verticillioides* culture was placed in the center of Petri plates containing a mixture of 50% PDA and 50% Luria Bertani agar (LBA). Next, 5 mm discs of Whatman (No. 1) filter paper were deposited at the four peripheric sides of the fungal plug and inoculated with 5 µL of each bacterial suspension (1.5×10^8 CFU/mL). Water was used instead of bacteria in the negative control plates. After 5 days of incubation, mycelial growth inhibition was calculated as given by formula (2):

$$I\ (\%) = [(C-T)/C] \times 100 \qquad (2)$$

where I (%) is the percentage of inhibition, C is the mycelial growth of *F. verticillioides* on the control plates, and T is the fungal growth in the presence of BCAs.

II.2.2.2. Determination of the antifungal potential of volatile organic metabolites

The inhibitory impact of volatile organic metabolites (VOMs) released by BCAs was evaluated **(Jayaswal *et al.*, 1993)**. Briefly, 24 h bacterial strains were streaked over LBA media. Then, mycelial plugs (5 mmϴ) taken from the growing edge of a three-day old *F. verticillioides* colony were centrally inoculated onto separate PDA plates. The BCAs and pathogen plates were superposed to place the organisms face to face, and sealed with parafilm tape. The control plates were made by superimposing uninoculated LBA media on *F. verticillioides* PDA plates. After 7 days of incubation at 25±2°C, mycelial growth inhibition was computed using formula (2) above.

II.2.3. Evaluation of the production capacity of hydrolytic enzymes by the BCAs

The ability of the twenty-five (25) bacterial strains to produce hydrolytic enzymes that can diffuse through the medium and destroy the principal components of the pathogen cell wall was determined by investigating cellulase, amylase, and protease synthesis.

II.2.3.1. Assessment of protease activity

The protocol outlined by **Saran *et al.*, (2007)** was used to show the capacity of the 25 BCAs to produce proteases for the hydrolysis of proteins present on the pathogen membrane. The culture medium consisted of a combination of 10 g of agar diluted in 100 mL of distilled water and 300 mL of 0.1 M citrate phosphate at pH 6.0. After autoclaving the mixture, 10 g of skim milk was aseptically added when the medium was still hot (50°C) to avoid coagulation and charring of the milk. The medium was then poured onto Petri plates to solidify, and the bacterial suspensions, which were set at 0.5 MacFarland standards, were spot (5 µL) inoculated onto skim milk agar media. After incubating the plates for four days, 10% tannic acid was added to the medium, and the formation of a clear zone surrounding the colonies indicated protease production. The diameters of the clear halos were measured, and the enzyme activity (EA) was computed according to the following formula (3)

$$EA = (HD-CD)/CD \quad (3)$$

where HD = diameter of the clear halo and CD = diameter of the clear BCA colony

II.2.3.2. Detection of cellulase activity

The capacity of bacterial strains to produce extracellular cellulase was evaluated using yeast peptone agar media, as reported by **(Gupta *et al.*, 2012)**. Culture medium was made by mixing 1 g of yeast extract with 0.5 g of peptone, 16 g of agar–agar, and 1000 mL of distilled water supplemented with 0.5% Nα-carboxymethyl cellulose (CMC). The mixture was sterilized at 120°C for 30 min before being poured onto Petri dishes. After the media had solidified, the bacterial strains were inoculated by placing the plates on the four edges of the plates, which were subsequently incubated for 4 days at 28°C. The formation of a clear zone surrounding the colony with the addition of 0.1% aqueous Congo red reagent and incubation for 15 min demonstrated positive cellulase activity. The width of the clear zone was measured, and the hydrolytic enzyme activity (EA) was calculated using formula (3) above.

II.2.3.3. Amylase production potential

The bacterial strain's capacity to produce amylase was evaluated using the procedure described by **Liu and Xu, (2008)**. Glucose yeast peptone agar was used for bacterial culture, consisting of a mixture of 1 g of glucose, 1 g of yeast extract, 0.5 g of peptone, and 16 g of agar and starch

(2%) dissolved in 1000 mL of distilled water. Following sterilization, the medium that flowed on the Petri dishes was used for bacterial strain culture for 4 days, after which the medium was flooded with iodine (1%), and the appearance of a clear zone around the colonies revealed a positive test.. The enzyme activity (EA) was calculated using the previous formula (3).

II.2.4. Determination of plant growth-promoting abilities

II.2.4.1. Determination of ammonia production

The ability of the bacterial strains to promote plant growth by producing a nitrogen source, which is essential for plant development, was evaluated by examining their ability to synthesize ammonia as described by **Ogbo and Okonkwo, (2012)**. In brief, a one-day-old bacterial strain culture was calibrated at 1.5×10^8 cells/mL, 30 µL of the bacterial suspension was distributed on a microplate containing 200 µL of peptone water (10%), and the plate was incubated for 72 h at 36 ± 2^0C. One microliter of Nessler reagent (potassium tetraiodomercurate (II)) was applied to the culture supernatant, and the development of a brown to yellow color indicated a positive test. Ammonia production was quantified using a microplate reader spectrophotometer (Infinite M200, Tecan) to measure the optical density at 450 nm. The experiment was conducted twice in duplicate. The ammonia concentration was determined using a $(NH_4)2SO_4$ standard curve at concentrations ranging from 1 to 10 mol/mL, and the results are expressed in mol/mL.

II.2.4.2. Evaluation of the phosphate solubilization ability

Phosphorous is a vital nutrient for plant development, and since soil acidity causes phosphate to complex, a biological control agent must be able to solubilize the complexed phosphate and enhance its availability to the plant. To assess this parameter, the method proposed by **Pikovskaya, (1948)** was used. Bacterial strains were cultured on solid pikovskaya agar and Tri-calcium phosphate (TCP) media, composed of $MgSO_4$. 7H2O (0.1 g), dextrose (10 g), yeast extract (0.5 g), glucose (13 g), $(NH_4)_2SO_4$ (0.5 g), KCl (0.2 g), $CaHPO_4$ (2.5 g), $MnSO_4$ (0.0002 g), NaCl (0.2 g), $FeSO_4$. $7H_2O$ (0.0002 g) and agar (15 g) in 1000 mL of distilled water. After five days of incubation, positive hydrolysis of complexed phosphate appeared as a clear zone around the colony whose diameter was measured and is expressed in millimeters (mm).

II.2.4.3. Determination of IAA potential

IAA (indole 3-acetic acid) is a phytohormone that is involved in practically every step of plant growth and development, including cell division, differentiation, and vascular bundle

formation, as well as the defense response. The protocol described by **Goswami *et al.*, (2013)** was followed to assess the bacterial strain's ability to produce this essential auxin. Bacteria were subsequently grown for 72 h in LB media supplemented with L-tryptophan (200 g/mL) and NaCl (2%). Following centrifugation, 150 µL of the supernatant was mixed with 100 µL of Salkowski reagent (12.5 mL of perchloric acid (70%), 0.5 mL of FeCl3 (0.5 M), and 12.25 mL of distilled water). The development of a pink color indicated successful IAA production, as measured by absorbance at 530 nm. A standard curve was used to determine IAA concentrations expressed as µg/ml.

II.2.4.4. Detection of siderophores production

The capacity of bacterial agents to produce iron-binding ligands known as siderophores that are able to bind ferric ions and make them available to the host plant under iron-deficient conditions was assessed by determining the production of three siderophores, hydroxamates, catecholates, and carboxylate, according to **Dave and Dube, (2000)**.

Briefly, bacterial strains were cultivated in liquid LB medium for 24 h and then centrifuged, and the supernatant filtrate was used to quantify siderophores. A mixture of culture filtrate and 2% ferric chloride was used for the quantification of hydroxamate and catecholate siderophores. The color shift from yellow to red revealed the presence of siderophores, as quantified by measuring the absorbance at 450 nm and 495 nm for the hydroxamate and catecholate siderophore types, respectively. For carboxylate quantification, the culture filtrate was mixed with 1 mL of CuSO4 and 2 mL of 250 µM acetate buffer, and the absorbance was measured at 280 nm to calculate the percentage of siderophores produced (Sid %), as described in formula (4):

$$\text{Sid (\%)} = [(ODt - ODc)/ODt] \times 100 \qquad (4)$$

II.2.5. Capacity of the bacterial agents to colonize maize seeds

Ahead of conducting an *in-planta* experiment to validate the biological potential of the BCA candidates against *F. verticillioides* on maize plants, the maize seed colonization potential of the *in vitro* selected strains was assessed.

II.2.5.1. Production of spontaneous resistant mutants

The selected bacterial strains were used to generate spontaneous rifampicin-resistant mutants by cultivating BCA on LB agar (LBA) plates supplemented with rifampicin. The minimal

inhibitory concentrations (MICs) of the bacterial strains were previously ascertained through their cultivation on solid media supplemented with various concentrations of antibiotics (400, 200, 100, 50, and 25 µg mL^{-1}). An MIC of 100 µg mL^{-1} was selected for further investigation. Initially, the bacterial strains were cultured in standard LBA media. Subsequently, a 1 mL suspension of mid-log phase bacteria was centrifuged at 8000 rpm for 3 min to pellet the cells, which were then resuspended in 3 mL of physiological saline solution. A 100 µL aliquot of the suspension was spread onto LBA plates containing 100 µg mL-1 rifampicin, and individual colonies were then transferred to fresh LBA plates supplemented with rifampicin. For subsequent use, the isolated mutants were kept at 4°C in LB-glycerol medium (v/v) supplemented with rifampicin at 100 µg mL^{-1} **(Enne *et al.*, 2004; Munjal *et al.*, 2016)**.

II.2.5.2. Determination of seed colonization by the bacterial strains

Under axenic conditions, sterilized maize seeds were inoculated with the respective mutant strains. To achieve this, the seeds were immersed in a bacterial solution calibrated at 3×10^8 cells/mL and incubated overnight, while saline water (0.9% NaCl) was used as a control. Afterward, seeds were placed on petri dishes lined with moistened tissue paper and allowed to germinate for 4 days. After the incubation period, young roots were harvested from the different treatments comprising the mutant strains and water for the control. The roots were surface disinfected with 2% sodium hypochlorite for 3 min and then rinsed three times with sterile distilled water. One gram of dried roots was mashed in 10 mL of sterile saline water using a sterile mortar and pestle and then diluted to 10^{-6} and 10^{-8}. Thereafter, 100 µL of each dilution was plated onto Petri dishes containing rifampicin-amended LBA media. The number of colony-forming units (CFU) was counted, and the colonization potential was quantified as CFU per gram of fresh roots **(Hallmann *et al.*, 2001)**.

II.2.6. *In planta* effect of bacterial treatments on *Fusarium* rot disease

Bacterial strains demonstrating promising *in vitro* biological activity against *F. verticillioides* growth and exhibiting plant growth-promoting characteristics were assessed for their ability to mitigate the impact of *F. verticillioides* on maize seedling stalks and root sections under greenhouse conditions.

II.2.6.1. Seed bacterization technic

Maize seeds of the CMS-8704 genotype were surface disinfected and pregerminated for two days in Petri dishes supplemented with two layers of humidified sterile filter paper.

Pregerminated seeds were subsequently immersed in a bacterial suspension calibrated at 3×10^8 CFU/mL for 6 h, whereas seeds designated for the control treatment were soaked in sterile saline water.

II.2.6.2. Substrate infestation, sowing and experimental layout

The *F. verticillioides* inoculum was prepared in sterile cornmeal sand and then mixed with garden soil at a rate of 10% (10 g of colonized cornmeal sand for 100 g of land soil), following the method outlined by **Sumner and Minton, (1989)** with slight modifications. The control substrates consisted of uninoculated cornmeal and garden soil. The inoculated and noninoculated substrates were then filled into plastic pots, with two maize seeds (previously treated with bacteria and water for the control) sown in each pot. Ten replicates were made up per treatment, resulting in a total of 120 pots. The pots were frequently watered for 28 days, after which the plants were harvested, and the data were recorded. The different treatments were as follows:

To: Control = uninoculated seeds and uninfected soil (plants grown under normal conditions);
T1: *F. verticillioides* = soil infected with *F. verticillioides* and uninoculated seeds;
T2: *Unidentified* LPR1+ = the soil was infected with *F. verticillioides*, while the seeds were colonized with the bacterial strain *unidentified* LPR1+;
T3: *L. minipressuralis* LGR12 = the soil was infected with *F. verticillioides*, while the seeds were colonized with the bacterial strain *L. minipressuralis* LGR12;
T4: *K. pneumoniae* RR19 = the soil was infected with *F. verticillioides*, while the seeds were colonized with the bacterial strain *K. pneumoniae* RR19;
T5: *B. megaterium* RR13 = the soil was infected with *F. verticillioides*, while the seeds were colonized with the bacterial strain *B. megaterium* RR13;
T6: *Unidentified* LPR3 = the soil was infected with *F. verticillioides*, while the seeds were colonized with the bacterial strain *Unidentified* LPR3;
T7: *B. amyloliquefaciens* RA37 = the soil was infected with *F. verticillioides*, while the seeds were colonized with the bacterial strain *B. amyloliquefaciens* RA37;
T8: *B. velezensis* BE1 = the soil was infected with *F. verticillioides*, while the seeds were colonized with the bacterial strain *B. velezensis* BE1;
T9: *C. indologenes* LPR17 = the soil was infected with *F. verticillioides*, while the seeds were colonized with the bacterial strain *C. indologenes* LPR17;

T10: *S. maltophilia* LPR6+ = the soil was infected with *F. verticillioides*, while the seeds were colonized with the bacterial strain *S. maltophilia* LPR6+;

T11: *B. amyloliquefaciens* BFL1 = the soil was infected with *F. verticillioides*, while the seeds were colonized with the bacterial strain *B. amyloliquefaciens* BFL1.

Experimental layout

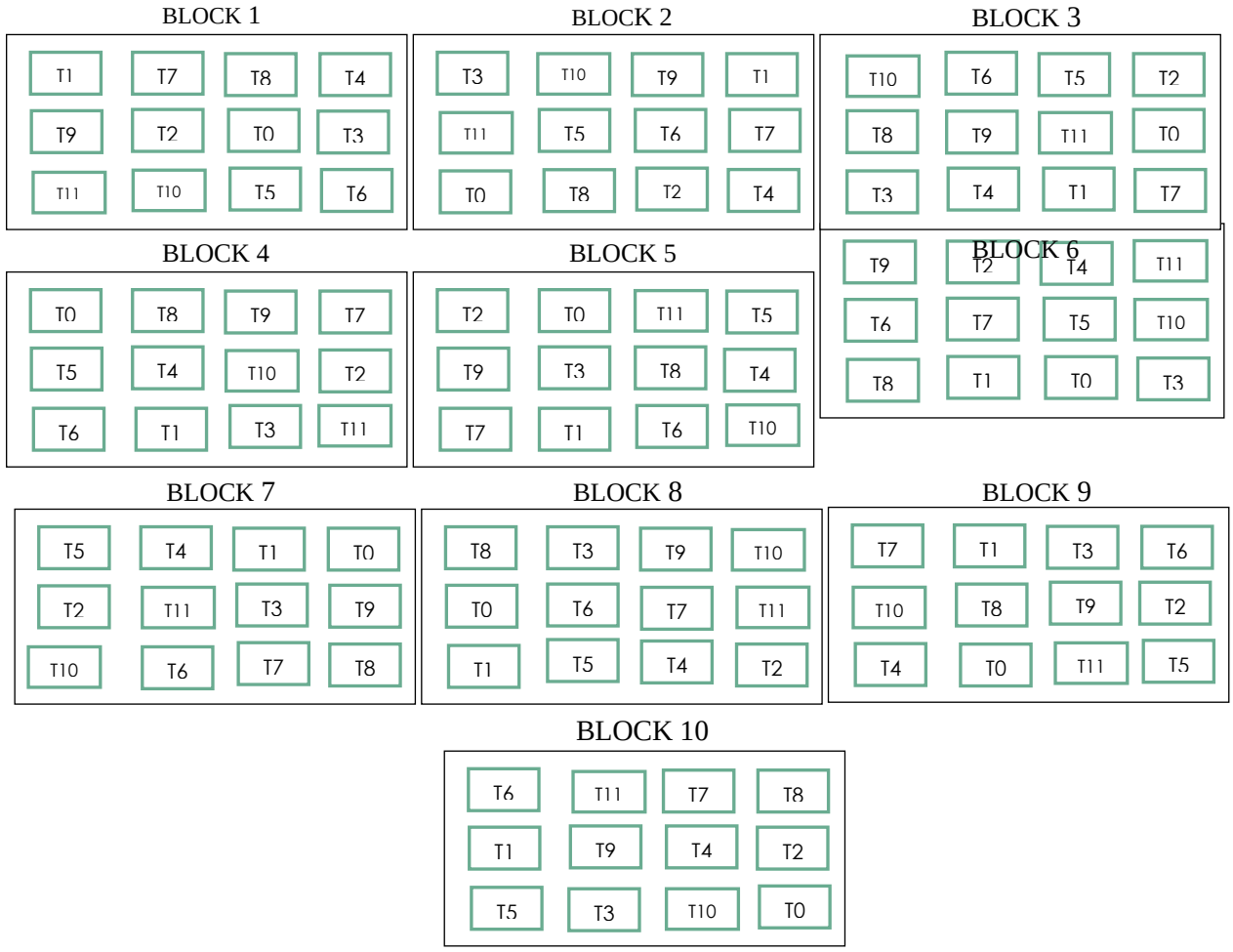


Figure 10: Experimental design assessing the protective potential of the bacterial agents against *Fusarium* rot in the greenhouse.

The experiment was carried out on a greenhouse bench utilizing a completely randomized block design, comprising 12 treatments. These treatments included an uninoculated control, the pathogen alone (*F. verticillioides*), and plants infected with *F. verticillioides* and treated with each of the ten biological control agents (BCAs). Each treatment was replicated 10 times. The experiment was divided into 10 blocks, denoted as Blocks 1 to 10, representing the different

repetitions. Each block contained all twelve treatments (To to T11) described above, which were arranged randomly.

II.2.6.3. Evaluation of the disease severity and agromorphological parameters of plants

Plants were carefully uprooted on days 11, 15, 19, 23, 27, 31, and 35 after sowing. Upon uprooting, they were cleaned with tap water, and the disease severity (DS) was determined using formula (5). The reduction in disease severity (RDS) was computed based on formula (6). Disease severity was evaluated using a six-class scale described by **Ferniah *et al.*, (2014)**, where 0 represents healthy roots and epicotyls, and 5 indicates either nongerminated seeds or completely rotted roots and shoots.

$$\text{Disease Severity (DS)} = \frac{\sum(\text{Severity scale} \times \text{Number of plant of each scale})}{\text{The highest scale} \times \text{Total number of plants}} \quad (5)$$

The reduction of the disease severity (RDS) was calculated using the following formula (6):

$$RDS = [(DS_{control} - DS_{treatment}) / DS_{control}] \times 100 \quad (6)$$

Morphological parameters such as plant root and shoot lengths (Cm) and dry weights (g) were recorded. The plant lengths were obtained using a graduated ruler while dry weights were obtained by drying the samples at 60°C for 72 h until a constant weight was achieved.

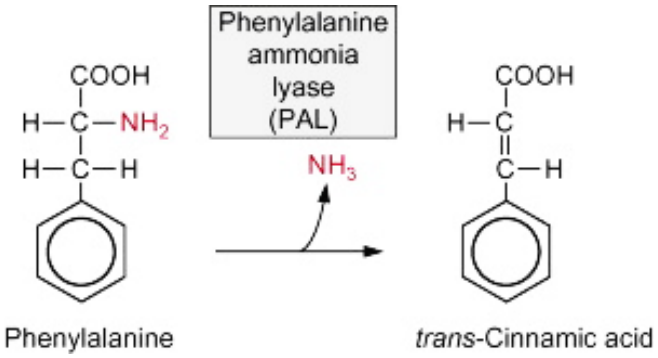
II.2.6.4. Assessment of some disease-related biochemical markers

- **Phenylalanine ammonia lyase activity (EC 4.3.1.24)**

The enzyme phenylalanine ammonia-lyase (PAL) plays an important role in the intersection of primary and secondary metabolism in plants. Its activity is typically triggered by plant pathogen infections, serving as part of the plant's defense mechanism.

Principle

Phenylalanine ammonia-lyase, catalyzes the deamination of phenylalanine, leading to the formation of a carbon-carbon double bound, resulting in trans-cinnamic acid and the release of ammonia (NH_3). The trans-cinnamic acid produced can be quantified spectrophotometrically at 290 nm.



Method

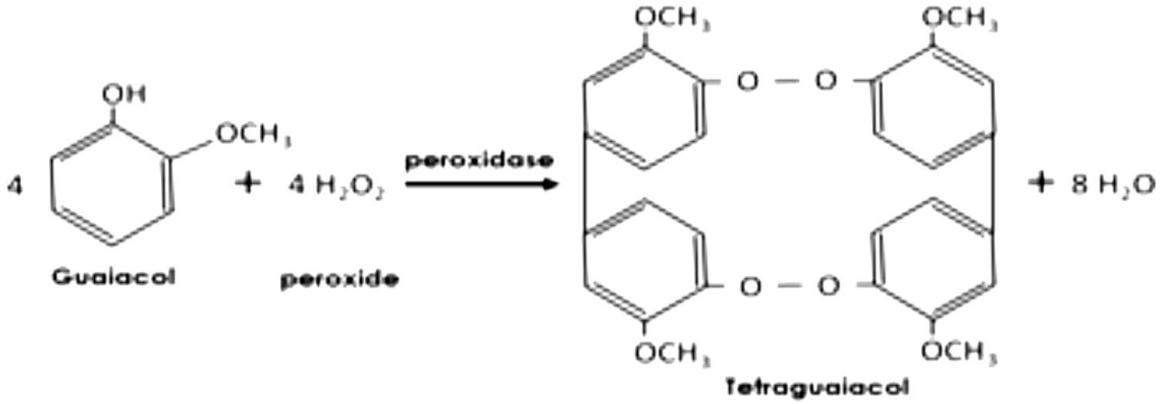
One gram of fresh root and shoot parts from plants subjected to different treatments was crushed in 50 mM potassium phosphate buffer (pH 7.2) and then centrifuged at 5,000 rpm/min at 4°C to obtain the enzymatic extract required for assessing PAL activity **(Whetten and Sederoff, 1992)**. Fifty microliters of the enzymatic extract was mixed with 150 µL of phosphate buffer (50 mM; pH 7.2) and 50 µL of phenylalanine (50 mM) followed by incubation at room temperature for 1 hour. The reaction was halted by adding 2 N HCL, and the optical density (OD) was measured at 290 nm. The PAL specific activity was measured in terms of an increase in OD and expressed as ΔOD)/minute/ gram fresh weight.

- **Guaiacol peroxidase activity (EC 1.11.1.7)**

The capacity of the BCAs to alleviate the stress induced by *F. verticiloides* invasion on plant cells was evaluated by assessing peroxidase-specific activity. The activity of guaiacol peroxidase (GPX), an antioxidant enzyme involved in detoxifying hydrogen peroxide (H_2O_2), was measured following the method described by **Paglia and Valentine, (1967)**.

Principle

Peroxidase, specifically guaiacol peroxidase catalyzes the oxidation of guaiacol (2-methyoxyphenol) or the reduction of hydrogen peroxide (H_2O_2) to water and tetraguaiacol, a brown product that can be quantified spectrophotometrically at 470 nm.



Method

To quantify the activity of guaiacol peroxidase, a solution consisting of 143 µL of guaiacol buffer (50 mM phosphate buffer at pH 7 and 9 mM guaiacol), 2.5 µL of hydrogen peroxide (H_2O_2), and 10 µL of enzymatic extract was prepared. Subsequently, the optical density (OD) of the produced tetraguaiacol was measured at 470 nm. The GPX activity was then expressed as the ΔOD. min-1 g-1 fresh weight.

II.2.7. Evaluation of the bacterial growth capacity under drought conditions

II.2.7.1. Drought tolerance capacity

The ability of bacterial isolates to grow under increasing concentrations of PEG 6000 as a water stress inducer was investigated as reported by **Forchetti *et al.*, (2007)**. In a microplate containing 100 µL of Lurea Broth (LB) medium supplemented with PEG 6000 at different concentrations (0, -0.05, -0.15, -0.49 and -1.79 MPa which correspond to 0%, 5%, 10%, 20% and 40% PEG respectively), 100 µL of a log phase bacterial culture calibrated at 0.5 McFarland (1.5×10^8 conidia/mL) was added and the plates were incubated under a constant shaker (150 rpm). Cell growth was evaluated every 2 h for 12 h by measuring the optical density at 600 nm using a spectrophotometer (Lecteur Infinite M200, Tecan).

II.2.7.2. Ability of the bacteria strains to form biofilms

To withstand severe environmental conditions, bacteria often create a matrix called a biofilm, which is composed of extracellular compounds such as proteins, DNA, and carbohydrates. The protocol described by **Ansari *et al.*, (2021)** was used with some modifications to assess the ability of bacterial agents to use the biofilm matrix as a drought resistance mechanism. In fact, early log phase bacterial cultures calibrated at 1 McFarland were added to each 96-well plate

containing 150 µL of LB culture medium supplemented with PEG 6000 at -1.79 MPa, and the mixture was cultured for 72 h. As a blank control, bacteria-free media was used. The supernatant was carefully discarded after the incubation period, and the wells were washed twice with sterilized distilled water to remove nonadherent cells. The plates were then treated with 100 µL of 0.1% crystal violet for 15 min before being removed. The wells were further washed twice with sterilized distilled water before 100 µL of 95% ethanol was added, and the optical density was measured at 560 nm to quantify the biofilms. Biofilm formation was defined as an OD $\geq$ 0.1, weak biofilm formation was defined as 0.1 $\geq$ OD 0.2, moderate biofilm formation was defined as 0.2 $\geq$ OD > 0.3, and strong biofilm formation was defined as OD $\geq$ 0.3 **(Naves *et al.*, 2008)**.

II.2.7.3. Salt tolerance capacity of the bacterial agents

The growth performance of the bacterial agents was assessed under salt stress conditions by monitoring the bacterial titer on LB (Luria Bertani) broth media supplemented with 200 mM NaCl in the test wells and 0 mM NaCl in the controls. One hundred microliters of LB broth medium supplemented with NaCl at the required concentrations was seeded into 96 wells of a microtiter plate. Next, 100 µL of bacterial cell suspensions with a titer of 10^8CFU/mL were added and incubated at 30°C. After 24 h, the optical density at 600 nm (OD_{600}) was measured **(Ali *et al.*, 2022b)**.

II.2.7.4. Ability to produce ACC deaminase encoding genes

PCR was used to detect the gene encoding ACC (1-aminocyclopropane-1-carboxylic acid) deaminase **(Jaya *et al.*, 2019)**. In brief, plasmid DNA was recovered from bacterial cultures by alkaline lysis and precipitation of DNA and proteins, after which the DNA was purified. Purified DNA (5 µL) was mixed with 2.5 µL of crystal buffer (10X), 2.5 µL of dNTPs (2 mM), 1.5 µL of each primers F and R (22 mM), 15 µL of distilled water, and 0.2 µL of Taq polymerase **(Lane, 1991)**. A thermal cycler was used for amplification, and 35 cycles of the following conditions (Table IV) were implemented:

Table IV: Steps and conditions of the PCR cycles used to detect the gene for ACC deaminase production

Steps	Temperature	Time
1. Predenaturation	95°C	3 min
2. Denaturation	95°C	45 s
3. Annealing	Variable	1 min
4. Polymerization	72°C	1.5 min
5. Extension	72°C	7 min

The temperature of the annealing step depends on the nucleotide chain of the primers and for this case, was 52°C.

- *Agarose gel electrophoresis*

At the end of the amplification phase, the PCR products were detected by migration on a 1% agarose gel, using electrophoresis with a constant voltage of 80 volts for 30 min. A DNA ladder ranging from 100 to 3000 base pairs was used as a marker. After migration, a geldoc Go image system was used to visualize migration bands. The ACC deaminase gene showed migration bands at 700 base pairs (bp).

II.2.8. Effect of bacterial agents on drought mitigation *in planta*

II.2.8.1. Culture substrate preparation and seed sowing

Soil sand provided by the Department of Plant Biology of The University of Yaounde 1 (Cameroon), was sifted, autoclaved twice at 121°C for 30 min, weighed and the same mass was added to each pot. The water holding capacity of each pot was determined by watering the pot containing soil, drop by drop until the first drop of water fell and, the entire amount of water used was measured and set as the total holding capacity of the pot.

Maize seeds (CMS-8704), from the seed bank of the National Institute for Agricultural Research and Development (IRAD) Yaounde, Cameroon were sorted, sterilized with 5% sodium chloride (2 min), rinsed three times with sterile distilled water and then dried. Sterile seeds were steeped for 12 h in a bacterial solution calibrated at 0.5 McFarland before being transferred to pots.

II.2.8.2. Drought stress imposition

The treatments consisted of inoculating seeds with the ten bacterial strains. Each treatment was replicated in 10 pots and uninoculated seeds were used as the control. Pots were well watered for 11 days to start with three leaf-stage seedlings which were then allowed to dry for 2 days. Drought treatment was implemented by maintaining the soil at 20% water holding capacity whereas the unstressed control was watered at 100% holding capacity. Plants were observed daily for signs of water stress (such as leaves chlorosis, rolling, and stunting) and 6 days later, physiological, morphological, and biochemical markers associated with stress were recorded as described by **Zhang *et al.*, (2018)** with slight modifications. The treatments included the following (Table V):

Table V: Treatments used in greenhouse assays for drought implementation and evaluation of the capacity of bacterial strains to induce tolerance in maize

Treatments	Watering	Seeds inoculation
T0: Unstressed	100% soil holding capacity	Uninoculated seeds
T1: Drought	20% soil holding capacity	Uninoculated seeds
T2: Drought + *Unidentified* LPR1+	20% soil holding capacity	Seeds inoculated with ***Unidentified* LPR1+**
T3: Drought + *L. minipressuralis* LGR12	20% soil holding capacity	Seeds inoculated with ***L. minipressuralis* LGR12**
T4: Drought + *K. pneumoneae* RR19	20% soil holding capacity	Seeds inoculated with ***K. pneumoneae* RR19**
T5: Drought + *B. megaterium* RR13	20% soil holding capacity	Seeds inoculated with ***B. megaterium* RR13**
T6: Drought + *Unidentified* LPR3	20% soil holding capacity	Seeds inoculated with ***Unidentified* LPR3**
T7: Drought + *B. amyloliquefaciens* RA37	20% soil holding capacity	Seeds inoculated with ***B. amyloliquefaciens* RA37**
T8: Drought + *B. velezensis* BE1	20% soil holding capacity	Seeds inoculated with ***B. velezensis* BE1**
T9: Drought + *C. indologenes* LPR17	20% soil holding capacity	Seeds inoculated with ***C. indologenes* LPR17**
T10: Drought + *S. maltophilia* LPR6+	20% soil holding capacity	Seeds inoculated with ***S. maltophilia* LPR6+**
T11: Drought + *B. amyloliquefaciens* BFL1	20% soil holding capacity	Seeds inoculated with ***B. amyloliquefaciens* BFL1**

Experimental layout

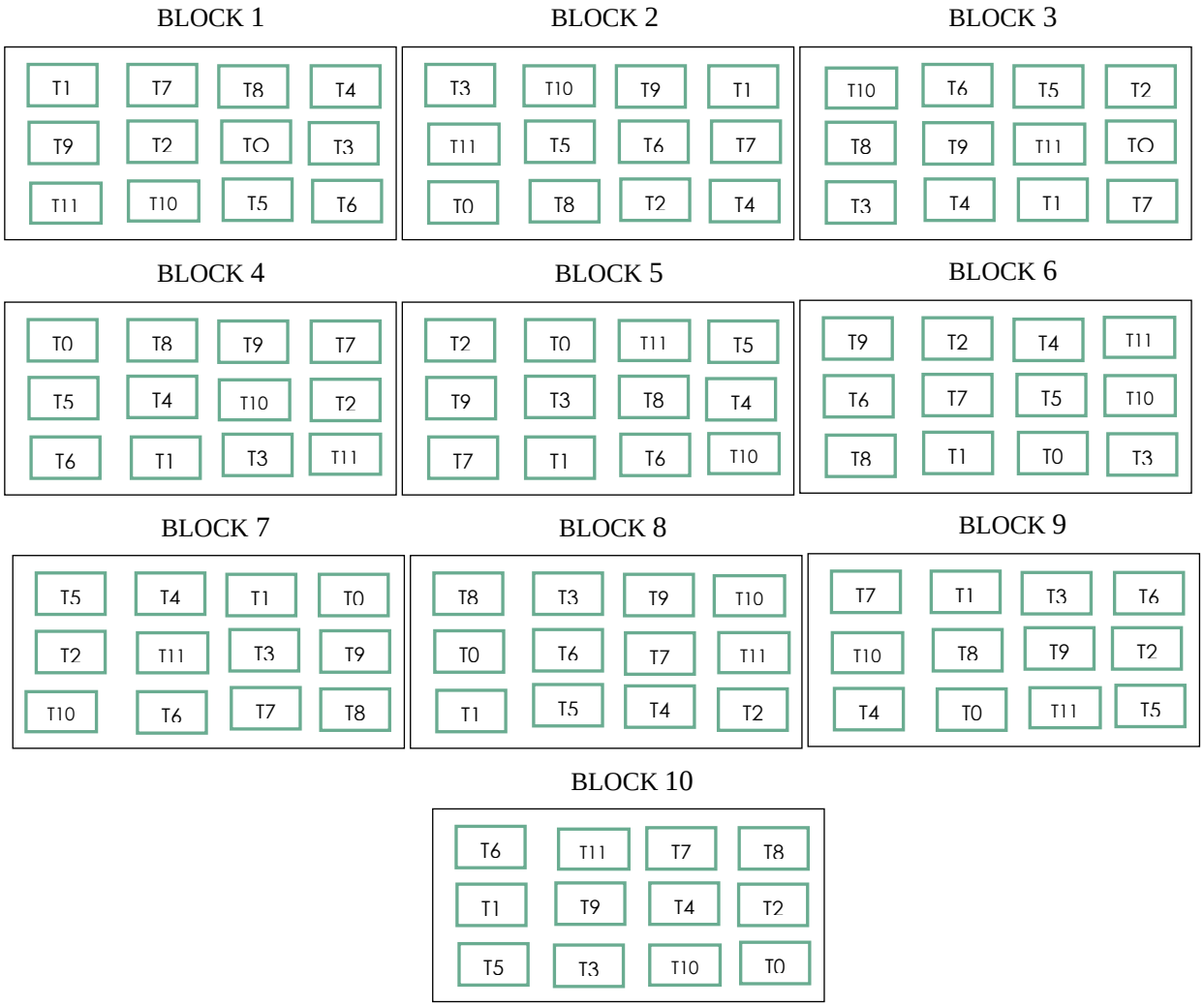


Figure 11: Experimental design for assessing the protective potential of bacterial agents against drought stress in a greenhouse

The experiment was carried out on a greenhouse bench using a randomized block design with 12 treatments, including a normal control, a drought control, and plants subjected to drought and treated with each of the 10 BCAs. The groups design consisted of 10 blocks with each of the various treatments, and each treatment consisted of two maize seeds in a pot, for a total of 120 pots.

II.2.8.3. Assessment of the morphological plant parameters under drought conditions

After the plants were harvested, agromorphological parameters, including plant height and fresh weight, were recorded as follows:

The plant height (Cm) was measured from the end of the principal root to the hypocotyl for the root height and from the hypocotyl to the top of the shoot part of the plant for the shoot height.

Fresh mass (g): obtained by weighing the freshly collected root and shoot material using a sensitive balance.

II.2.8.4. Assessment of physiological parameters under dought conditions

- **Leaf area, rolling and chlorosis**

At the end of the experimental days, five plants were randomly selected from each treatment and leaf physiological parameters such as chlorosis were recorded, which consisted of counting the number of leaves on each plant with intervene or entire yellowing. The number of rolled leaves on each plant leaf was also recorded. The length and width of the third fully expanded leaf (from the top) of each of the five plants in the various treatments were measured **(Mananze *et al.*, 2018)**, and the leaf area (LA) was calculated using formula (7) below **(Montgomery, 1911)**.

$$LA = \alpha \times L \times W \quad (7)$$

where: LA = leaf area; L = leaf length, W = leaf width and α = rectangle weight factor equal to 0.75.

- **Chlorophyll estimation**

The impact of drought on leaf chlorophyll content was evaluated using three distinct sides of the third fully established leaf (from the top) of each of the five plants taken under the various treatments. For chlorophyll extraction, 0.5 g of fresh leaves were ground, homogenized in 5 mL of 80% (v/v) acetone solution and incubated in the shade for 48 h. The homogenate was centrifuged at 500×g for 5 min and optical densities were measured at 645, 663 and 470 nm to quantify the total chlorophyll, chlorophyll a, b and carotenoid contents using **(Lichtenthaler and Wellburn, 1983)** equations (8, 9, 10 and 11).

Total chlorophyll (mg g^{-1} FW) = ((20.2 × A_{646} + 8.02 × A_{663}) × V) / (W × 1000)	(8)
Chl a (mg^{-1} ml) = [(12.21A_{663} - 2.81 A_{646}) × V)/W]	(9)
Chl b (mg^{-1} ml) = [(12.21A_{646} - 5.03A_{663}) × V)/W]	(10)
Carotenoids (mg^{-1} ml) = [((1000A_{470} - 3.27Chl a -104Chl b) /227) × V)/W]	(11)

were: A = absorbance at specific wavelengths; V = volume of chlorophyll extract (5 mL) and W = leave fresh weight (500 mg).

- **Relative water content**

The procedure described by **(González and González-Vilar, 2003)** was used to calculate the relative water content of the plant leaves. Ten leaves were taken from five plants in each treatment and weighed immediately to obtain the fresh weight (FW). The leaves were subsequently soaked in distilled water (4°C) for 24 h before being weighed again to determine the turgor weight (TW). Finally, the leaves were dried for 24 h at 50°C to yield the dry weight (DW), and the relative water content (RWC) was calculated using formula (12):

RWC (%) = (FW – DW/TW – DW) × 100	(12)

II.2.8.5. Biochemical parameters

- **Effect of bacterial priming on enzyme activity**

The previously prepared enzyme extracts were used to determine the specific activity of the antioxidant enzymes catalase (CAT) and guaiacol peroxidase (GPX).

- ✓ ***Enzymatic extract preparation***

After the plants were harvested, the root and shoot parts were washed and smashed and enzymes were extracted using the methodology described by **Tarafdar and Marschner, (1994)**. In brief, 5 mL of phosphate buffer (50 mM; pH 7) was added to 1 g of ground plant tissue and centrifuged at 5000 rpm for 10 min. The recovered supernatant served as the whole enzymatic extract for the dosage of specific enzymes.

✓ ***Catalase specific activity (EC 1.11.1.6)***

Principle

Catalase activity can be measured by following the decomposition of H_2O_2 by catalase to water and oxygen. This can be followed directly by a decrease in extinction per unit time at 240 nm.

Method

The capacity of catalase content in the enzymatic extract to reduce H_2O_2 in water molecules was evaluated according to the method described by **(Aebi, 1984)**. The reaction consisted of a mixture of 100 µL of potassium phosphate buffer (50 mM; pH 7), 30 µL of enzyme extract and 2 µL of H_2O_2 (0.3%). The reduction of H_2O_2 was measured at 240 nm and the specific activity was expressed as OD/min/g FM.

✓ ***Guaiacol peroxidase (EC 1.11.17)***

Guaiacol peroxidase (GPX) is an enzyme that facilitates the oxidation of guaiacol by H_2O_2 to tetraguaiacol. The enzyme activity was measured by spectrophotometric measurement of tetraguaiacol produced at 470 nm. The solution contained 150 µL of guaiacol buffer (95% potassium phosphate buffer, 5% guaiacol), 2.5 µL of H_2O_2, and 10 µL of enzyme extract. Compared with that in the unstressed control, the increase in the amount of tetraguaiacol produced by plants indicated an increase in GPX synthesis, and enzyme activity was quantified as the change in absorbance per time unit per g of fresh material **(Paglia and Valentine, 1967)**.

✓ ***Effect of bacterial priming on total phenols and flavonoids concentration***

Phenolic compounds are the most extensively distributed secondary metabolites involved in the plant response to stress and were extracted from plants using a modified approach of **Conde *et al.*, (1995)**.

Principle

Phenol estimation using Folin-Ciocalteu reagent (a mixture of phosphomolybdate and phophotungstate), is a colorimetric method based on the reduction of Folin-Ciocalteu with phenolic compounds in an alkaline medium to form a blue-colored complex that increases the absorbance between 550 and 750nm.

Method

In brief, 0.5 g of dried samples (from stressed plants, plants stressed and treated with the 10 bacterial agents, and unstressed plants) was added to methanol (80%) under acidified conditions (0.1% HCl) for 2 h and centrifuged at 1800 × g for 15 min. The collected supernatant served as enzymatic extracts for total phenol and flavonoid quantification **(Magwaza *et al.*, 2016)**.

The total phenol content was determined using the Folin-Ciocalteu reagent, as described by **Akkol *et al.*, (2008)**. A combination of 20 µL of enzyme extract, 100 µL of Folin Ciocaltheu reagent (10%), and 80 µL of sodium carbonate solution (20%) was used in the process. The absorbance was measured at 725 nm after 2 h of incubation. A gallic acid standard curve was used and the phenol content was expressed as the OD µg equivalent gallic acid/mg dry weight.

Flavonoid quantification consisted of their precipitation in methanol extract using formaldehyde (8 mg/mL) and 50% HCl in proportions of 2:1:1, and the previous methodology of **Akkol *et al.*, (2008)** was used to quantify nonflavonoid phenols. The flavonoid content was calculated by subtracting total phenols from nonflavonoid phenols and was expressed as the OD µg of Eq gallic acid/mg DW.

II.2.9. Effect of bacterial agents on *Fusarium* rot under drought stress conditions

To assess the ability of BCAs to induce resistance against *F. verticillioides* rot under drought stress conditions, BCAs with the ability to induce maize resistance against *F. verticillioides* infection and to stimulate resistance against drought were selected. Maize seeds were surface disinfected as previously stated, pregerminated, and soaked in the bacterial suspension before being seeded in soil previously infected by *F. verticillioides*, and the preliminary drought stress protocol was followed. The various treatments are presented in Table VI:

Table VI: Treatments used in the greenhouse assay to evaluate the capacity of bacterial strains to protect maize against combined drought stress and *F. verticillioides* infection

Treatments	**Watering**	**Seeds inoculation**	**Soil infestation**
T0: Control	100% soil holding capacity (SHC)	Uninoculated seeds	Uninfected soil
T1: Drought	20% SHC	Uninoculated seeds	Uninfected soil
T2: *F. verticillioides*	100% SHC	Uninoculated seeds	With *F. verticillioides*
T3: Drought + *F. verticillioides*	20% SHC	Uninoculated seeds	With *F. verticillioides*
T4: D+Fv+ Unidentified LPR1+	20% SHC	Seeds inoculated by **Unidentified LPR1+**	With *F. verticillioides*
T5: D+Fv+ *B. megaterium* RR13	20% SHC	Seeds inoculated by ***B. megaterium* RR13**	With *F. verticillioides*

D+Fv+Unidentified LPR1+ and D+Fv+ *B. megaterium* RR13 represent the plants submitted to drought, inoculated with *F. verticillioides*, and treated with the bacterial agents Unidentified LPR1+ and *B. megaterium* RR13 respectively; SHC is the soil water holding capacity, the proportion of water in soil as referring to the total holding capacity of each pot.

Experimental layout

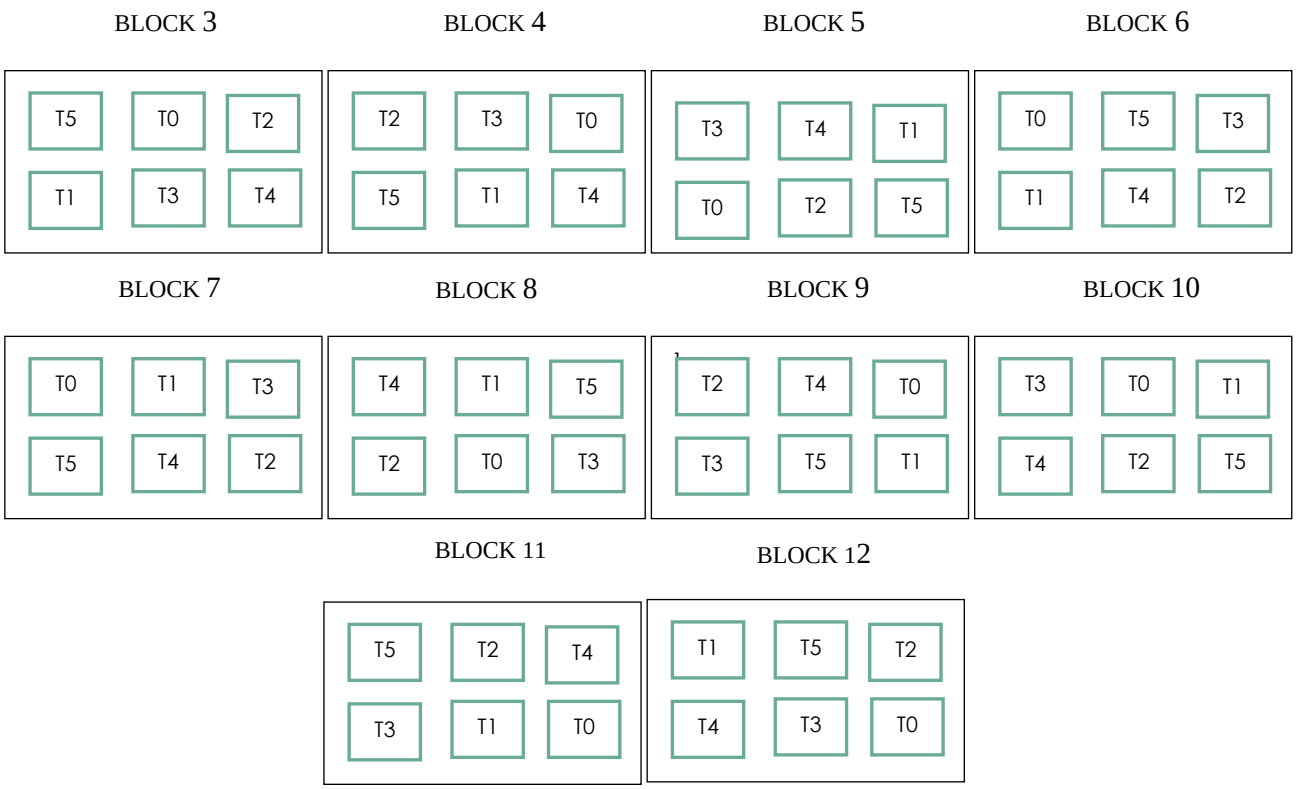


Figure 12: Experimental design for determining the protective potential of the bacterial agents against combined drought stress and *Fusarium* rot in greenhouse

The experiment was carried out on a greenhouse bench using a totally randomized block design with 6 treatments: a normal control; a drought control; an *F. verticillioides* control, a control of infected (by *F. verticillioides*) and drought-stressed plants; and plants infected by *F. verticillioides*, stressed and treated with the bacterial strains *B. megaterium* RR13 and *unidentified* LPR1+. The groups design consisted of 10 blocks with each of the various treatments, and each treatment consisted of two maize seeds in a pot, for a total of 60 pots.

II.2.9.1. Morphological physiological and biochemical parameter recording

Plants were harvested at the end of the drought period and all the different parameters previously collected for *F. verticillioides* infection and for drought resistance were recorded. Additionally, proline content and electrolyte leakage were determined:

- ✓ Physiological parameters: leaf area, curling, chlorosis, chlorophyll content, relative water content (recorded as described previously) and electrolyte leakage

- ✓ Biochemical parameters: PAL, catalase, GPX activities (determined as described previously) and proline content
- ✓ Morphological parameters: included shoot and root length and fresh and dry mass (recorded as described previously).

II.2.9.2. Electrolyte leakage

One of the damages caused by drought in plants is the destabilization of the cell membrane which can be monitored by the quantification of electrolytes released into solution. Electrolyte leakage (EL) was evaluated according to **Dionisio-Sese and Tobita, (1998)**.

Principle

Plant tissues are immersed in water and the level of electrolytes that leak from cells to the water can be measured by the conductivity of the solution.

Method

Inside test tubes containing distilled water, 200 mg leaf fresh weight of each treatment (Table VI) was introduced at 25°C for 2 h and the conductivity owing to electrolyte extraction from extracellular spaces (EC1) was measured using an electrical conductivity meter. In another test tube, 200 mg of fresh leaf weight was added to distilled water, boiled for 30 min, and then cooled to 25°C, and the conductivity owing to the extraction of all the electrolytes contained within the cells (EC2) was calculated. The electrolyte leakage was calculated as EL = (EC1/EC2) × 100.

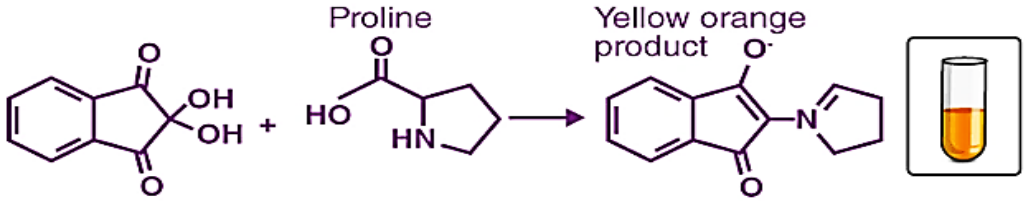
II.2.9.3. Estimation of proline content

Proline was quantified as an important trait for assessing the ability of bacterial strains to induce drought tolerance in maize after *Fusarium* infection. Free proline accumulation was detected in the fresh parts (roots and shoots) of plants infected with *F. verticillioides* and subjected to drought stress using **Bates *et al.*, (1973)** method.

Principle

Proline extracted with sulfosalicylic acid reacts with acid ninhydrin to form a yellow–orange complex that absorbs at 520 nm. In brief, ninhydrin can react with an amino acid via oxidative deamination to produce CO2, ammonia and an aldehyde associated with hydrantin. Thus, the

released ammonia starts a second reaction with another ninhydrin molecule to form a blue complex or yellow orange if it involves an iminoacid such as proline.



Method

In brief, 0.05 g of fresh material from each treatment (Table VI) was homogenized with sulfosalicylic acid and incubated for 72 h to facilitate proline release. After filtering through Whatman paper No. 2, the homogenate was treated with 200 µL of acid ninhydrin and 200 µL of glacial acetic acid. The mixture was boiled for 1 h and cooled in an ice bath, and 400 µL of toluene was added to extract the chromophore from the aqueous phase. The absorbance was read at 520 nm using a UV–visible spectrophotometer. A standard curve was built using proline solutions ranging from 0.5 to 2.5 mM, which helped to determine the proline concentration in the various samples, as shown in formula (13):

$$\text{µmoles proline/g fresh weight material} = \frac{[(\text{µg proline/ml} \times \text{ml toluene})/115.5\ \text{µg/µmole}]}{[(\text{g sample})/5]} \quad (13)$$

II.2.10. Statistical analysis

Raw data collected from the experiments were analyzed using analysis of variance (ANOVA) with OriginLab statistical and GraphPad Prism 8 softwares. The difference between the means was compared by Tukey's multiple comparison-based tests at the 5% level of significance. Principal component analysis and Pearson correlation were used to analyze the relationships between drought and infection-related parameters. The correlation coefficient (r) determined the strength of the relationships. Data were presented as the average mean ± standard deviation (SD) of experiments performed in triplicate.

CHAPTER III:

RESULTS AND DISCUSSION

CHAPTER III: RESULTS AND DISCUSSION

III.1. RESULTS

III.1.1. BIOLOGICAL CONTROL POTENTIAL OF DESERT-SPURGE (*E. ANTIQUORUM*) DERIVED ENDOPHYTIC BACTERIA AGAINST *F. VERTICILLIOIDES*.

III.1.1.1. Virulence of the isolated fungi strains

A total of five fungi isolates were obtained from maize rotted stems and roots with morphological characteristics of the *Fusarium* genus, including white, yellow and pink mycelia with growth rates ranging from five to six days necessary to cover a 90 mm PDA plate and the development of banana-like macroconidia during microscopic observation. Virulence towards a susceptible maize variety was assessed to determine the virulence of *Fusarium* isolates. Key symptoms of the *Fusarium* stem, and root rot disease were recorded as follows: seed germination inhibition (Figure 13a), sudden post-emergence seedling death (Figure 13b), stunted seedlings, leaf yellowing (Figure 13c), and reduced root system, were recorded. After a final count of 28 DAS, the isolate that presented very high virulence with 100% mean disease incidence and a severity score of 4 was selected for further studies (Figure 13).

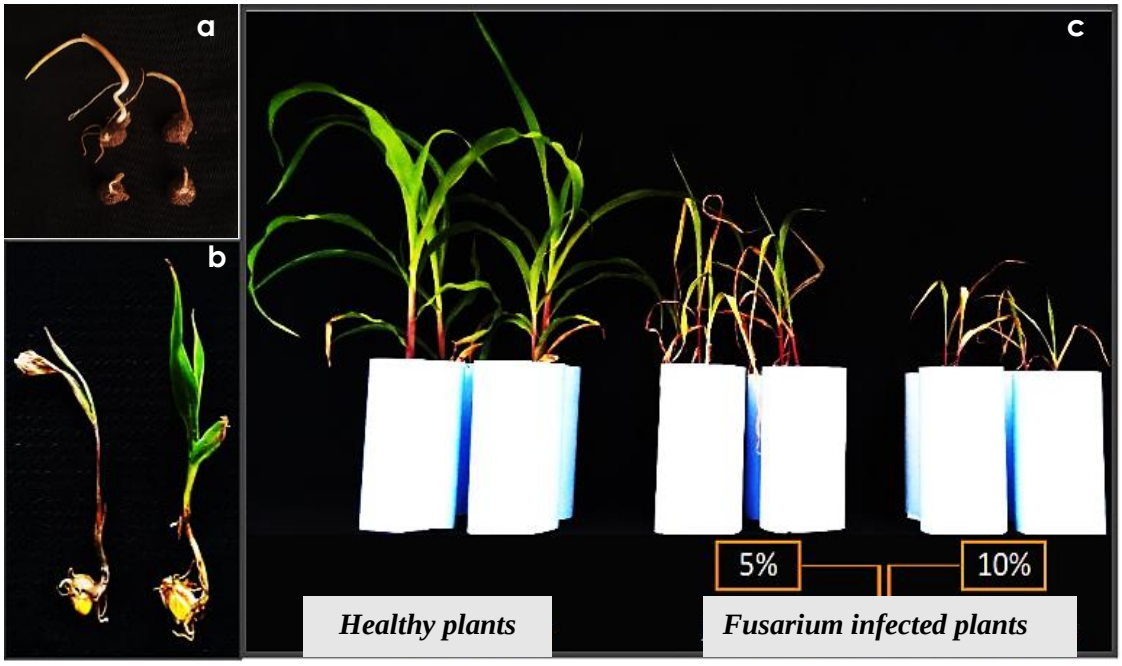


Figure 13: Symptoms of *F. verticillioides* on maize emergence and vegetative growth stages.
a) *F. verticillioides*-infested seeds with inhibited germination. b) Preemergence damping-off, c) Stunted growth, leaf chlorosis and ear rot at 5% and 10% inoculum loads.

III.1.1.2. Identification of the selected pathogen

III.1.1.2.1. Morphological identification

The pathogenic isolate recovered from maize tissues displayed a hyaline to whitish young mycelium that turned pink or violet after 5 days on PDA (Figure 14A). On carnation leaf agar (CLA), macroconidia were thiny-walled and straight, with 4 to 5 septate and foot-shaped basal cells (Figure 14C). *In situ* preparations displayed predominant mono-celled and club-shaped microconidia in chains branched on long single phialides (Figure 14B and D). These characteristics led to the presumption of *F. verticillioides* and *F. proliferatum* according to the Leslie and Summerell, (2006) identification manual.

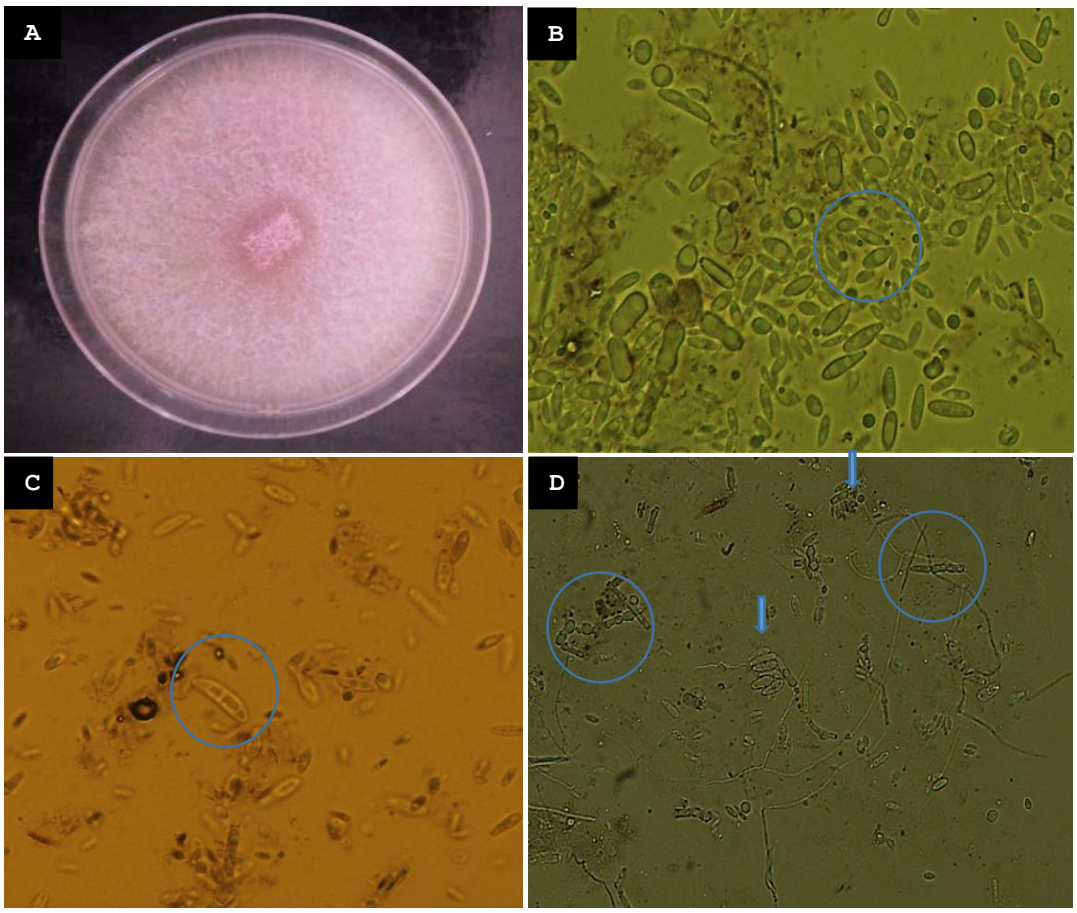


Figure 14: Macroscopic and microscopic characteristics of the pathogenic isolate.
Based on the violet pigment on PDA plates (A), an abundance of microconidia with 0 to 1 septate (B), macroconidia with four septate (C) and microconidia in long chains and small aggregates (D).

III.1.1.2.2. Identification based on 5.8S rDNA and TEF-1α gene sequencing

Due to the difficulty of determining the exact identity of *Fusarium* species through morphological tools, genome-based identification was performed. The ITS rDNA region and translation elongation factor 1-alpha (TEF-1α) of the pathogenic isolate were sequenced, and identification was performed by comparison with published sequences in GenBank. The results from the BLAST search revealed that the obtained ITS sequence displayed 98% similarity with sequences from *F. verticillioides*, *F. proliferatum*, and *F. oxysporum* in the NCBI database. However, the results for TEF-1α were similar to those for only sequences of *F. verticillioides* species. To infer the evolutionary history of our isolate, the maximum likelihood method was used for phylogenetic analysis. The Jukes-Cantor model with discrete gamma distribution rates was implemented for the ITS analysis (Figure 15), and the Kimura 2-parameter model with a discrete gamma distribution rate was implemented for TEF-1α analysis (Figure 16). According to the results, the delimitation power of the ITS gene was lower. Our isolate was found to be closely related to *F. verticillioides*, *F. proliferatum*, *F. mangiferae* and *F. circinatum*, which belong to the *F. fujikuroi* species complex. However, the TEF-1α-based phylogenetic analysis showed that our pathogenic isolate was very distinct from *F. proliferatum*, *F. mangiferae* and *F. circinatum* but closely related to *F. verticillioides* species in a well-supported clade of 97 bootstrap supports. Therefore, our isolate was confirmed to be *F. verticillioides* FV.

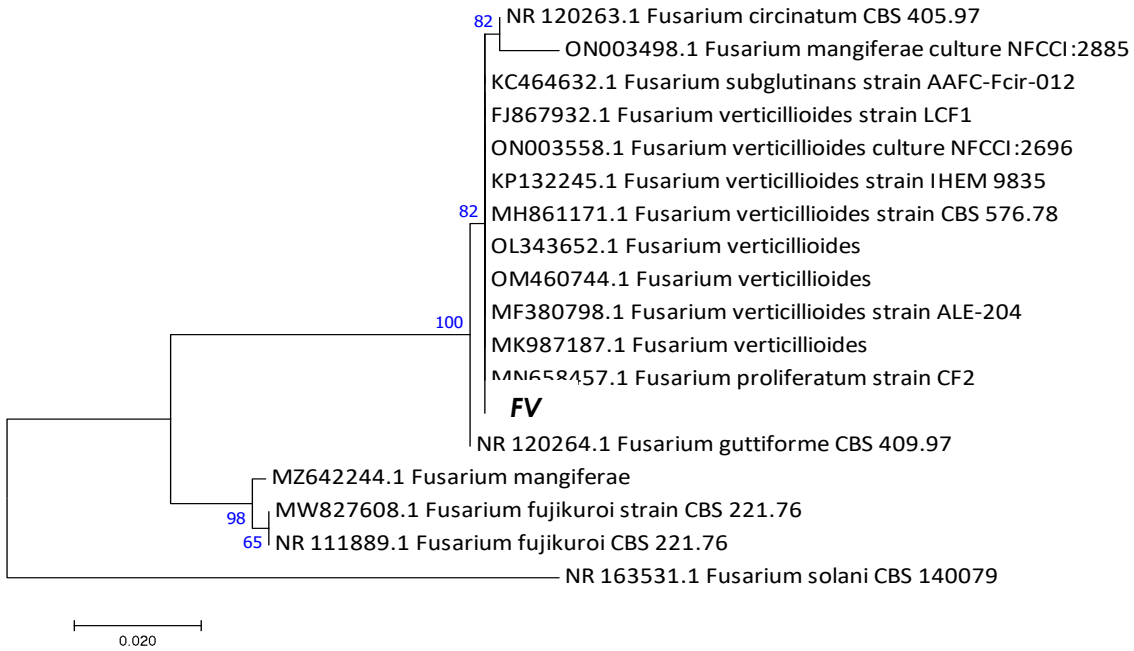


Figure 15: Molecular phylogenetic analysis of *F. verticillioides* F.V. based on the ITS gene sequence

The evolutionary history was inferred by using the maximum likelihood method based on the Jukes–Cantor model [1]. The tree with the highest log likelihood (- 972.93) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the maximum parsimony method. A discrete gamma distribution was used to model evolutionary rate differences among sites (2 categories (+*G*, parameter = 0.0500)). The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 437 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

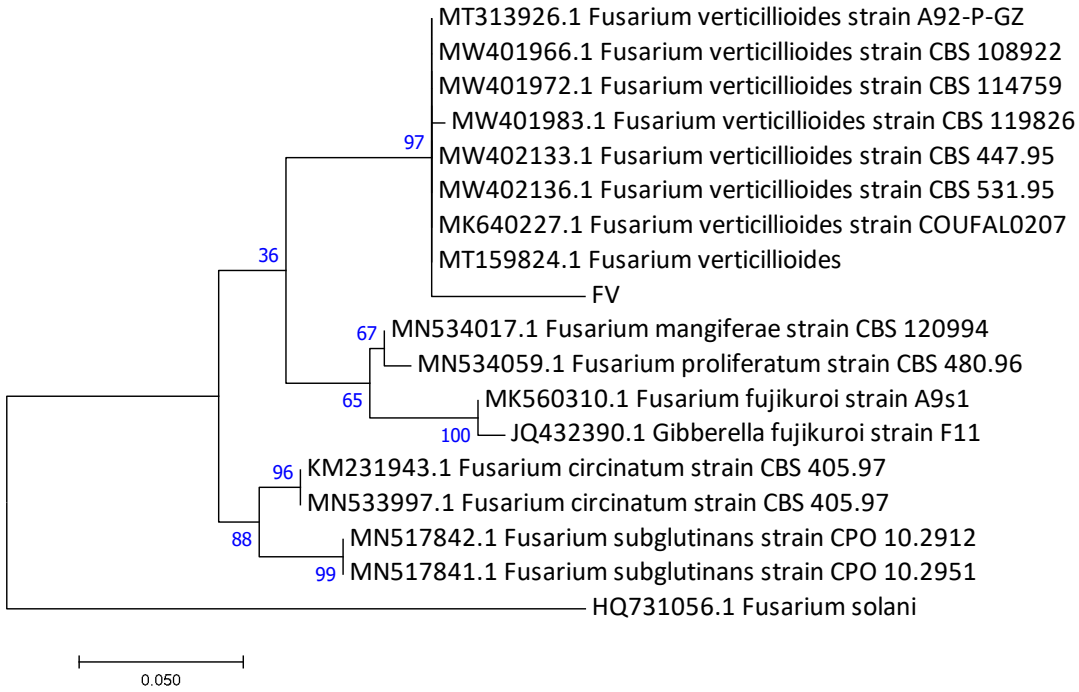


Figure 16: Molecular phylogenetic analysis of *F. verticillioides* F.V. based on the TEF 1α gene sequence

The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model [1]. The tree with the highest log likelihood (-798.84) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the maximum parsimony method. A discrete gamma distribution was used to model evolutionary rate differences among sites (2 categories (+*G*, parameter = 0.1845)). The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 252 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

III.1.1.3. *In vitro* antagonism

III.1.1.3.1. Inhibition of the mycelial growth of F. verticillioides in dual culture

F. verticillioides growth inhibition ranging from 4.26 to 68.6% was recorded (Figure 17). Five of the 25 BCAs, *B. amyloliquefaciens* RA37 (68.6%), *B. velezensis* BE1 (65.4%), *B. megaterium* RR13 (59.0%), *L. minipressuralis* LGR12 (53.9%) and an *unidentified* LPR1+ (53.2%), displayed remarkable inhibitory potential (inhibition > 50%). The best BCAs were *B. amyloliquefaciens* RA37 and *velezensis* BE1, with inhibition percentages of 68.6% and 65.4%, respectively.

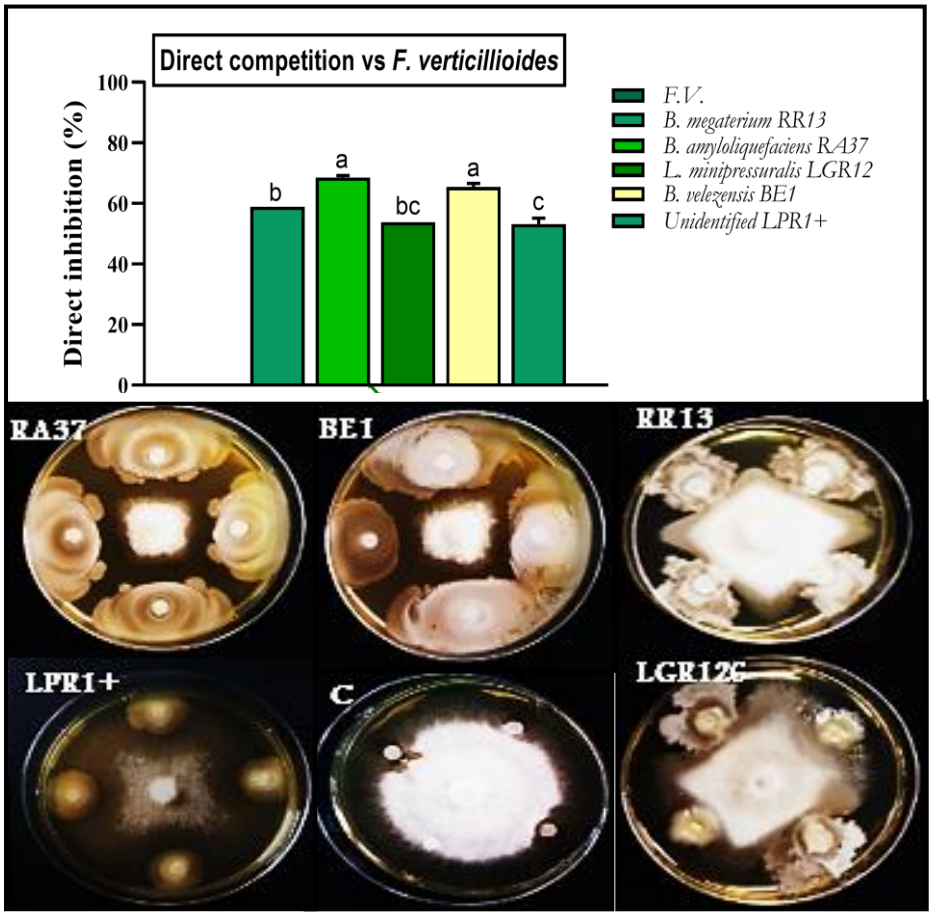


Figure 17: Inhibitory profiles of outstanding BCAs in dual culture against *F. verticillioides*. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$

III.1.1.3.2. Volatile organic metabolites (VOMs) from BCAs inhibit F. verticillioides growth

The volatile organic metabolites (VOMs) released by the BCAs differentially restricted the linear growth of *F. verticillioides*, with inhibitions ranging from 8.49 to 71.7% (Figure 18), except for the *P. peneri* RA24 strain, which had no activity. Eight out of the 25 bacterial strains (32%), namely, *B. amyloliquefaciens* BFL1 (71.7%), *C. indologenes* LPR17, unidentified LPR3 (66.9%), *S. maltophilia* LPR6+ (62.3%), *C. indologenes* LPR11+, *K. pneumoneae* RR19, *B. xiamenensis* RA4 (61.3%) and *B. megaterium* RR13, exhibited more than 50% inhibition. *B. amyloliquefaciens* BFL1, unidentified LPR3 and *C. indologenes* LPR17, with inhibition percentages of 71.7%, 66.9% and 66.9% respectively, produced more active antifungal VOMs.

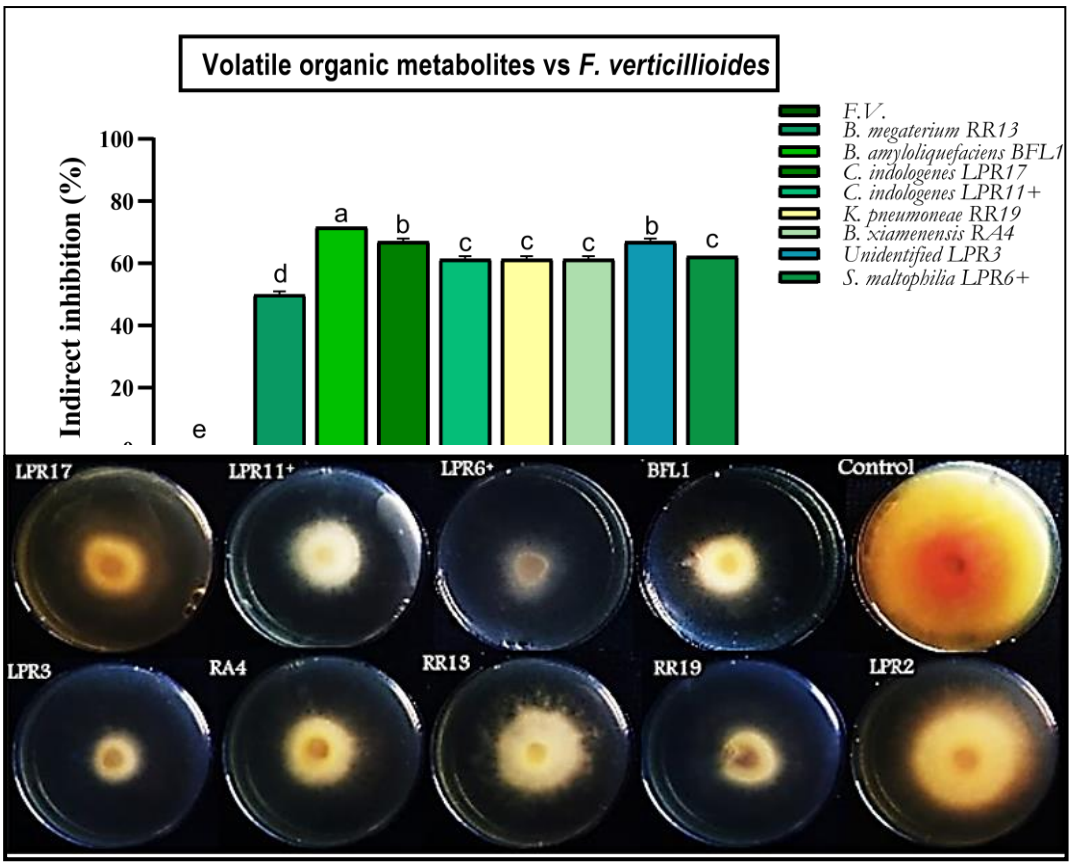


Figure 18: Inhibitory profiles of VOMs from outstanding BCAs in dual culture against *F. verticillioides*. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

III.1.1.4. Production of extracellular defense enzymes by the BCAs

To understand the mechanism by which biocontrol agents inhibit the growth of *F. verticillioides*, their abilities to produce hydrolytic enzymes, protease, cellulase, and amylase, were studied (Table VII).

All the tested bacteria were able to produce at least two of the three hydrolytic enzymes evaluated (Table VII). All 25 strains presented the ability to produce cellulase with a clear halo on yeast peptone agar (Figure 19c). The enzymatic activities varied from 0.1 to 1.5 IU, and the best agents were *L. minipressuralis* PA2+ and *L. fusiformis* LPR19, with enzyme activities of 1.5 and 1.3 IU, respectively (Table VII).

For protease production, 88% of the strains (22 bacteria) tested positive for clear halos (Figure 19a) on skin milk agar (except for *P putida* PA3+ and *B. amyloliquefaciens* RA37, which lacked

protease activity), whose activities ranged from 0.2 to 1.1 IU. The best agents were unidentified LPR1+, *S. maltophilia* LPR6+ and *P. peneri* RA24, with protease activities of 1.1, 1.1 and 1.01 IU, respectively.

Twenty-one (84%) of the bacterial strains tested positive for amylase production with a clear halo (Figure 19b) on yeast peptone agar (*P. peneri* RA24, *C. indologenes* LPR17, unidentified LPR3 and *S. maltophilia* LPR6+ with no inhibition zone), and their activities ranged from 0.31 to 1.7 IU. With activities of 1.7 and 1.6 IU, respectively, *B. velezensis* BE1 and *S. maltophilia* RA33 performed well.

Table VII: Values for hydrolysis diameters produced by the bacterial strains in the respective media for protease, cellulase, and amylase

Bacteria strains	Protease (IU)	Amylase (IU)	Cellulase (IU)
***P. peneri* RA24**	1.0 ± 0.0 ^{ab}	0.0 ± 0.0 ^{f}	0.6 ± 0.1 ^{cd}
***B. megaterium* RR13**	0.5 ± 0.0 ^{cd}	1.3 ± 0.3 ^{bc}	0.7 ± 0.1 ^{c}
***E. cloacae* LPR26**	0.9 ± 0.1 ^{b}	0.8 ± 0.2 ^{d}	0.6 ± 0.0 ^{cd}
***B. Amyloliquefaciens* BFR2**	0.7 ± 0.1 ^{bc}	0.9 ± 0.2 ^{d}	0.5 ± 0.0 ^{cd}
***B. Amyloliquefaciens* RA37**	0.0 ± 0.0 ^{f}	0.6 ± 0.1 ^{de}	0.7 ± 0.1 ^{c}
***Paracoccus sp* LPR20**	0.4 ± 0.0 ^{d}	0.8 ± 0.0 ^{d}	0.5 ± 0.1 ^{cd}
***Unidentified* LPR1+**	1.1 ± 0.2 ^{a}	1.4 ± 0.5 ^{b}	0.8 ± 0.1 ^{c}
***B. amyloliquefaciens* BFL1**	0.2 ± 0.0 ^{def}	0.3 ± 0.1 ^{e}	0.3 ± 0.0 ^{de}
***L. fusiformis* LPR19**	0.9 ± 0.2 ^{b}	0.4 ± 0.0 ^{e}	1.3 ± 0.1 ^{ab}
***L. minipressuralis* PA2+**	0.7 ± 0.1 ^{bc}	0.9 ± 0.1 ^{cd}	1.5 ± 0.4 ^{a}
***P. aeruginosas* RR4**	0.6 ± 0.1 ^{c}	1.2 ± 0.0 ^{c}	1.2 ± 0.0 ^{ab}
***C. indologenes* LPR17**	0.6 ± 0.0 ^{c}	0.0 ± 0.0 ^{f}	0.9 ± 0.1 ^{bc}
***K. pneumoneae* RR19**	0.3 ± 0.0 ^{de}	0.9 ± 0.1 ^{cd}	0.8 ± 0.1 ^{c}
***L. minipressuralis* LGR12**	0.8 ± 0.1 ^{b}	0.4 ± 0.0 ^{e}	1.0 ± 0.4 ^{b}
***Unidentified* LPR3+**	0.4 ± 0.0 ^{d}	0.6 ± 0.1 ^{de}	0.7 ± 0.0 ^{c}
***B. velezensis* BE1**	0.2 ± 0.0 ^{def}	1.7 ± 0.4 ^{a}	0.8 ± 0.1 ^{c}
***B. xiamenensis* RA4**	0.7 ± 0.3 ^{bc}	0.5 ± 0.1 ^{de}	1.3 ± 0.4 ^{ab}
***Unidentified* LPR3**	0.6 ± 0.0 ^{c}	0.0 ± 0.0 ^{f}	0.1 ± 0.0 ^{e}
***P. putida* RR20**	0.9 ± 0.1 ^{b}	1.0 ± 0.1 ^{d}	0.2 ± 0.0 ^{de}
***C. indologenes* LPR11+**	0.7 ± 0.0 ^{c}	1.3 ± 0.0 ^{c}	0.5 ± 0.0 ^{cd}
***S. maltophilia* LPR6+**	1.1 ± 0.0 ^{a}	0.0 ± 0.0 ^{f}	0.5 ± 0.0 ^{cd}
***P. putida* PA3+**	0.0 ± 0.0 ^{f}	0.9 ± 0.1 ^{cd}	0.9 ± 0.1 ^{bc}
***C. fruendii* LPR4+**	0.3 ± 0.1 ^{de}	1.2 ± 0.1 ^{c}	0.3 ± 0.1 ^{d}
***L. minipressuralis* LGR12+**	0.8 ± 0.0 ^{b}	1.2 ± 0.2 ^{c}	0.4 ± 0.0 ^{cd}
***S. maltophila* RA33**	1.0 ± 0.2 ^{ab}	1.6 ± 0.3 ^{ab}	0.7 ± 0.0 ^{c}
P values	<0.0001	<0.0001	<0.0001

IU = International unit. The results are expressed as the means ± SDs, and values in each column with different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

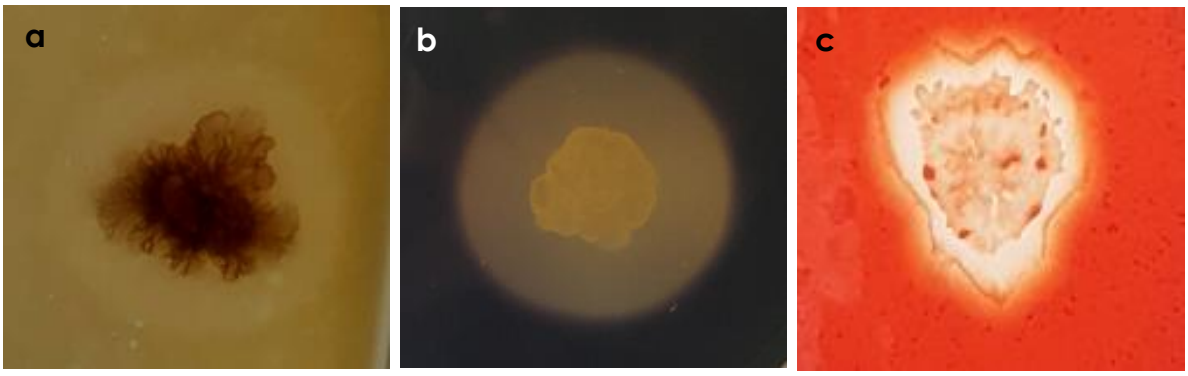


Figure 19: Inhibition halos produced by bacterial strains in protease (a), amylase (b) and cellulase (c) specific media.

III.1.1.5. Plant growth-promoting (PGP) traits

The bacterial agents demonstrated the capacity to produce growth promoters able to stimulate nutrient uptake by the plant, and the results are presented in Table VIII. Among the 25 tested bacteria, 12 (48%) were able to solubilize complex phosphate to make it available for the plant, with a clear halo around the bacterial colonies on Pikovskaya agar. The hydrolysis diameters ranged from 0.5 to 6 mm, and the most active strains were *B. velezensis* BE1 and *C. indologenes* LPR11+ with hydrolysis diameters of 6 and 4.5 mm, respectively.

All the tested bacteria presented the potential to produce ammonia in peptone water, with activities ranging from 0.7 to 6.4 µmol/ml. The best activities were observed for 11 bacteria, which produced more than 5 µmol/ml ammonia (*L. fusiformis* LPR19, *B. velezensis* BE1, *unidentified* LPR3+, *E. cloacae* LPR2+, *L. minipressuralis* PA2+ and LGR12, *C. indologenes* LPR17, *B. amyloliquefaciens* BFR2, *B. xiamenensis* RA4, *Paracoccus* sp. LPR20 and *L. minipressuralis* LGR12+), in decreasing order of their production potential.

IAA was produced by all the tested bacteria, and its production ranged from 20.9 to 57.4 µg/ml. Two of the strains were able to produce more than 50 µg/ml auxin, including unidentified LPR1+ and LPR3, with production potentials of 53.4 and 57.4 µg/ml, respectively.

Regarding siderophore production, all the strains tested positive for the production of the three types of siderophores, and the most produced siderophore was catecholate, followed by hydroxamate. Catecholate production ranged from 9.5% to 26.3%, and the most potent candidates were *B. amyloliquefaciens* BFL1 and *B. megaterium* RR13, which produced 26.3% and 24.2%, respectively. Hydroxamate production ranged from 8.7% to 31.2%, and *S. maltophilia* RA33 and unidentified LPR3+ were the most prominent, with percentages of 31.2

and 27.7%, respectively. Concerning the carboxylate type of siderophores, the production varied from 6.8 to 12.9%, and the most efficient strains were from the *Bacillus* genus, represented by *B. megaterium* RR13, *B. xiamenensis* RA4, *B. amyloliquefaciens* BFL1 and BFR2, with 12.9% production for all of them.

Out of the twenty-five bacteria strains tested, twelve demonstrated the ability to produce all four evaluated growth-promoting traits (phosphate solubilization, ammonia production, IAA production, and siderophores production), with productive capacities varying among the strains. These strains are *E. cloacae LGR26*, *B. amyloliquefaciens BFR2*, *B. amyloliquefaciens RA37*, *Paracoccus LPR20*, *C. indologenes LPR17*, *K. pneumoneae RR19*, *unidentied LPR3+*, *B. velezensis BE1*, *unidentified LPR3*, *C. indologenes LPR11+*, *S. maltophilia LPR6+*, *L. minipressuralis LGR12+*.

Table VIII: Recapitulation of the plant growth-promoting parameters (ammonia, indole acetic acid, phosphate and siderophores) produced by the twenty-five bacterial agents

Bacteria strains	Phosphate Solubilization (mm)	Ammonia Production (µmol/mL)	IAA Production (µg/ml)	Siderophore catecholate (%)	Siderophore hydroxamate (%)	Siderophore carboxylate (%)
P. peneri RA24	0.0 ± 0.0 ^{e}	2.7 ± 0.6 ^{d}	23.8 ± 0.7 ^{fg}	24.1 ± 0.6 ^{b}	15.4 ± 2.9 ^{cd}	11.2 ± 2.2 ^{b}
B. megaterium RR13	0.0 ± 0.0 ^{e}	4.3 ± 0.4 ^{bc}	49.0 ± 2.5 ^{b}	24.2 ± 0.0 ^{b}	15.9 ± 1.1 ^{cd}	13.0 ± 0.0 ^{a}
E. cloacae LGR26	2.5 ± 0.7 ^{c}	0.9 ± 0.6 ^{hi}	26.2 ± 1.6 ^{ef}	22.6 ± 2.2 ^{bc}	15.2 ± 1.0 ^{cde}	8.9 ± 1.9 ^{cd}
B. amyloliquefaciens BFR2	3.0 ± 1.4 ^{c}	5.5 ± 0.6 ^{b}	25.3 ± 1.6 ^{f}	17.8 ± 0.0 ^{cd}	24.5 ± 0.0 ^{ab}	12.9 ± 0.0 ^{ab}
B. amyloliquefaciens RA37	4.0 ± 1.4 ^{bc}	1.1 ± 0.3 ^{f}	44.0 ± 0.0 ^{c}	17.9 ± 3.4 ^{cd}	12.5 ± 1.2 ^{de}	11.8 ± 0.0 ^{ab}
Paracoccus LPR20	2.0 ± 0.0 ^{d}	5.5 ± 0.5 ^{b}	23.2 ± 1.4 ^{fg}	15.0 ± 2.6 ^{e}	8.7 ± 3.4 ^{h}	10.8 ± 1.6 ^{b}
unidentifiedLPR1+	0.0 ± 0.0 ^{e}	4.1 ± 1.1 ^{bc}	52.0 ± 2.6 ^{ab}	15.7 ± 0.0 ^{e}	10.9 ± 0.0 ^{fg}	9.5 ± 2.9 ^{c}
B. amyloliquefaciens BFL1	0.0 ± 0.0 ^{e}	4.2 ± 0.4 ^{bc}	28.6 ± 3.0 ^{e}	26.3 ± 0.0 ^{a}	18.7 ± 0.0 ^{bc}	12.9 ± 0.0 ^{ab}
L. fusiformis LPR19	0.0 ± 0.0 ^{e}	6.4 ± 0.6 ^{a}	20.9 ± 0.9 ^{gh}	23.9 ± 0.0 ^{b}	14.1 ± 1.2 ^{d}	10.7 ± 17 ^{b}
L. minipressuralis PA2+	0.0 ± 0.0 ^{e}	5.8 ± 0.3 ^{b}	22.6 ± 0.5 ^{fg}	14.7 ± 0.8 ^{e}	10.4 ± 0.9 ^{fg}	8.2 ± 0.0 ^{cd}
P. aeruginosas RR4	0.0 ± 0.0 ^{e}	3.9 ± 0.5 ^{c}	21.9 ± 0.7 ^{g}	15.4 ± 0.3 ^{e}	10.6 ± 1.5 ^{fg}	11.3 ± 1.2 ^{b}
C. indologenes LPR17	0.5 ± 0.0 ^{de}	5.6 ± 0.0 ^{b}	25.0 ± 0.8 ^{f}	19.0 ± 5.1 ^{c}	17.2 ± 0.0 ^{c}	11.1 ± 0.0 ^{b}
K. pneumoneae RR19	3.0 ± 0.0 ^{c}	3.3 ± 0.9 ^{cd}	25.4 ± 1.1 ^{f}	23.4 ± 3.1 ^{b}	16.4 ± 2.0 ^{cd}	8.5 ± 0.9 ^{cd}
L. minipressuralis LGR12	0.0 ± 0.0 ^{e}	5.8 ± 0.3 ^{b}	38.5 ± 0.0 ^{d}	22.9 ± 1.1 ^{b}	19.7 ± 1.6 ^{b}	9.7 ± 3.0 ^{c}
unidentied LPR3+	4.0 ± 0.0 ^{bc}	5.9 ± 1.0 ^{b}	23.6 ± 0.7 ^{fg}	15.3 ± 0.0 ^{e}	27.7 ± 0.0 ^{a}	8.4 ± 0.4 ^{cd}
B. velezensis BE1	6.0 ± 1.4 ^{a}	6.3 ± 0.1 ^{ab}	23.3 ± 0.7 ^{fg}	22.6 ± 3.7 ^{bc}	21.0 ± 1.8 ^{b}	12.0 ± 1.0 ^{ab}

B. xiamenensis RA4	0.0 ± 0.0 ^{e}	5.5 ± 0.4 ^{b}	24.9 ± 1.2 ^{f}	19.2 ± 0.0 ^{c}	15.4 ± 0.0 ^{cd}	12.9 ± 0.0 ^{ab}
unidentifiedLPR3	3.0 ± 0.0 ^{c}	4.9 ± 0.3 ^{b}	57.4 ± 1.9 ^{a}	16.6 ± 1.5 ^{d}	10.9 ± 1.5 ^{fg}	12.5 ± 0.0 ^{ab}
P. putida RR20	0.0 ± 0.0 ^{e}	0.8 ± 0.5 ^{i}	21.4 ± 3.7 ^{g}	19.5 ± 0.1 ^{c}	13.8 ± 0.3 ^{d}	6.8 ± 2.1 ^{d}
C. indologenes LPR11+	4.5 ± 2.1 ^{b}	4.4 ± 0.4 ^{bc}	23.1 ± 0.1 ^{fg}	17.0 ± 0.0 ^{d}	9.0 ± 0.0 ^{gh}	9.1 ± 1.2 ^{c}
S. maltophilia LPR6+	2.0 ± 0.0 ^{d}	4.7 ± 0.7 ^{bc}	24.3 ± 0.5 ^{f}	23.6 ± 0.0 ^{b}	19.0 ± 3.7 ^{bc}	11.2 ± 2.0 ^{b}
P. putida PA3+	0.0 ± 0.0 ^{e}	1.7 ± 0.8 ^{e}	24.2 ± 1.2 ^{f}	23.4 ± 0.0 ^{b}	27.5 ± 0.0 ^{a}	8.3 ± 0.1 ^{cd}
C. fruendii LPR4+	0.0 ± 0.0 ^{e}	2.3 ± 0.3 ^{de}	22.8 ± 1.5 ^{fg}	22.8 ± 0.0 ^{b}	14.8 ± 3.2 ^{d}	11.0 ± 2.6 ^{b}
L. minipressuralis LGR12+	3.0 ± 1.4 ^{c}	5.1 ± 0.3 ^{b}	23.6 ± 1.0 ^{fg}	19.8 ± 2.5 ^{c}	17.6 ± 0.0 ^{c}	10.5 ± 0.6 ^{bc}
S. maltophilia RA33	0.0 ± 0.0 ^{e}	3.4 ± 0.8 ^{cd}	25.7 ± 0.8 ^{f}	9.5 ± 0.0 ^{f}	31.2 ± 0.0 ^{a}	9.5 ± 2.3 ^{c}
P values	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

The results are expressed as the means ± SDs, and values in each column with different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

- After demonstrating the antagonistic potential of the bacterial agents against mycelial growth of *F. verticillioides* using *in vitro* biological assays, the capacity to produce hydrolytic enzymes against pathogen cell wall components and the ability to produce some plant growth-promoting elements, the different performances of the bacterial agents were compared, and the most promising candidates were selected for further study. This guided us to select ten strains (Figure 20): ***B. megaterium* RR13; *B. amyloliquefaciens* RA37 and BFL1; *unidentified* LPR1+ and LPR3; *C. indologenes* LPR17; *K. pneumoneae* RR19; *L. minipressuralis* LGR12; *B. velezensis* BE1; and *S. maltophilia* LPR6+**.

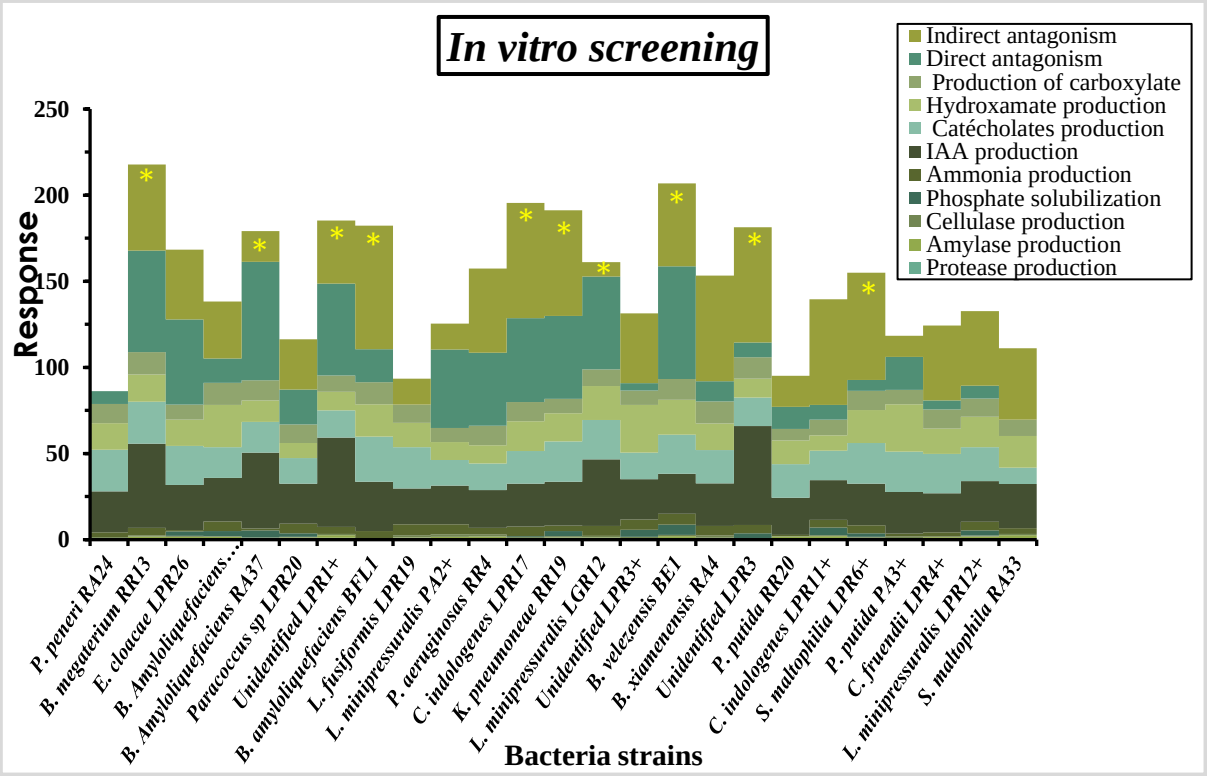


Figure 20: Recapitulation of the *in vitro* biological performances demonstrated by the twenty-five tested bacterial agents against the evaluated parameters. * Indicates selected candidates based upon overall response to the studied parameters.

III.1.1.6. Confirmation of the biocontrol and PGP traits of promising BCAs on maize plants

III.1.1.6.1. Reduction in seedlings blight severity

Ten (10) bacterial strains with promising *in vitro* biological potential were assessed for their protective effects against *F. verticillioides* under greenhouse conditions. In agreement with the

in vitro results, all the bacterial strains demonstrated biological control activities against *F. verticillioides* through disease mitigation.

The number of seedlings with blight symptoms was recorded eleven days after infection, which was considered as post-emergence-damping effect. Treatments involving only *Fusarium* and associated with eight bacteria (*B. megaterium*, RR13, *B. amyloliquefaciens* BFL1, *unidentified* LPR1+, *C. indologenes* LPR17, *K. pneumonia* RR19, *L. minipressuralis* LGR12, *B. velezensis* BE1 and *S. maltophilia* LPR6+) caused at least 1 to 3 plants to die or wilt before emergence. After this period, the disease severity was recorded for each treatment every four days for 24 days to determine the extent of the disease and the ability of bacterial agents to reduce the development of ear and root rot on maize seedlings. For all the collected results, we observed a considerable reduction in disease severity in response to all the treatments, which decreased with time (Figure 21). At the end of the 35 days of the experiment, we obtained a significant reduction in seedlings blight severity of up to 45%. The most effective seed treatments were *C. indologenes* LPR17, *K. pneumoneae* RR19 and *E. clocae* LPR2+, with ear and root rot reduction percentages of 45% for the first two and 42.5% for the last one, respectively. Figure 21 below shows the reduction potential of the strains.

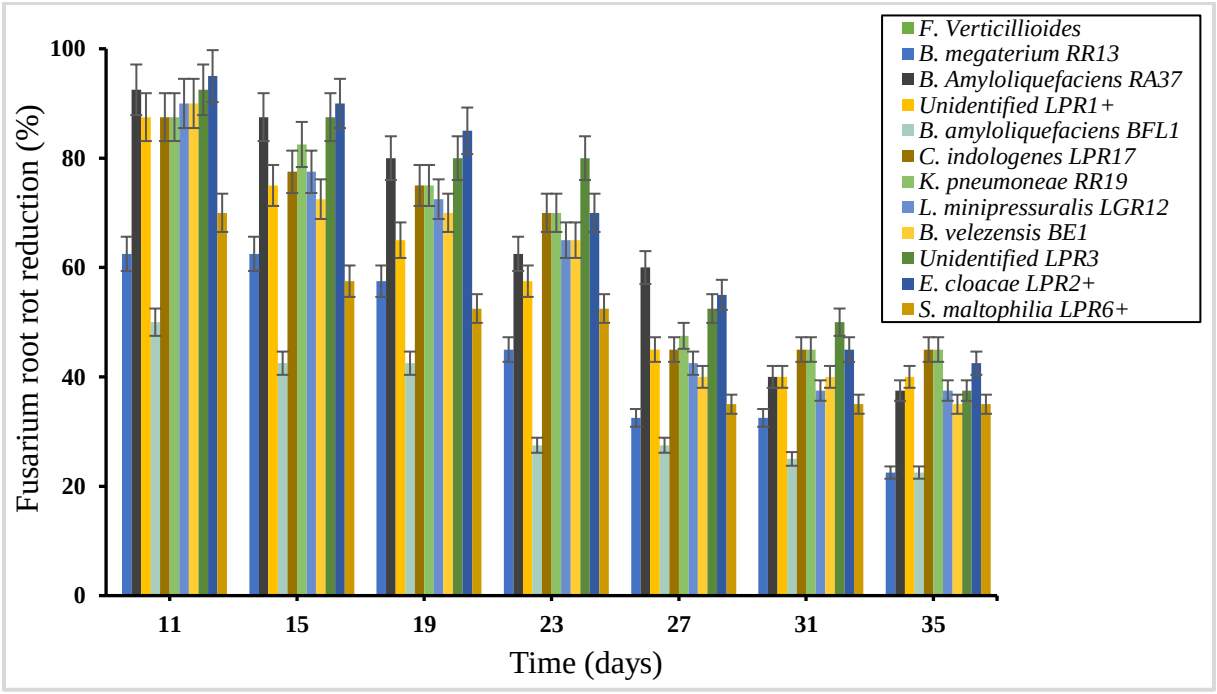


Figure 21: Effect of bacterial inoculation on the reduction of Fusarium rot progress in maize seedlings.

III.1.1.6.2. Effects of seed bacterization and pathogen infection on several growth parameters

An evaluation of the effect of bacterial seed colonizations on the reduction of disease through plant development revealed that the different bacterial colonizations had a positive effect on maize growth compared to the *Fv* control (Figure 22). In fact, almost all bacterial agents maintained or significantly increased the root and shoot development of maize compared to those of the infected control by up to 129.4% and 31.5%, respectively. Treatment with *B. amyloliquefaciens* BFL1 repressed shoot development compared to that of the single *Fv*-treated plants. The shoot length increased from 0.48% to 31.5% in response to the bacterial treatments, and unidentified LPR3 demonstrated the greatest increase of 31.5% (79.3 cm of shoot length compared to 60.3 cm obtained with a single *Fv*). On the other hand, the bacterial treatments increased the root length of maize plants, with percentages varying from 50.8% to 129.4%. The greatest activity was exhibited by *K. pneumoniae* RR19 (129.4%), whose root length was 33.8 cm, while that of the single-Fv control was 14.8 cm. More interestingly, compared to the normal (uninfected) control, up to seven of the ten bacterial strains increased maize root growth to a value higher than the normal growth of the plant under uninfected conditions, and the percentage of growth increase varied from 10% to 35.3% (Figure 23).

The dry weights of the shoot dry masses of the infected plants significantly increased in response to the five bacterial treatments: *C. indologenes* LPR17, *K. pneumoneae* RR19, *B. amyloliquefaciens* RA37, *B. velezensis* BE1 and unidentified LPR3 compared to those in plants inoculated with only Fv. The shoot dry mass of plants inoculated with these strains were 55.3%, 44.7% and 31.6% greater than that of plants in the Fv control group. However, the shoot dry mass of the *B. megaterium* RR13 strain was significantly lower than that of the Fv control (Figure 23). In addition, the root dry mass significantly increased with response to the two treatments, *K. pneumoniae* RR19 and *B. amyloliquefaciens* RA37, with an increase of 49.5% obtained for *K. pneumoniae* RR19.

Figure 22: Effect of *F. verticillioides* infection and BCAs application on the development of *Fusarium* rot disease on the aerial and root parts of young maize plants.

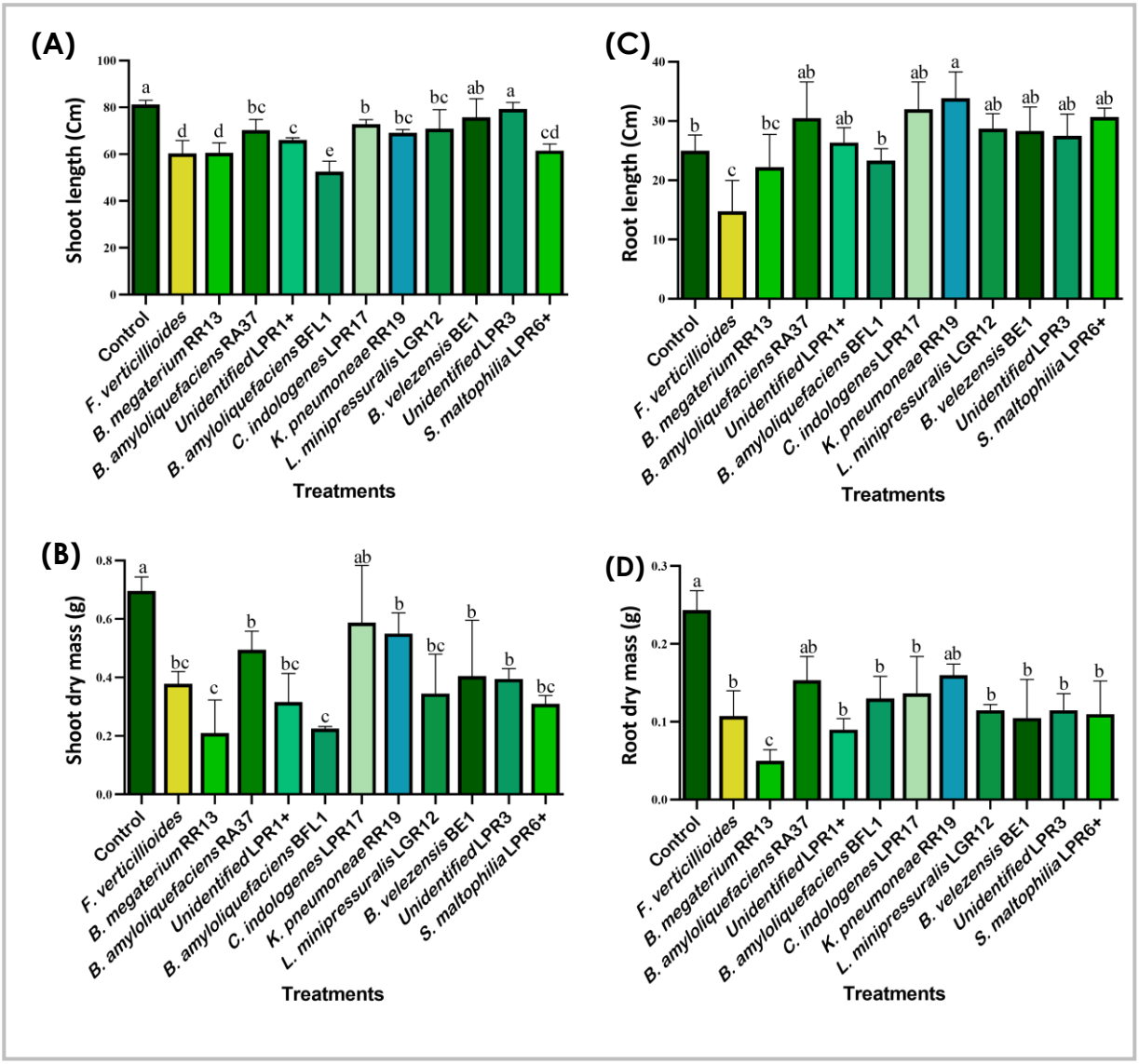


Figure 23: Effect of *F. verticillioides* infection and BCA application on shoot length (A), shoot biomass (B), root length (C), and root biomass (D) of maize under greenhouse conditions. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

III.1.1.6.3. Effect of seed bacterization and pathogen infection on the specific activities of some defense-related enzymes

Gaiacol peroxidase (GPX) and phenylalanine ammonia-lyase (PAL) are among the most important enzymes involved in plant defense against pathogen attack. The ability of bacterial agents to enhance enzyme activity in the presence of *F. verticillioides* was evaluated (Figure 24). The results revealed that compared with the normal control treatment, the *Fv* treatment significantly increased GPX activity by 108.9%. Similarly, compared with the Fv treatment alone, the four bacterial treatments significantly increased the enzyme activity in the presence of *Fv* from 15.4% to 126.9%. In fact, the enzyme activity increased from 604.9 OD/min/g FM

in the uninoculated control to 1263.8 OD/min/g FM after *Fv* inoculation and increased to 2867.8, 2066.2, 1539.1 and 1458.6 OD/min/g FM when the seeds were treated with the bacterial agents *K. pneumoneae* RR19, *S. maltophila* LPR6+ *C. indologenes* LPR17 and unidentified LPR3, respectively.

Compared with the uninoculated control, single inoculation with *Fv* had a nonsignificant impact on PAL activity. Therefore, compared with the *Fv* control, seven of the ten bacterial treatments significantly increased PAL activity by 20.4% to 70.7% (Figure 24). Among these strains, *S. maltophila* LPR6+ exhibited the most important activity, followed by *C. indologenes* LPR17, with increases in enzyme activity of 70.7% and 66.5%, respectively.This increase in GPX and PAL activities can explain the ability of endophytic bacteria to stimulate the maize defense system to respond to external attack.

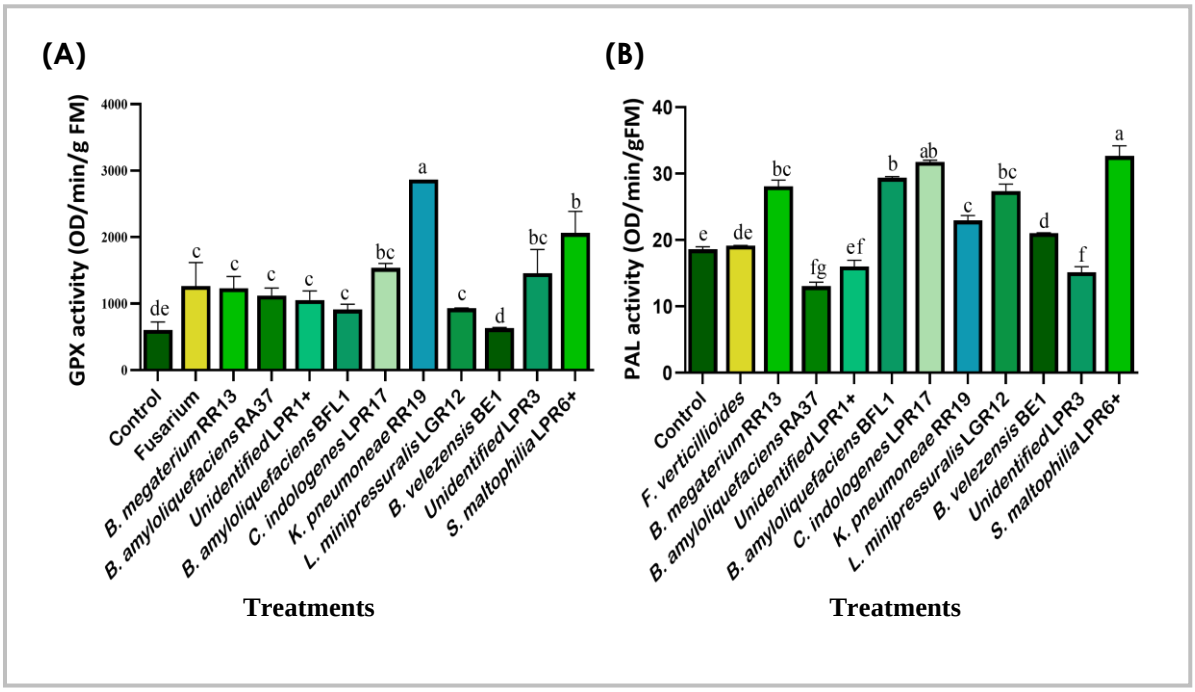


Figure 24: Differential responses of bacterized and infected (*Fv*) maize seedlings to the specific activities of GPX (A) and PAL (B) in greenhouse conditions. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

- Compared with the single inoculation, all ten bacterial strains tested for their ability to protect young maize plants against *Fusarium verticillioides* infection significantly reduced the development of the disease and improved the growth performance of the plants. Additionally, the BCAs improved plant immunity by increasing the activity of the defense enzyme PAL and/or the antioxidant GPX. The ten bacterial agents will

therefore be evaluated further for their capacity to induce drought tolerance in young maize plants.

III.1.2. ANTAGONISTIC BACTERIAL CANDIDATES INDUCED DROUGHT TOLERANCE IN MAIZE SEEDLINGS UNDER *IN VITRO* AND GREENHOUSE CONDITIONS

III.1.2.1. *In vitro* drought tolerance capacity of the BCAs

The bacterial strains were assessed for *in vitro* drought tolerance in polyethylene glycol (PEG 6000)-supplemented LB media. Drought stress substantially inhibited the growth of BCAs at all PEG 6000 concentrations tested (-0.05 MPa, -0.15 MPa, -0.49 MPa, and -1.79 MPa). In fact, the strain growth rate decreased with increase in the matrix stress (Figure 25). Compared with the unstressed control, the investigated strains *B. megaterium* RR13, *B. velezensis* BE1, and *L. minipressuralis* LGR12 exhibited tolerance to low stress levels with similar cell densities at -0.05 MPa. The four strains, *B. megaterium* RR13, *B. amyloliquefaciens* RA37, unidentified LPR1+, and *C. indologenes* LPR17, were able to thrive at the highest water potential (-1.79 MPa). Overall, regardless of the PEG concentration, the *B. megaterium* RR13 strain exhibited the greatest drought tolerance, with a growth rate almost identical to that of the unstressed control.

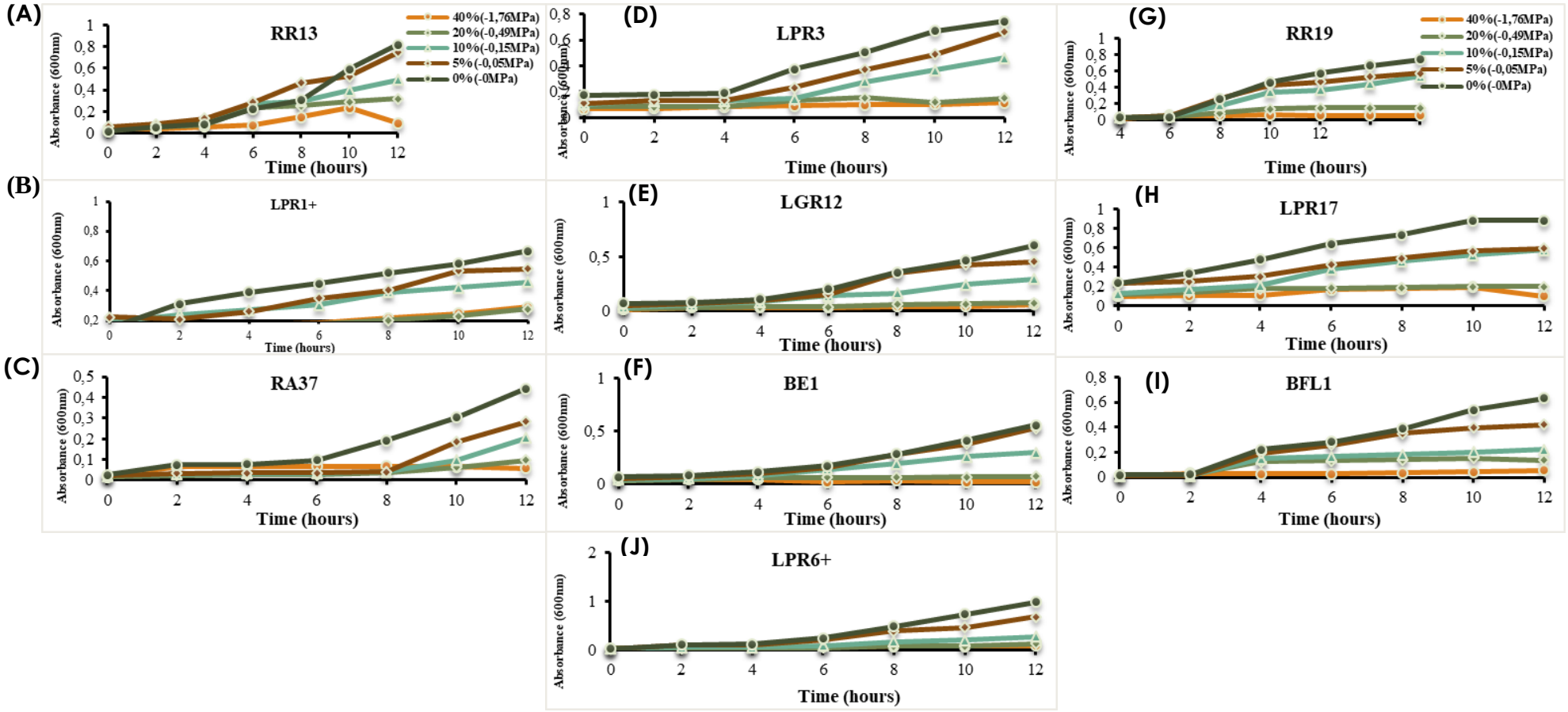


Figure 25: Growth kinetics of antagonistic bacterial strains under various drought stress conditions induced by different PEG concentrations (-1.76 MPa; -0.49 MPa; -0.15 MPa and -0.05 MPa). The figures (A), (B), (C), (D), (E), (F), (G), (I) and (J) respectively present the growth kinetics of the strains *B. megaterium* RR13, Unidentified LPR1+; *B. amyloliquefaciens* RA37; Unidentified LPR3; *L. minipressuralis* LGR12; *B. velezensis* BE1; *K. pneumoneae* RR19; *C. indologenes* LPR17; *B. amyloliquefaciens* BFL1 and *S. maltophilia* LPR6+, under the different PEG concentrations after 2, 4, 6, 8, 10 and 12 hours.

III.1.2.2. Biofilm formation

Interestingly, all of the bacterial strains produced biofilms in PEG 6000-induced drought media (-1.76 MPa, -0.49 MPa, -0.15 MPa, and -0.05 MPa). At 570 nm, the bacterial optical densities ranged from 0.5 to 2.5, indicating the ability of these bacteria to form biofilms (Figure 26). *Unidentified* LPR1+ and LPR3, with OD values of 2.5 and 2.1, respectively, had the highest production potentials.

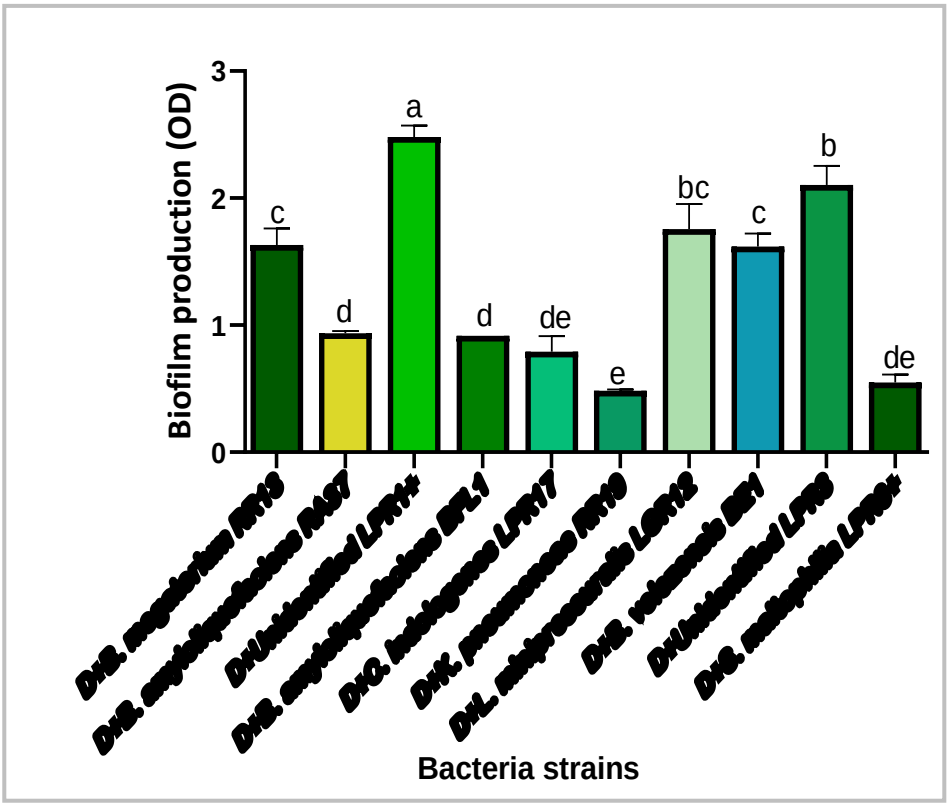


Figure 26: Variability in BCA biofilm formation under PEG 6000-induced stress (-1.76 MPa). Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

III.1.2.3. Salinity stress tolerance ability of the bacterial strains

The salinity stress tolerance of the ten selected BCAs was tested at 200 mM NaCl. Five of the ten strains, namely, *B. velezensis* BE1, *unidentified* LPR1+, LPR3, *C. indologenes* LPR17, and *S. maltophilia* LPR6+ were not affected by this salt concentration when compared with their respective unstressed treatments (Figure 27). It was unexpected, therefore, that *K. pneumoneae* RR19 showed greater growth potential under salt stress than under unstressed conditions. In contrast to unstressed controls, we found a significant decrease in cell growth and that stress had a substantial impact on the growth of the four other strains.

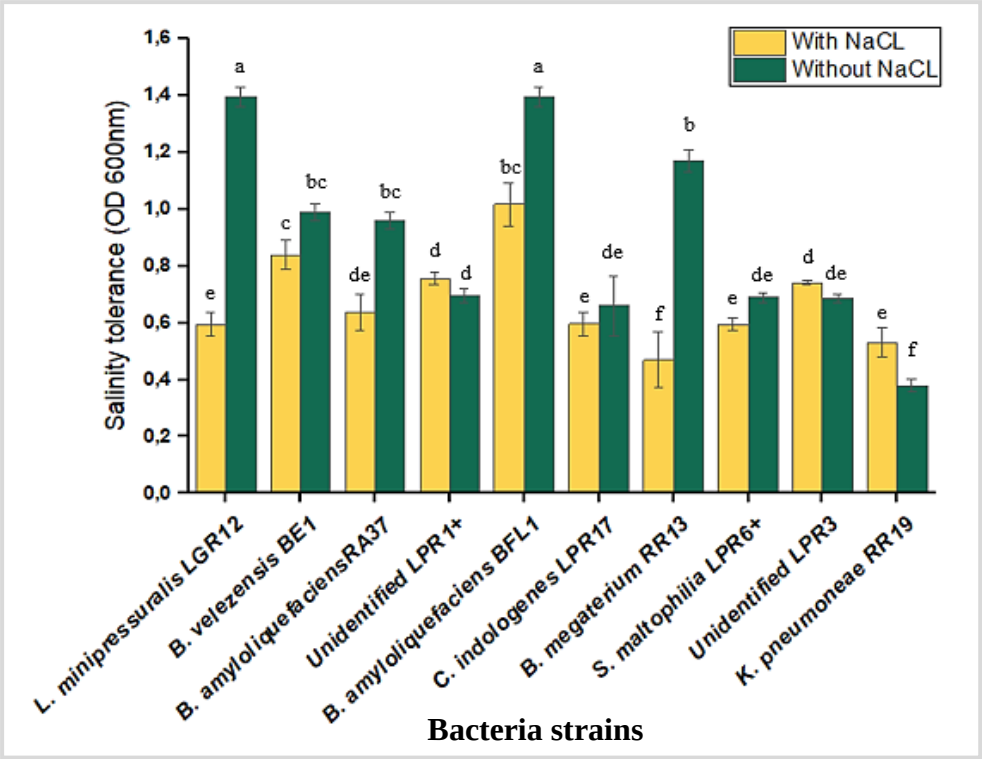


Figure 27: Optical densities reflecting the growth profiles of the bacterial strains read after 24 hours at 600 nm, both in the presence and absence of salt stress induced by 200 mM NaCl. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

III.1.2.4. Amplification and detection of the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase gene

The presence of the ACC deaminase (ACCd) gene in the 10 bacterial strains was tested by amplifying the ACCd gene using PCR and gene-specific primers. Among the examined strains, four (*B. velezensis* BE1, *B. amyloliquefaciens* BFL1, *C. indologenes* LPR17, and *S. maltophilia* LPR6+) were proven to harbor the ACCd synthesis gene (Figure 28), as evidenced by agarose gel electrophoresis of amplification of the 700 bp product.

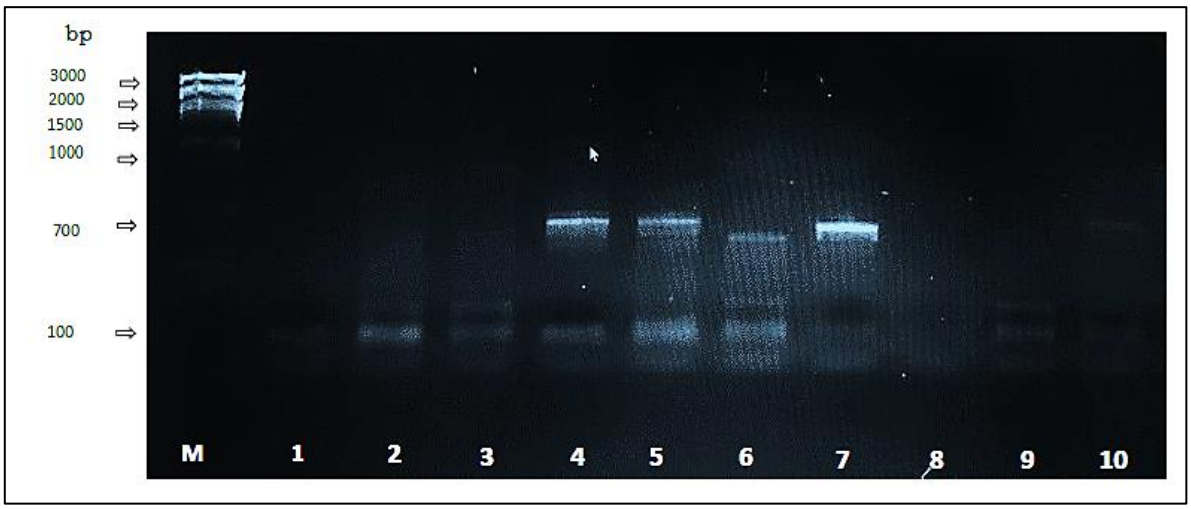


Figure 28: Electrophoretic profile displaying migration bands of the ACC deaminase gene from the ten bacterial strains. M=molecular weight marker; 1=Control; 2=*L. minipressuralis* LGR12; 3=*B. amyloliquefaciens* RA37; 4=*B. velezensis* BE1; 5=*B. amyloliquefaciens* BFL1; 6=*B. megaterium* RR13; 7=*C. indologenes* LPR17; 8=*Unidentified* LPR1+; 9=*Unidentified* LPR1+ and 10=*S. maltophilia* LPR6+.

- The bacterial strains *K. pneumoniae* RR19 and *B. amyloliquefaciens* RA37 were eliminated from the evaluated agents owing to their extremely poor capacity to survive drought stress and related factors (Figure 29). The ability of the bacterial agents to promote drought tolerance in maize plants was evaluated using eight strains: ***L. minipressuralis* LGR12, *B. velezensis* BE1, *B. amyloliquefaciens* RA37, *B. megaterium* RR13, *unidentified* LPR1+ and LPR3, *C. indologenes* LPR17, and *S. maltophilia* LPR6+**.

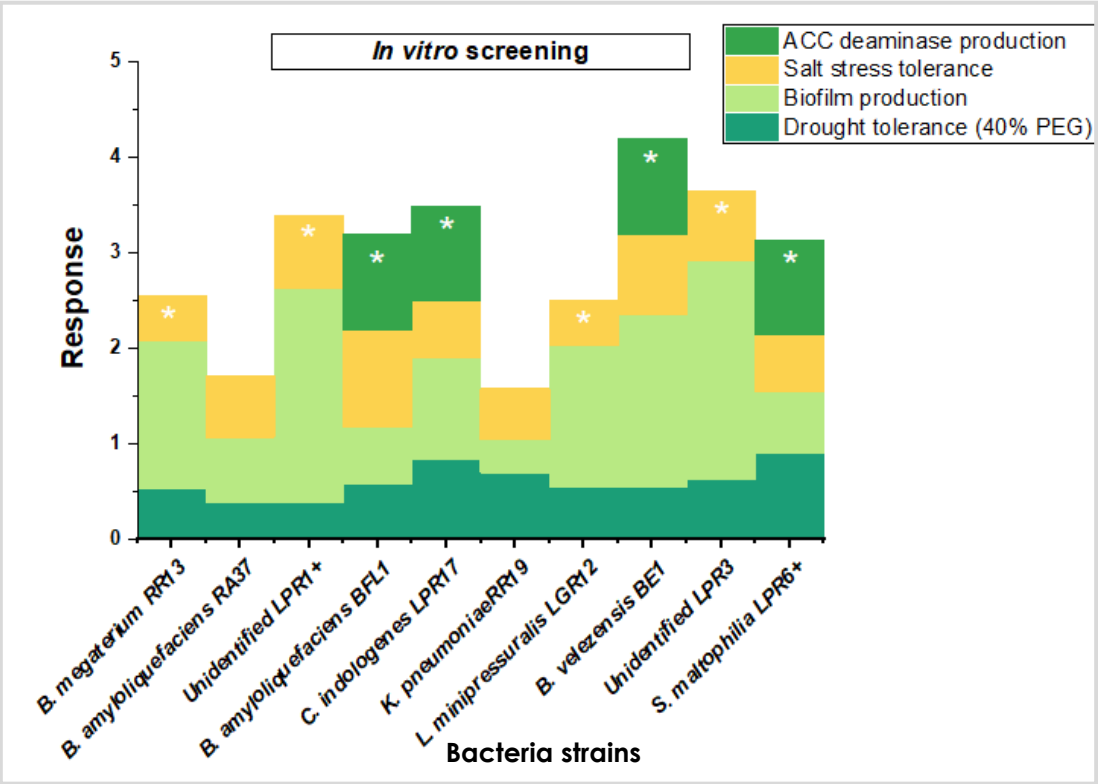


Figure 29: *In vitro* drought tolerance of the ten tested bacterial agents. The ability of the bacterial strains to grow on PEG-supplemented media, to produce biofilms, to express the ACC deaminase encoding gene, and to grow on salt-supplemented media were evaluated. * Indicates selected candidates based upon overall response to the studied parameters.

III.1.2.5. Protective effect of the bacterial candidates against the impact of drought on young maize plants

III.1.2.5.1. In planta drought tolerance

The findings displayed in Figure 30 demonstrate the capacity of bacterial candidates to heal drought damage in maize. Drought severely decreased maize length and biomass compared to those in the unstressed plants, but several bacterial treatments increased growth metrics under drought conditions. In fact, the shoot length decreased by 31.1% in response to drought stress. On the other hand, drought did not significantly affect the root length compared to the unstressed control. However, the bacterial treatment *B. megaterium* RR13 significantly improved the root length under drought stress, exceeding the root length of unstressed plants (38.5%). In comparison to the single drought treatment, *B. megaterium* RR13 increased the root length by 85.3%. Drought had a considerable impact on the plant shoot and root fresh mass, with up to 88.7% and 92.6% decreases in shoot and root mass, respectively. *B. megaterium* RR13 and unidentified LPR1+ bacterial treatments greatly enhanced shoot biomass during

drought. However, the bacterial agents had a negative effect on root weight, except for the strain unidentified LPR1+, which showed at least a 15.2% increase in root mass compared to the single drought control.

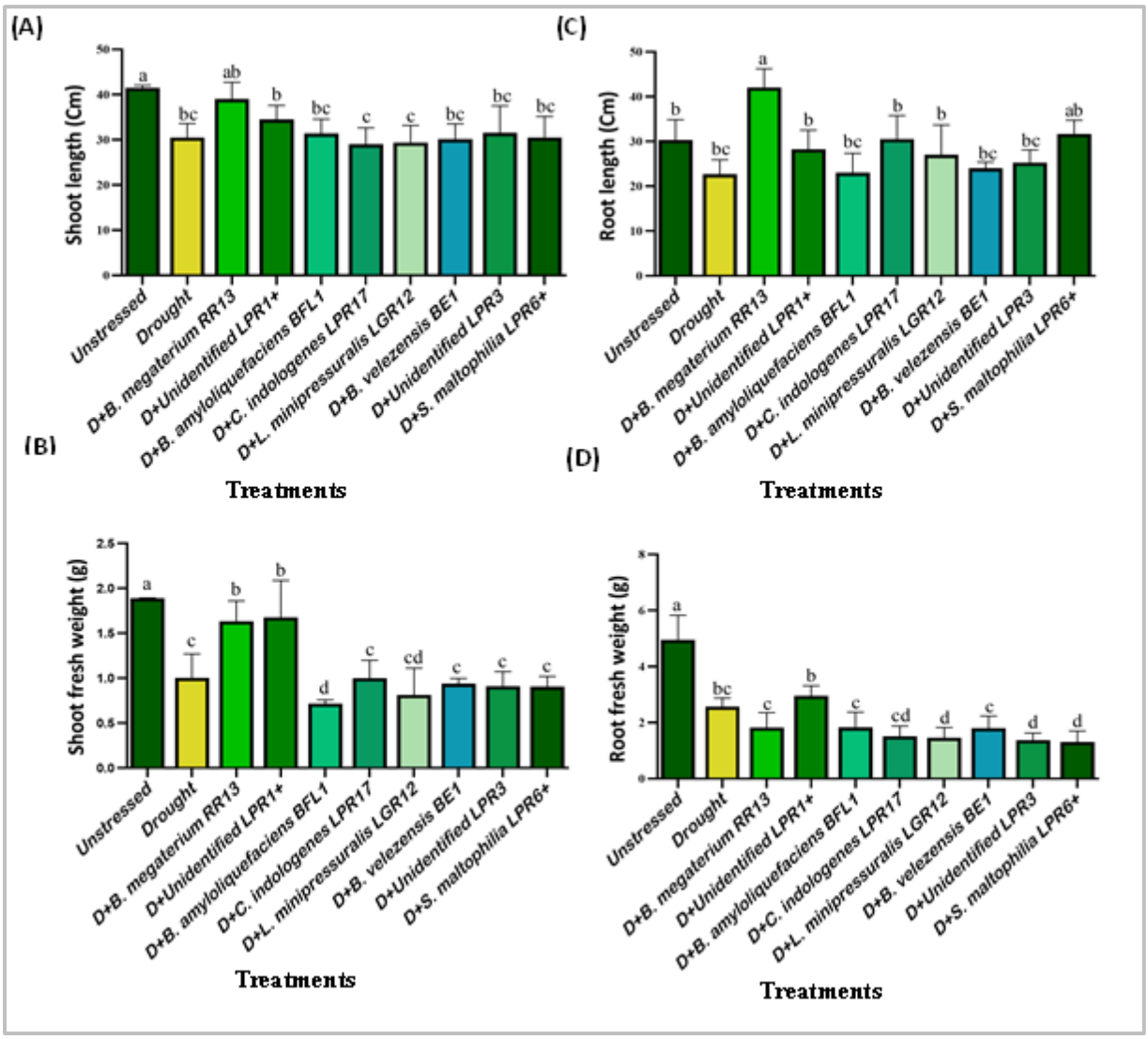


Figure 30: Effect of bacterial treatments on shoot length (A), shoot biomass (B), root length (C), and root biomass (D) of drought-stressed maize under greenhouse conditions. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

III.1.2.5.2. Effect of bacterial inoculation on maize physiological parameters under drought conditions

Drought, as predicted, decreased leaf area and water content while increasing leaf rolling and chlorosis (Figure 31). Compared with the drought control, *unidentified* LPR1+ and LPR3 reduced leaf rolling, while no significant effect on leaf chlorosis was detected. Drought drastically decreased the leaf area (256.9%); however, compared with the uninoculated drought control, the presence of the bacterial agent *unidentified* LPR1+ enhanced this parameter

(127.7%). Compared with the unstressed treatment, drought reduced the relative water content (RWC) of maize leaves by 277.2%, whereas the *L. minipressuralis* LGR12 and *B. megaterium* RR13 bacterial treatments increased the RWC by 90.7% and 141.8%, respectively.

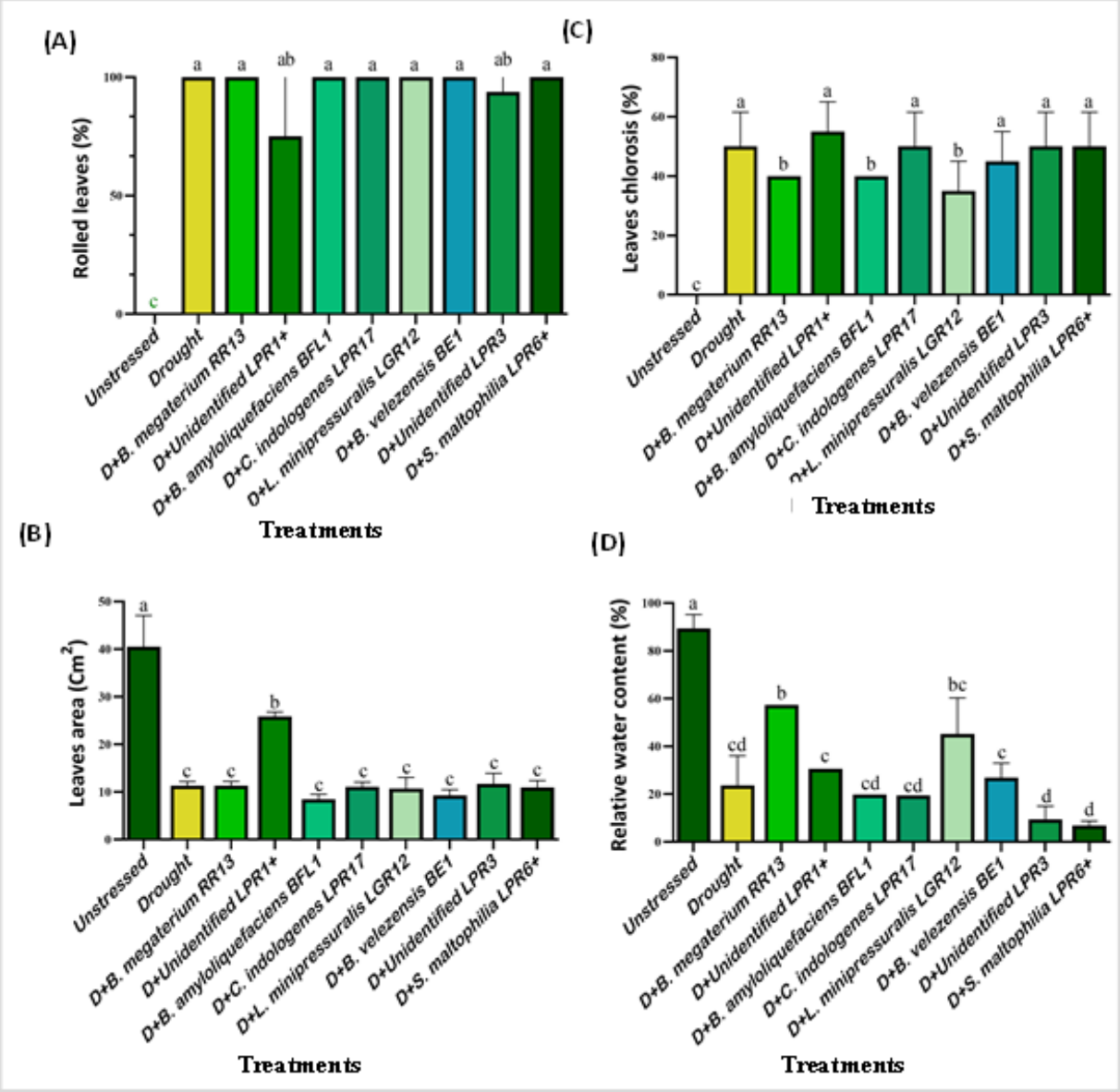


Figure 31: Effect of bacterial treatments on leaf rolling (A), area (B), chlorosis (C), and relative water content (D) of maize under drought stress. Unstressed = plants watered at 100% holding capacity; D = Drought = plants watered at 20% holding capacity. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

Drought dramatically decreased the chlorophyll a, b, total chlorophyll, and carotenoid contents in maize leaves compared to those in the unstressed control. However, compared with the uninoculated drought control, several bacterial strains greatly increased the chlorophyll a (216.6%), b (39.7%), total (146.5%), and carotenoid (35.8%) contents (Figure 32). Compared

with the uninoculated drought control, *B. megaterium* RR13 and *S. maltophilia* LPR6+ performed best in terms of chlorophyll a (216.6% and 203.9%, respectively), b (39.7% and 29.1%, respectively), and total chlorophyll (146.5% and 136%, respectively), while *B. amyloliquefaciens* BFL1 increased the carotenoid content (35.8%).

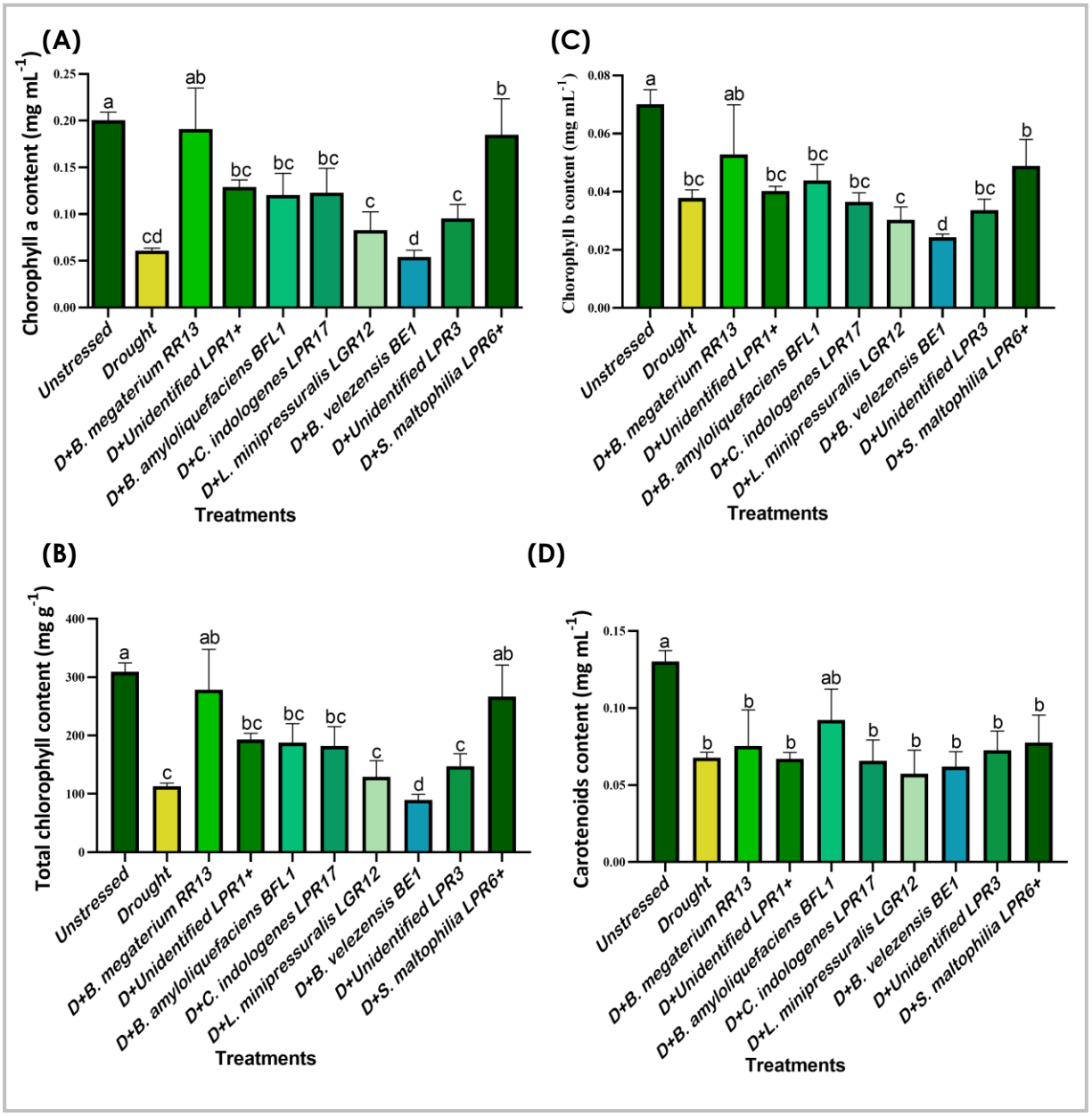


Figure 32: Chlorophyll a (A), total chlorophyll (B), chlorophyll b (C), and carotenoid (D) contents of maize leaves under drought stress and after or without bacterial treatments. Unstressed = plants watered at 100% holding capacity; D = Drought = plants watered at 20% holding capacity. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

III.1.2.5.3. Effects of bacterial candidates on maize biochemical parameters under drought stress

Compared with that of the unstressed control, the water deficit-induced increased in catalase activity, as shown in Figure 33. Plants inoculated with the bacterial strains *unidentified* LPR1+, *C. indologenes* LPR17, *L. minipressuralis* LGR12, *unidentified* LPR3, and *B. velezensis* BE1 showed a substantial decrease in catalase activity ranging from 25.3% to 137.5% compared to the uninoculated drought control. Compared to the unstressed control, *C. indologenes* LPR17 and *unidentified* LPR3 had lower CAT activity. The percentage of plants treated with *B. velezensis* BE1 decreased the most (137.5%). Drought stress had little effect on guaiacol peroxidase (GPX) activity in almost all the bacterial treatments.

Drought conditions resulted in a considerable decrease in the total phenol and flavonoid contents in maize compared to those in the unstressed control (Figure 33). Furthermore, bacterial treatments reduced plant phenolic metabolism under drought stress. Bacterial inoculation significantly increased the total phenol accumulation from 18% to 106.9% and the flavonoid content from 20.2% to 74.2%. Compared with the uninoculated drought control, the strains unidentified LPR1+, LPR3, and *B. megaterium* RR13 showed the greatest total phenol accumulation, with corresponding increases of 77.7, 84.0, and 106.9%, respectively. More intriguingly, these bacterial treatments increased phenol production to levels greater than those of the unstressed control. Furthermore, the maximum flavonoid concentrations were recorded when plants were inoculated with *unidentified* LPR1+ (61.7%), LPR3 (52.3%), or *B. megaterium* RR13 (74.2%), referred to as the total phenolic content.

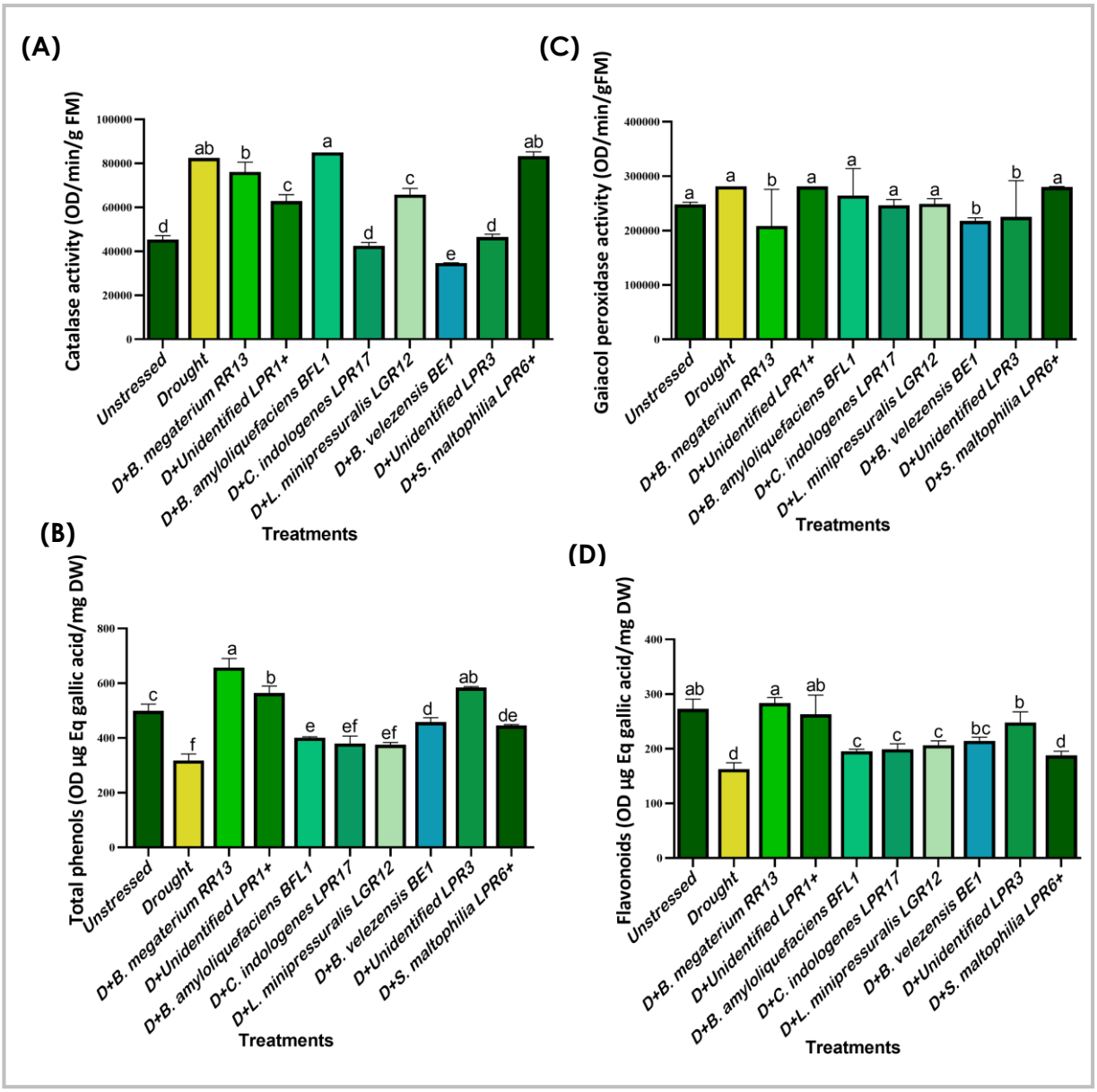


Figure 33: Effect of bacterial treatments on catalase (A), total phenols content (B), guaiacol peroxidase (C), and flavonoid content (D) in maize seedlings subjected to drought stress. Unstressed = plants watered at 100% holding capacity; D = Drought = plants watered at 20% holding capacity. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

- Concerning the investigated parameters, the eight biological agents demonstrated varying abilities to induce drought tolerance in young maize plants. However, the two bacterial strains displayed the strongest potential to protect maize plants from extreme drought stress conditions (Figure 35). ***Unidentified* LPR1+** and ***B. megaterium* RR13** BCAs were therefore chosen and their ability to induce tolerance against the combined impact of *F. verticillioides* rot and drought stress in maize were tested.

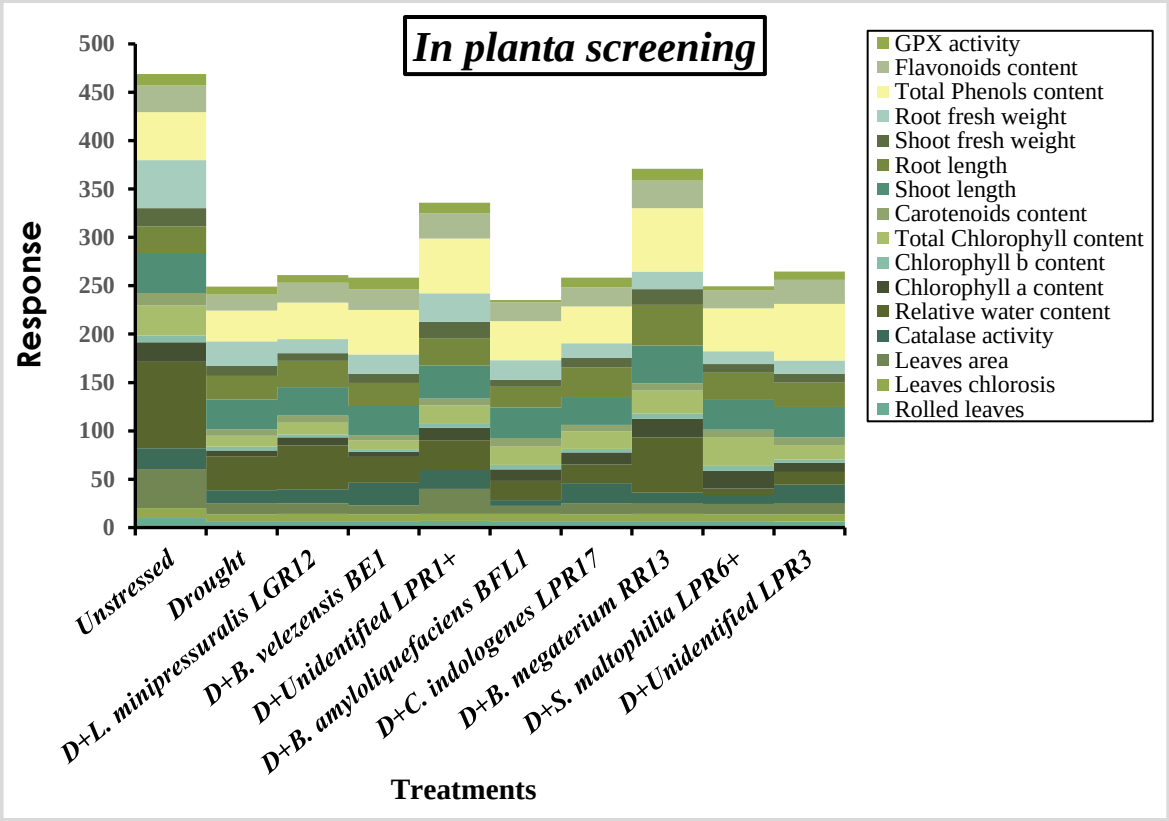


Figure 34: Cumulative *in planta* parameters evaluated for the drought tolerance of the eight tested bacterial agents. * Indicates selected candidates based upon overall response to the studied parameters.

III.1.3. CAPACITY OF SELECTED AGENTS TO IMPROVE MAIZE TOLERANCE AGAINST THE COMBINED EFFECTS OF DROUGHT AND *F. VERTICILLIOIDES* ROT.

III.1.3.1. Effect of bacterial inoculation on maize morphological parameters under combined drought and *F. verticillioides* rot

Combined, drought stress and *F. verticillioides* rot greatly increased the effects of each stress on maize morphological parameters. In fact, *F. verticillioides* infection considerably decreased plant shoot and root lengths (by 35.5% and 142.5%, respectively, compared to those of the normal control), and the same phenomenon was observed with drought, which had a lesser effect (Figure 35). Consequently, the combined stressors considerably reduced the shoot length compared to a single *F. verticillioides* plant (29.3%), the shoot and root lengths in comparison to drought (49.9% and 130.2%), and the shoot and root lengths compared to the normal control (75.14% and 140.1%). However, compared to the stressed and infected treatments, application of the bacterial strain *unidentified* LPR1+ under combined stress conditions significantly improved shoot length (18.5%), and root length increased upon application of both *unidentified* LPR1+ (64.7%) and *B. megaterium* RR13 (13.4%). *Unidentified* LPR1+ showed the most promising potential.

Furthermore, the shoot and root fresh weights of the plants greatly decreased under drought (by 102.4% and 97.2%, respectively), Fv rot (by 191.8% and 167.1%), or a combination of the two stressors (Figure 35). The two stresses had the most severe influence on plant biomass. Shoot fresh weight decreased under the combined treatment compared with the Fv (117.8%), drought (214.1%), and the normal control (535.6% and 251.7%). Compared with the stressed and infected control, all the bacterial treatments did not affect the root weight, while the bacterial agent *unidentified* LPR1+ greatly enhanced the plant shoot weight (11.7%).

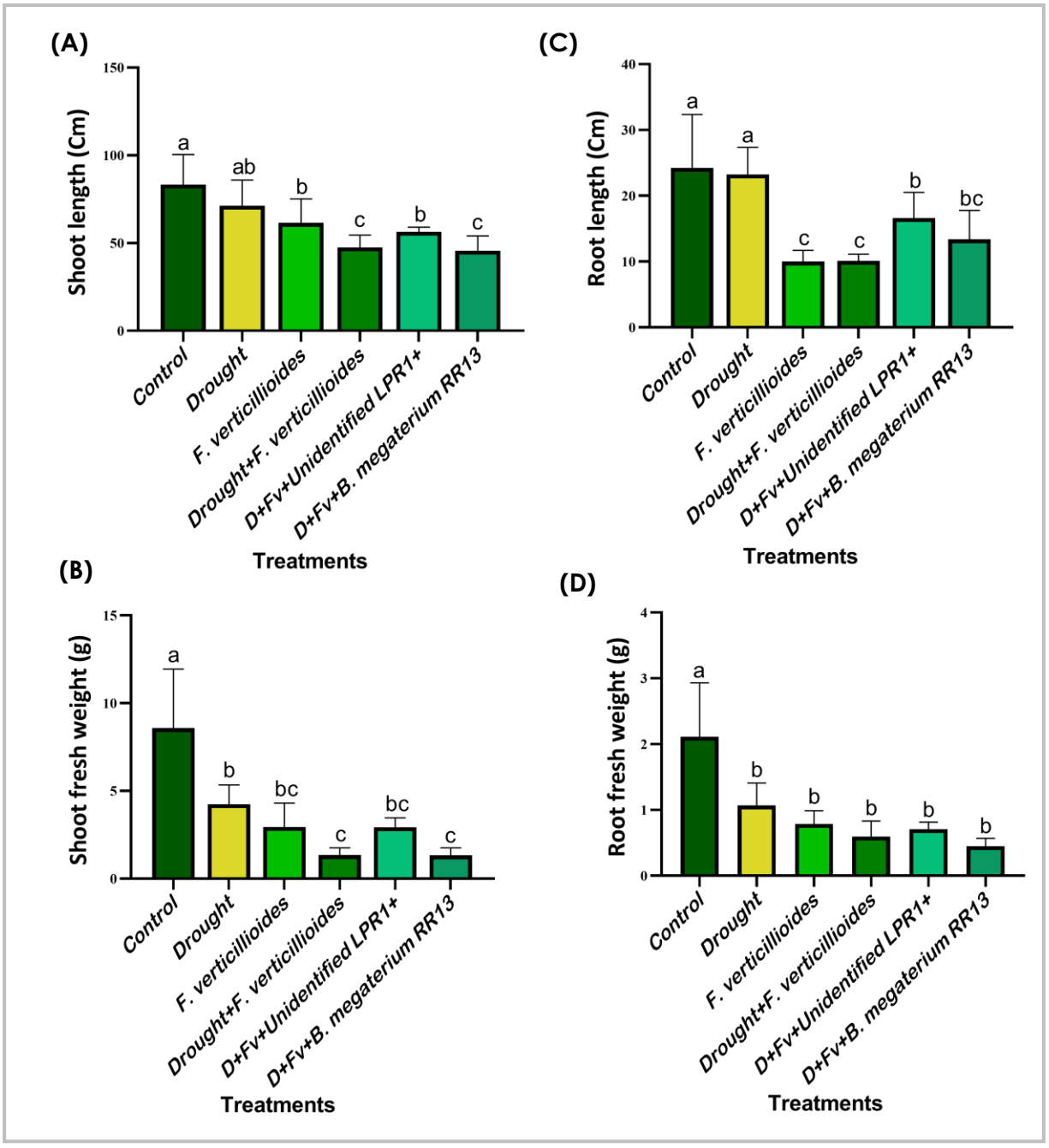


Figure 35: Effect of bacterial treatments (unidentified LPR1+ and *B. megaterium* RR13) on the shoot length (A), shoot biomass (B), root length (C) and biomass (D) of maize plants under the combined effect of drought stress and *F. verticillioides* rot. Control = uninoculated plants watered at 100% holding capacity; D = drought = plants watered at 20% holding capacity. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

III.1.3.2. Effect of bacterial inoculation on maize physiological parameters under combined drought and *F. verticillioides* rot

Drought, *F. verticillioides* inoculation, and, most crucially, the combined impacts of drought and Fv infection had substantial impacts on maize physiological parameters such as leaf chlorosis and leaf area. Inoculation of the bacterial strains, on the other hand, greatly improved various parameters. Drought and Fv infection enhanced leaf chlorosis, and the combination of the two specified conditions increased it approximately threefold (Figure 36). Under combined stress, we observed approximately 88% leaf chlorosis compared with that under single-Fv infection and 34.3% greater leaf chlorosis compared with that under drought treatment. Inoculation with the bacterial strains unidentified LPR1+ and *B. megaterium* RR13 reduced maize leaf chlorosis by 24.3% and 42.4%, respectively, compared to the unbacterized infected and stressed treatment. Therefore, no significant change in leaf rolling was observed upon bacterial treatment.

The maize leaf area dramatically decreased following drought (by 79.6%), Fv infection (137.2%), and the combination of drought and Fv infection (325.8%) compared to that of the normal control. However, compared to the infected and stressed treatments, the unidentified LPR1+ bacterial agent greatly increased the plant leaf area by 84.4%. The relative water content was not significantly affected by drought, Fv infection, or their combination.

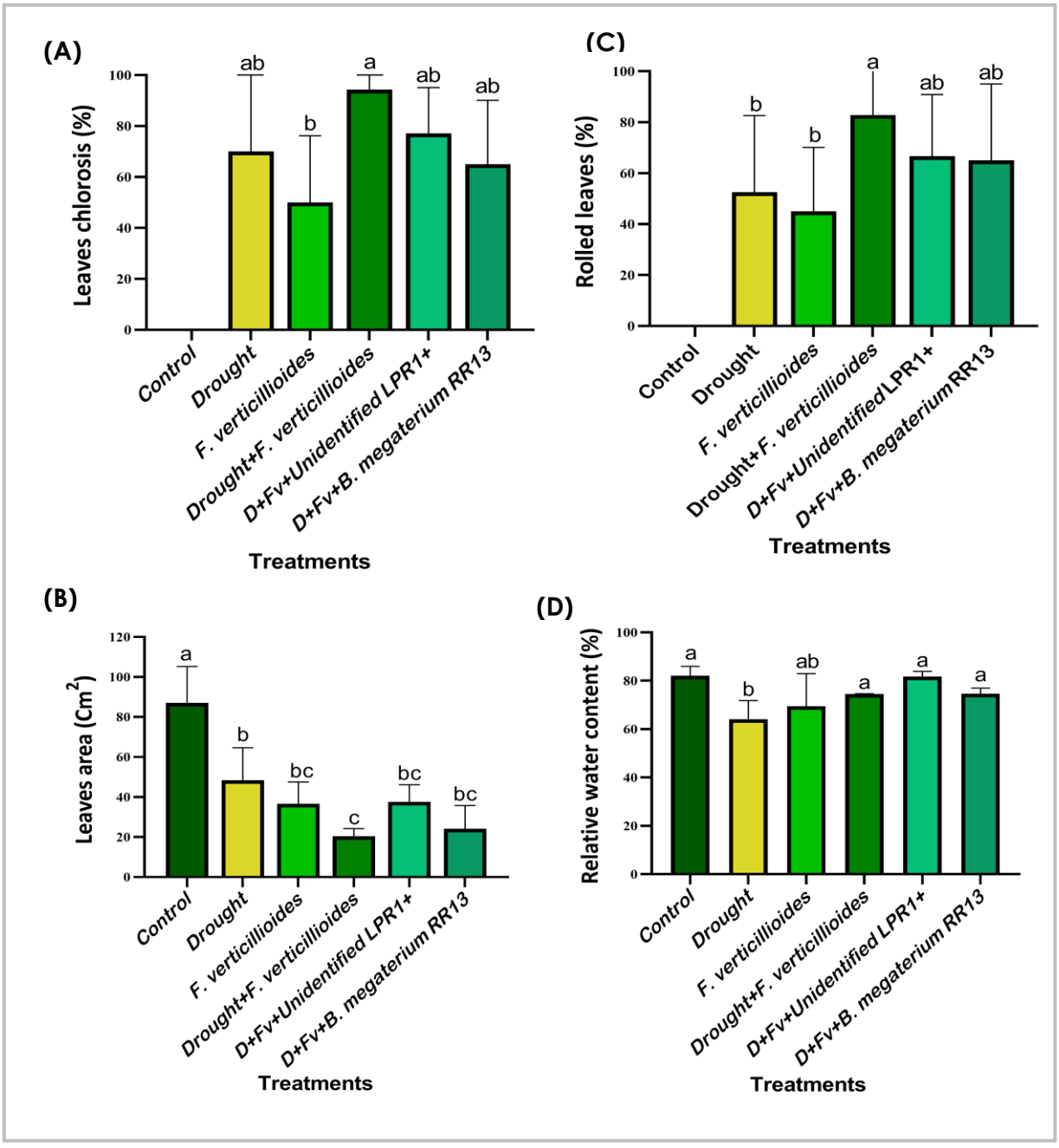


Figure 36: Effect of the bacterial treatments unidentified LPR1+ and *B. megaterium* RR13 on maize leaf chlorosis (A), area (B), rolling (C), and relative water content (D) under the combined effect of drought stress and *F. verticillioides* rot. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

In addition to these physiological characteristics, drought and Fv rot had considerable impacts on the chlorophyll and carotenoid levels of maize leaves, and the combination of both stressors had the greatest impact (Figure 37). Indeed, compared with the normal control, drought and Fv infection decreased the chlorophyll a content by 23.1% and 14.3%, respectively, but the combination of the two stressors reduced the chlorophyll a content by 106.5%. However,

compared to the unbacterized stressed and infected treatments, inoculation of the bacterial strains unidentified LPR1+ and *B. megaterium* RR13 considerably enhanced the chlorophyll a concentration by 29.0% and 61.3%, respectively.

Drought had little effect on the total chlorophyll concentration; however, Fv infection drastically decreased it. Drought and Fv infection together decreased the total chlorophyll content by 47.9% and 24.3%, respectively, compared to their independent effects (Figure 37). Under stressful and infected conditions, compared with the unbacterized treatment, the bacterial treatment with *B. megaterium* RR13 considerably increased the total chlorophyll content. In terms of carotenoid content, drought had no effect, while the Fv decreased by 52.9% compared to that of the normal control. As a result, compared with the Fv treatment, the combined stress treatment decreased the carotenoid content by 59.3%, the drought treatment decreased it by 159.3%, and the normal control decreased it by 140.7%. However, treatments with the two bacterial agents improved the parameter, and *B. megaterium* RR13 restored it to the normal control level. Chlorophyll b content did not change in the different treatments.

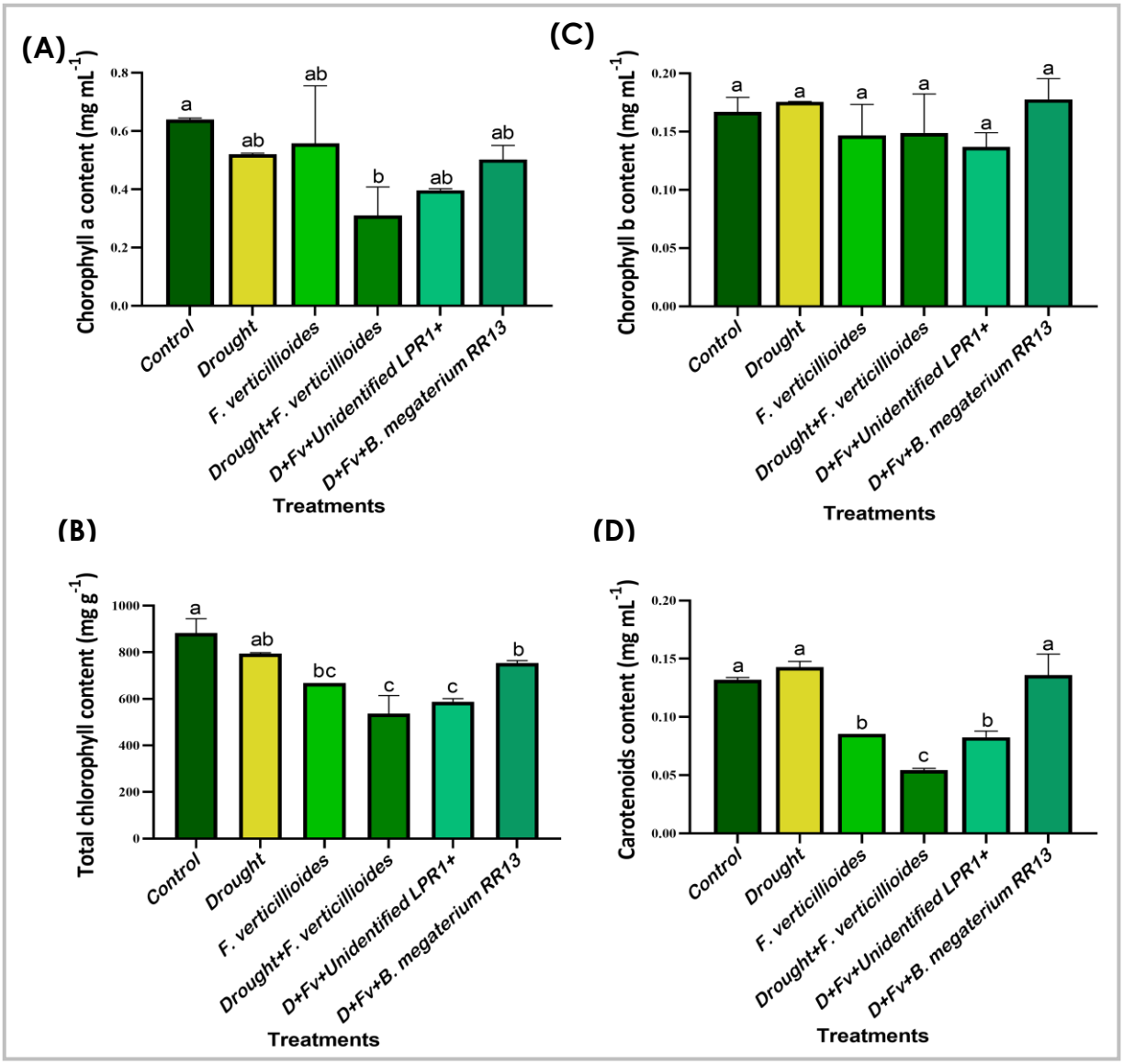


Figure 37: Effect of bacterial treatments (unidentified LPR1+ and *B. megaterium* RR13) on maize chlorophyll a (A), total chlorophyll (B), chlorophyll b (C), and carotenoid contents under the combined effect of drought stress and *F. verticillioides* rot. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

Electrolyte leakage in stressed and bacterized maize leaves was tested to evaluate cell membrane stability as a stress tolerance mechanism. Both drought and Fv rot dramatically increased electrolyte loss, especially when combined. As a result, the use of bacterial agents significantly decreased electrolyte release compared to the unbacterized infected and stressed treatments, as well as the solitary drought or Fv treatments (Figure 38). More intriguingly, the electrolyte leakage of the two bacterial strains decreased less than that of the control, suggesting their ability to reinforce the cell membrane.

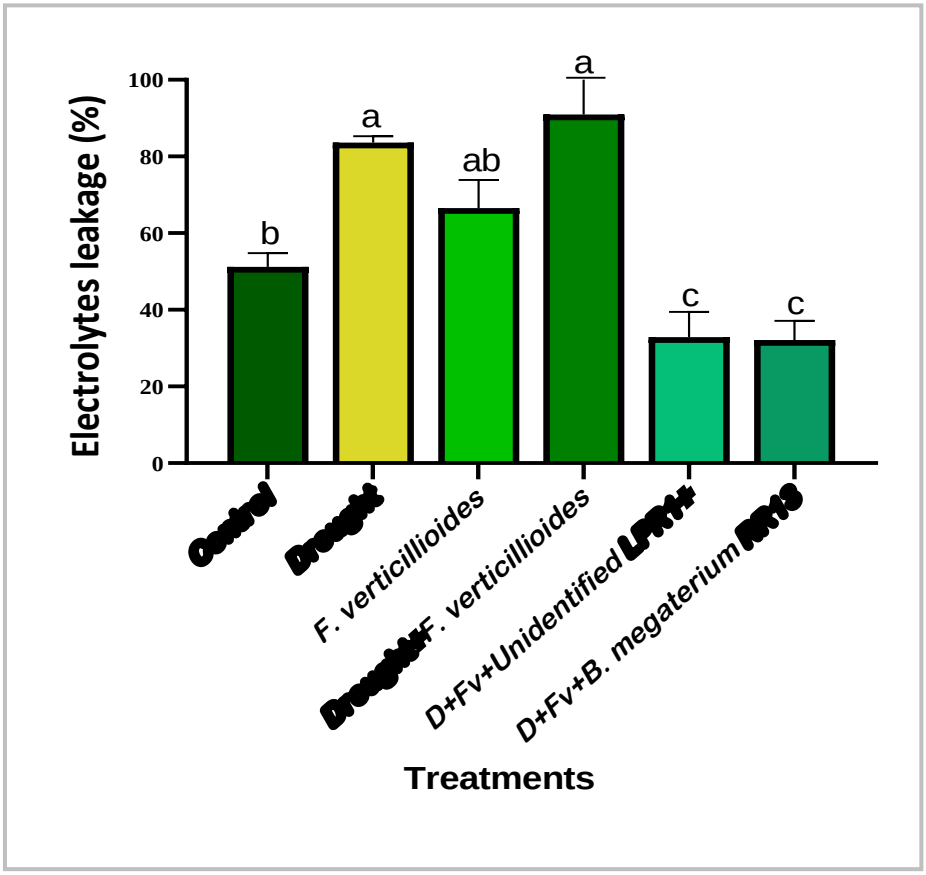


Figure 38: Effect of bacterial treatments (unidentified LPR1+ and *B. megaterium* RR13) on electrolyte leakage in maize leaves under the combined effect of drought stress and *F. verticillioides* rot. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

III.1.3.3. Effect of bacterial inoculation on biochemical parameters expressed by maize under combined effect of drought and *F. verticillioides* rot

The various changes recorded in the synthesis of specific enzymes after the individual and combined impacts of Fv rot, drought stress, and bacterial treatment are shown in Figure 40 below. Fv rot and drought stress enhanced PAL enzyme activity (Figure 39). Additionally, the combination of both pressures resulted in a considerable increase in enzyme activity as a result of the combination of each single stress. Compared with the unbacterized treatment, *B. megaterium* RR13 exhibited a considerable increase in PAL activity, indicating an improvement in the plant defense system. However, unidentified LPR1+ showed lower enzyme activity than the unbacterized treatment, demonstrating the bacterial strain's ability to considerably lower the impact of combined stress on the plant. In terms of catalase activity, drought caused the greatest increase, whereas combined stress caused no change in enzyme

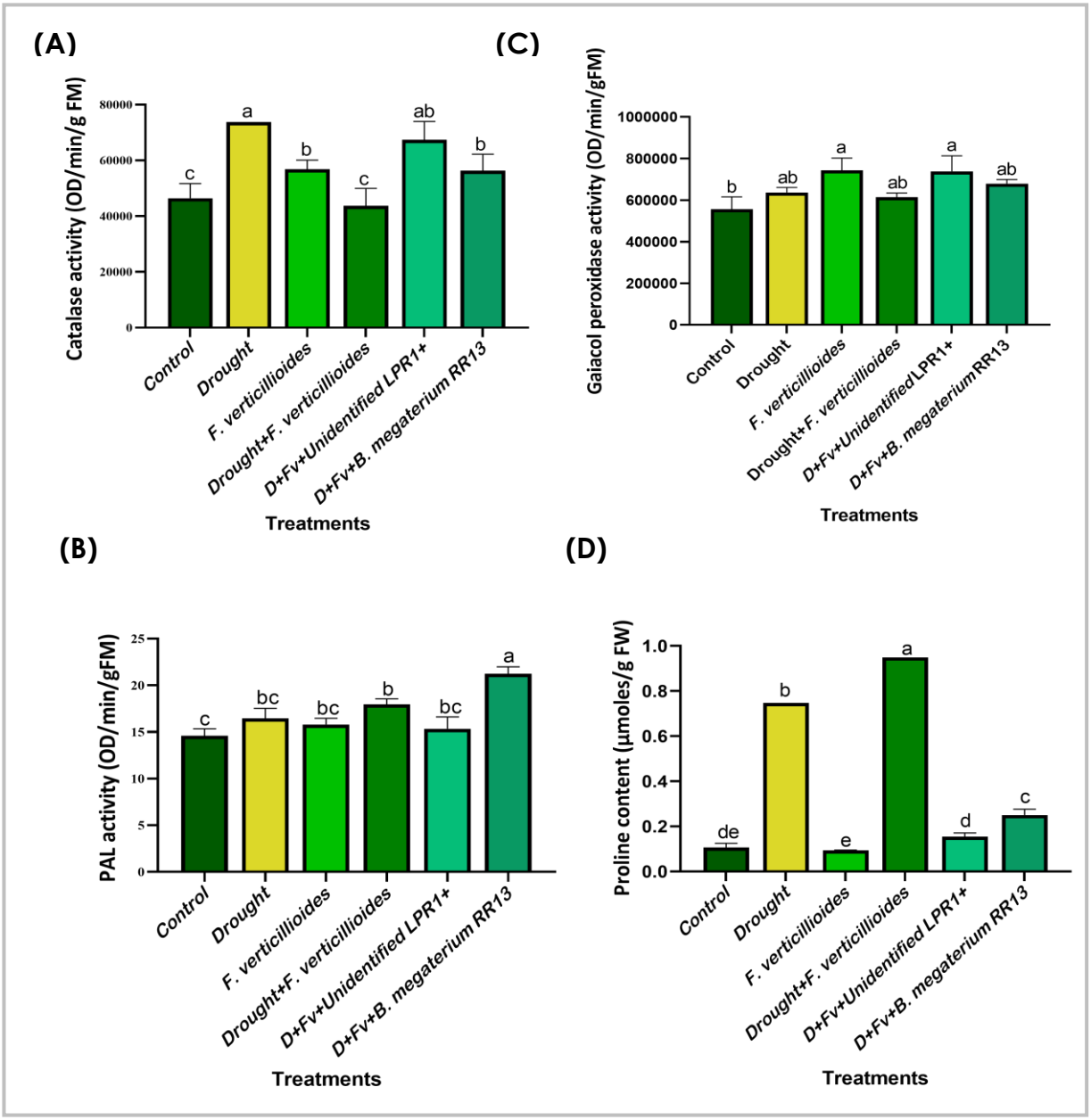


Figure 39: Effect of bacterial treatments (unidentified LPR1+ and *B. megaterium* RR13) on catalase (A), phenylalanine ammonia lyase (B), guaiacol peroxidase (C), and proline (C) activities under the combined effect of drought stress and *F. verticillioides* rot. Bar charts bearing different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$

III.1.2.6. Principal component analysis and correlations among the studied parameters

The correlations between morphological, physiological, and biochemical parameters evaluated in maize following pathogenic infection, drought, and their combination are reported (Figure 40). There was a strong negative correlation between the combined drought treatment and *Fusarium* rot and between the drought treatment and the control, while there was a correlation

between the individual treatments and the control. This reveals that plants subjected to the combined stress are more affected than plants subjected to the other stresses compared with the control. Compared with individual stresses, stress combinations were significantly negatively correlated with plant growth parameters (root and shoot length and weight), as well as with chlorophyll content and leaf area. We observed a positive relationship between combined stress and leaf rolling and chlorosis. Taken together, these observations indicate that the photosynthesis and respiration damage caused by stress are more important because these processes are responsible for the green color of leaves and plant growth and development. Therefore, the bacterial treatments reduced the distance between the combined stress treatment and the control. This represents a reduction in damage or progress to the health state of treated plants. In addition, combined stress was less negatively correlated with *F. verticillioides* than drought. This observation may be supported by the fact that drought conditions were established in the plant prior to the onset of the disease. Research suggests that when abiotic stress precedes biotic stress, the plant's defense mechanisms primarily target abiotic stressors, potentially hindering its ability to effectively defend against biotic stress. Compared with the other treatments, the bacterial treatment unidentified LPR1+, which demonstrated the greatest ability to improve maize tolerance against combined stress, presented a greater negative correlation with drought. This bacterial treatment acts by enhancing the plant defense process, which principally consisted of fighting against drought. The bacterial treatment of unidentified LPR1+ mediated plant protection mainly by improving the relative water content of the leaves. This mechanism could be investigated as an important issue to solve the problem of combined stress in maize plants.

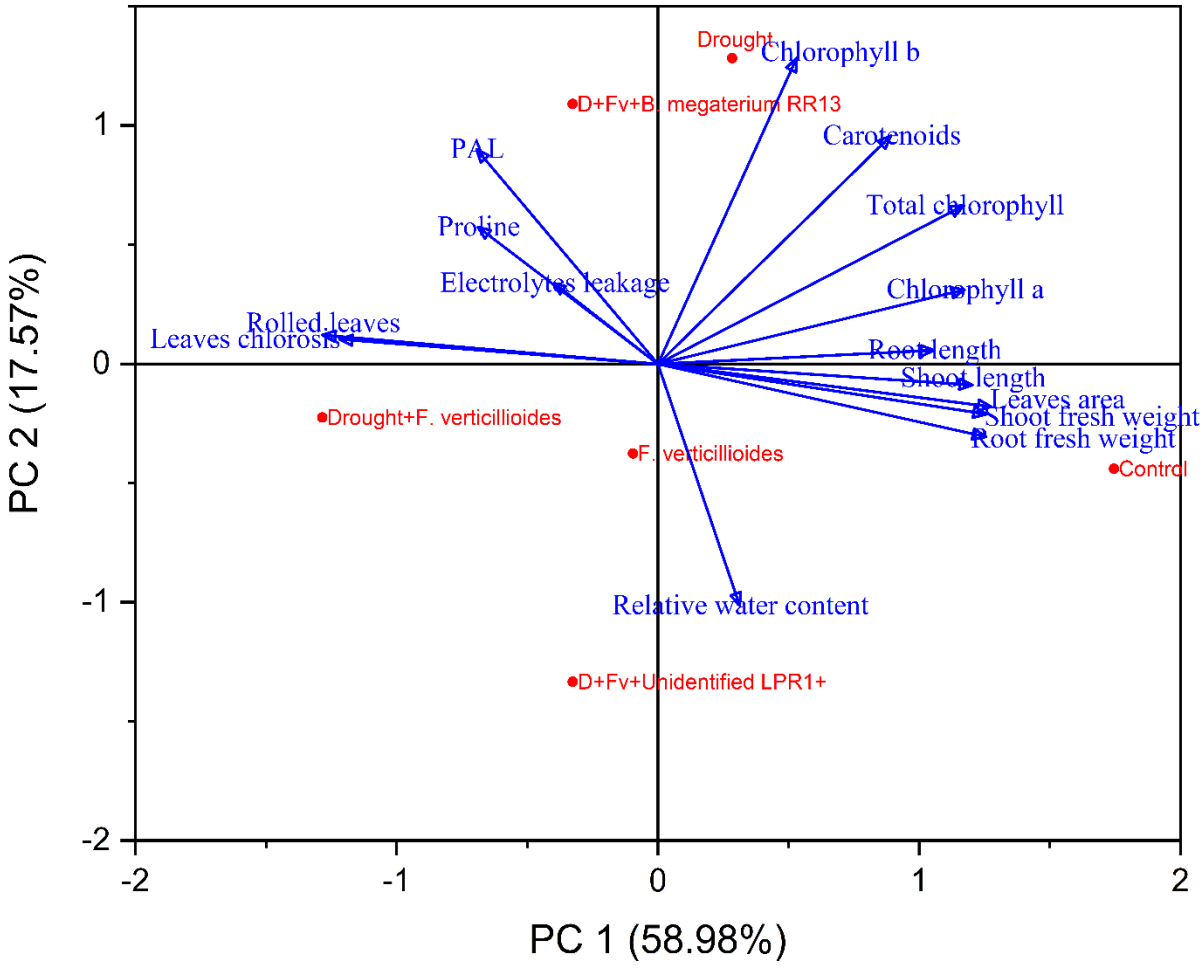


Figure 40: Principal component analysis (PCA) of the parameters of maize subjected to drought, *Fv* infection, combined drought and *Fv* infection and treated with bacterial agents under combined stress. The red points represent the different treatments, and the blue points represent the recorded parameters.

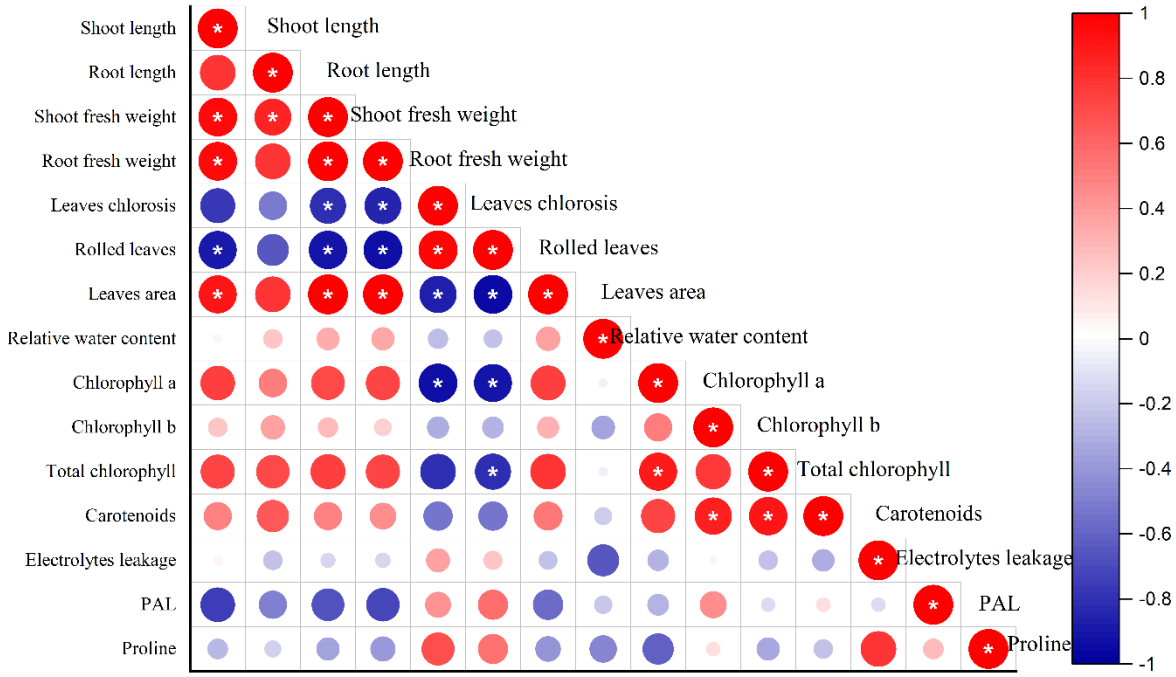


Figure 41: Pearson correlation intensities between the evaluated parameters in maize subjected to drought, pathogenic infection, or their combination and treated with the bacterial agents under combined stress.

Blue indicates a negative correlation, and red indicates a positive correlation between parameters. The color intensity and circle size are directly correlated with the Pearson correlation coefficient, as presented in the diagram scale. * Indicates a significant difference at $p<0.05$.

III.2. DISCUSSION

Recently, the scientific community has focused on microbial technology in the quest for ecologically sound and cost-effective alternatives to agrochemicals. However, cumulative evidence has pinpointed the limited ability of beneficial microorganisms to impart desirable traits to their hosts, due to climate change and putative catastrophic drought events **(Goswami *et al.*, 2014)**. As the result of the ongoing climate changes conditions experienced by the planet, the management of plant diseases requires various adaptive and alleviation approaches in which stress-adaptive beneficial microbes are the most effective and appropriate approaches for ensuring plant protection and alleviating drought stress in agriculture **(Mahanty *et al.*, 2017)**. Transferring microbial endophytes from desert plants that thrive in low nutrient and high-stress conditions to maize plants, for instance, may aid in the resolution of *F. verticillioides* ingress. Therefore, the present study was designed to evaluate the potential of bacterial endophytes from *Euphorbia antiquorum* L. (Cactus), a plant that grows in arid and semiarid soils against *Fusarium verticillioides*, the causative agent of maize ear, stem, and root rot in one part, drought stress in another, and subsequently, their combined effect.

Several *Fusarium* isolates were obtained from symptomatic maize plants and their virulence against a local commercial maize variety (CMS 8704) evaluated. The most virulent isolate was identified via morphological and molecular techniques. Taxonomic characterization revealed banana-like and septated macroconidia as well as the absence of chlamydospores and cotton-like vegetative structures (mycelia). These outcomes, along with others, led to the presumption of members of the *Fusarium* genus. Sequencing of the internal transcribed spacer (ITS) region revealed 100% homology with the strains *F. proliferatum* and *F. verticillioides*. Clear demarcation was observed between the two strains upon sequencing of the translation elongation factor 1 (TEF-1) region, which is a more specific gene sequence for the differentiation of *Fusarium* species. Indeed, *F. verticillioides* is one of the most common fungal pathogens of maize in Cameroon's key producing basins **(Ngoko *et al.*, 2001; Tagne *et al.*, 2021)** and worldwide.

The studied BCAs inhibited *F. verticillioides* mycelial growth by up to 68% and 71% in direct and indirect confrontation assays, respectively. *Bacillus*, *Pantoea*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, *Streptomyces*, *Enterobacter*, *Klebsiella*, and many other biocontrol bacteria have demonstrated inhibitory effects against a broad range of phytopathogens **(Raaijmakers and Mazzola, 2012; Ali *et al.*, 2022a)**. Compared with those of the other BCAs, members of

the *Bacillus* genus showed outstanding inhibitory effects in this study. This is presumably due to their high capacity to produce broad-spectrum antibiotics and/or develop endospores **(Karimi *et al.*, 2016; Figueroa-López *et al.*, 2016)**. Thus, antibiosis, which refers to the formation of antimicrobial compounds, has been promoted as one of the most common predicted properties of potential BCAs and has been widely documented. According to **Caulier *et al.*, 2019; Jacoby and Kopriva, (2019)**, the genome of *B. velezensis* LDO2 contains approximately 32 gene clusters involved in the biosynthesis of antimicrobials such as cyclic lipopeptides, polyketides, and volatile compounds in response to the phytopathogen *A. flavus*. In addition to antibiosis, the starvation of surrounding pathogens via low molecular weight compounds such as siderophores that are capable of binding soil nutrients, rendering them inaccessible to the pathogen, is cited as one of the most important attributes used by BCAs to suppress pathogen growth **(McNeely *et al.*, 2017)**.

In addition, the synthesis and release of hydrolytic enzymes with the ability to lyse cell wall components that cause fungal death have been extensively reported as some of the most interesting mechanisms employed by BCAs **(Jadhav *et al.*, 2017b)**. The studied bacterial agents exhibited a strong ability to produce cellulases, amylases, and/or proteases. Hence, fungi have a unique cell wall structure mainly composed of chitin, glucans, mannans, and glycoproteins, which are needed for nutrient transport, extracellular degradation of nonpermeable substrates, communication, and modification of cell wall structures. Cell wall damage might result in the degradation of signal molecules, receptors, and structural molecules that alter physio-pathological processes or cause direct cell death. The same phenomenon was reported with cellulase through the hydrolysis of 1,4-β-D-glycosidic linkages in cellulose that make up the cell wall membranes of plants, improving the colonization ability of the bacterial strains **(Jadhav *et al.*, 2017a)**.

Another well-documented mechanism employed by bacterial agents to counteract the effects of fungal pathogens is plant growth promotion; a phenomenon that involves compensation of damaged cells and provision of metabolic precursors for the main biochemical processes in the plant. The requirement for energy (ATP) to fuel pathways implicated in plant defense responses has extensively been shown to link primary and secondary metabolism. Hence, the plant defense system is primarily involved in key steps in plant development, such as seed germination and seedling development **(Mengiste *et al.*, 2003)**. There are a plethora of *in vitro* traits expected from promising BCAs, including but not limited to the ability to produce and

release IAA, siderophores, ammonia, and solubilization of inorganic phosphate. In this study, the 25 tested bacterial agents presented the ability to produce IAA, ammonia, the three types of tested siderophores (catecholate, carboxylate, and hydroxamate), and, for some, the capacity to solubilize inorganic phosphate. Thus, bacteria produce low-molecular-weight chelators known as siderophores to acquire Fe^{3+}, reduce them to Fe2+, and then release them into cells, leading to improved plant growth **(Pahari *et al.*, 2017)**. Siderophores are classified based on the coordinating groups that chelate the Fe^{3+} ion, and the most common coordinating groups are catecholates, hydroxamates, and carboxylates **(Ali and Vidhale, 2013)**. Endophytic growth promoters solubilize inorganic phosphate (P) with the help of low molecular weight organic acids to make it available for the plant **(Rawat *et al.*, 2021)**. This potential has been widely reported for endophytic bacteria from the genera Bacillus, Enterobacter, Klebsiella, and many others **(Prabhu *et al.*, 2019)**. Our data further confirmed this assertion, as bacterial agents able to solubilize inorganic phosphate also demonstrated good plant growth effects *in planta*. In addition, iron is the major component for various vital functions of plants, such as photosynthesis; the synthesis of nucleosides, redox reagents, amino acids, and enzyme cofactors; and the presence of $Fe^{3+,}$ which is inaccessible to both plants and microorganisms **(Sah and Singh, 2015)**. Another plant growth mechanism used by biocontrol agents is indole acetic acid (IAA) production. This auxin is implicated in almost all plant growth processes, such as plant cell division, seed germination, and vegetative growth. By producing IAA, endophytic bacteria increase root length and area, thereby increasing the accessibility of soil nutrients to plants **(Ahemad and Kibret, 2014)**.

The bacterial candidates with the best *in vitro* profiles were evaluated directly on young maize plants for their ability to reduce or inhibit the effects of *F. verticillioides*. Compared with single-Fv inoculation, all ten tested bacteria reduced the severity of maize rot caused by *Fv* and increased plant length. The potential of RDS could be correlated with the different antagonistic mechanisms employed by bacterial agents, such as direct or indirect inhibition of pathogen growth, cell death due to the action of hydrolytic enzymes or indirect effects of plant growth promotion. On the other hand, it could be assumed that the reduction in disease severity by bacterial strains creates more favorable growing conditions that lead to better plant growth. In fact, plant defense against pathogens is triggered by a wealth of mechanisms. In the case of resistance induced by bacterial agents, there could be a direct effect of the bacteria against the pathogen, as previously mentioned; on the other hand, the bacteria can induce resistance in the host, called induced systemic resistance (ISR) **(Fadiji and Babalola, 2020)**. For ISR, priming

plants by BCAs, referring to plant preparation by inoculation of protective agents prior to pathogen attack, has emerged as an important cellular process in many types of biologically and chemically induced systemic immunity. This is characterized by an enhanced level of resistance due to faster and/or stronger activation of cellular defenses upon pathogenic invasion **(Conrath *et al.*, 2015)**. This ISR is regulated by jasmonic acid (JA) and ethylene (ET). However, there is a type of systemic resistance induced by BCAs called systemic acquired resistance (SAR) that is dependent on salicylic acid (SA) **(Pieterse *et al.*, 2014)**. This overall systemic resistance is accompanied by the synthesis of defense compounds such as chitinases, ß-1,3-glucanases, pathogenesis-related (PR) proteins, callose, enzymes of the phenylpropanoid pathway (PAL), peroxidases (GPX), and phenolics.

Furthermore, some biological agents significantly increased PAL- and GPX-specific activities ($p<0.05$). This may correlate with their ability to suppress the development of ear and root rot diseases. These results suggested that GPX and PAL are implicated in distressing plants inoculated with the best BCA strains. The GPX burst is one of the early responses of host plant cells to pathogen infection **(Afzal *et al.*, 2014)** and reduces pathogenesis. In addition, peroxidases are important scavengers that act as electron donors, reducing reactive oxygen species (ROS), particularly O_2 and peroxyl radicals, to less or no harmful molecules **(Bhattacharya, 2015)**. Moreover, the PAL enzyme possesses antimicrobial activity and increases the rate of polymerization of phenolic compounds into lignin-like substances that are deposited in cell walls and papillae and restrict pathogen invasion **(Bhardwaj *et al.*, 2014)**.
Ten bacterial endophytes with the most promising biocontrol potential were further examined for their ability to confer drought resistance in maize. The strains exhibited growth capacities at moderate water potentials but proceeded at lower water potentials except for *B. megaterium* RR13, unidentified LPR1+, *C. indologenes* LPR17, and *B. amyloliquefaciens* RA37. Indeed, **Eke *et al.*, (2019b) and (2021)** have previously documented the drought tolerance ability of these strains. Furthermore, **Wang *et al.*, (2014)** reported similar findings with bacteria isolated from *Populus euphratica* growing under moisture stress conditions, with severe repression of cell growth (-1.02 MPa). Furthermore, recent works from **Sandhya *et al.*, (2017) and Siddique *et al.*, (2022)** demonstrated that bacterial endophytes can sustain water stress as high as -1.02 MPa. Bacterial drought stress resistance can be attributed to numerous mechanisms, including the production of exopolysaccharides (EPSs), which are hydrophilic compounds released into the microenvironment outside cells that can act as water reservoirs. Additionally, in soil, EPS act through the activation of particle aggregation, resulting in more free water molecules

(Mandal *et al.*, 2022). Biofilm production is another important strategy that bacteria use to prevent drought stress **(Fazeli-Nasab *et al.*, 2022)**. In plants, biofilm production promotes bacterial cell survival at the seed surface under drought conditions and increases root colonization for optimum plant protection **(Singh and Chauhan, 2017)**. Several studies have shown the critical role that biofilms play in providing protection against biotic and abiotic challenges, including drought stress **(Wang *et al.*, 2019)**. For example, *Planomicrobium chinense*, *Bacillus cereus*, *Bacillus subtilis*, and *P. fluorescens* have been found to possess biofilm-like structures in roots and the ability to improve drought tolerance in some plants **(Bounedjah *et al.*, 2012; Furlan *et al.*, 2017)**. **Jaleel *et al.*, (2007)** also reported that a mutant strain of *P. fluorescens* with increased biofilm production is thought to be more effective for water budget and carrot colonization than its wild-type parent. This is due to the ability of biofilms generated at the root surface to increase soil accumulation, improve water status, and increase microbial biomass, hence boosting root exudates under stress **(Lugtenberg and Kamilova, 2009)**. The ability of the bacterial strains in this study to form biofilms in the face of extreme drought may be related to their capacity for stress resistance, as some of the most efficient biofilm producers, *Bacillus megaterium* RR13 and *unidentified* LPR1+, were also the most resilient to this high-stress situation.

Bacterial strains also promote drought tolerance in plants by producing ACC deaminase, which hydrolyzes ACC to α-ketobutyrate and ammonia **(Ngumbi and Kloepper, 2016)**. ACC activity increases during drought, increasing ethylene levels that negatively affect plant development and cause senescence. It has been discovered that inoculating plants with bacteria that contain ACC deaminase can lessen the harmful effects of ethylene stress by catalyzing the breakdown of ACC, a precursor to ethylene synthesis, into α-ketobutyrate and ammonia. This, in turn, can encourage water conservation, plant growth, and development in times of water scarcity **(Khan *et al.*, 2020)**. Furthermore, under conditions of water stress, bacterial agents may promote the production of osmoprotectants, including trehalose, fructans, sorbitol, polyols, proline, mannitol, and oligosaccharides, which safeguard cells from cellular osmotic shock. Osmolytes create hydrogen bonds with macromolecules to inhibit the formation of intramolecular hydrogen bonds in these molecules **(Kumari *et al.*, 2015; Vurukonda *et al.*, 2016)**.

This section aimed to evaluate the ability of the bacterial strains to thrive under drought stress. Two strains were eliminated due to their poor ability to withstand drought. The remaining eight strains were tested on maize plants for their capacity to confer drought tolerance. Generally,

water scarcity leads to plant growth and yield losses in many cereal crops **(Moreno-Galván *et al.*, 2020)**. Drought adversely affects maize shoot and root development. However, seed treatments with bacterial endophytic strains significantly boosted plant growth. Notably, treatments with *Bacillus megaterium* RR13 and unidentified LPR1+ led to a marked increase in shoot and root length, as well as shoot biomass. **Shirinbayan *et al.*, (2019)** reported that inoculating maize plants with Bacillus strains isolated from semiarid areas increased nutrient absorption, plant height, and root and shoot biomass under drought stress. This is linked to microbial hormone production, which is thought to be the most likely mechanism influencing plant growth and development **(Naveed *et al.*, 2014a)**. Drought stress at the root level stimulates the synthesis of ABA, which induces stomatal closure on the leaf surface. This results in a reduced rate of transpiration, decreased CO2 assimilation, and reduced photosynthetic pigments, ultimately lowering growth capacity and plant biomass. Furthermore, the most immediate indicator of dryness is a halt in cell growth and cell division, leading to hindered plant turgor. This mechanism is utilized to avoid drought by lowering water use at the expense of plant growth and development **(Beneová *et al.*, 2012)**.

Endophytic bacteria have been extensively demonstrated to enhance maize plant tolerance to drought stress **(Yang *et al.*, 2009; Vardharajula *et al.*, 2011; Kavamura *et al.*, 2013; Naveed *et al.*, 2014c; Azeem *et al.*, 2022)**. The response of plants to water scarcity has been studied using genetic, biochemical, and morphophysiological features, including but not limited to the relative water content (RWC), leaf area, chlorophyll content, and electron transport regulation **(Maccaferri *et al.*, 2011; Bürling *et al.*, 2013)**. A reduction in total leaf area, caused by a decrease in the number and/or size of leaves affected by leaf rolling, is, therefore, an essential morphological alteration. This change combined with a decrease in stomatal conductance effectively reduces water loss via evapotranspiration **(Wang *et al.*, 2008)**. Therefore, an increase in the photosynthetic rate is a key stress tolerance mechanism mediated by bacterial endophytes. According to **Huseynova *et al.*, (2009) and Anjum *et al.*, (2020)**, water deficit results in the deterioration of thylakoid membranes, lowering the plant's capacity for photosynthetic processes. Interestingly, we observed in this study that drought reduced the contents of chlorophylls a and b and total chlorophyll. However, the chlorophyll content was markedly increased by seed inoculation with bacterial agents, especially *Bacillus megaterium* RR13, which clearly improved maize photosynthetic performance. These findings are consistent with those of **Rashid *et al.*, (2022)**, who reported that under drought stress, *Bacillus megaterium* increased the production of photosynthetic pigments (chlorophyll a, b, and

carotenoids), relative water content, and leaf area. In addition, the decrease in chlorophyll b under drought compared with that in chlorophyll a might be attributed to an adaptation mechanism in maize plants to enable drought tolerance. Maize, as a C4 plant, can tolerate drought to some extent, and chlorophyll b is known to be the most effective for drought tolerance, allowing the plant to absorb a broader range of wavelengths of light and convert a large range of energy from the sun into chemical energy **(Martin, 2019)**.

Similarly, drought stress increases the production of reactive oxygen species (ROS), known for their harmful effects, such as membrane injuries caused by lipid peroxidation, protein degradation, and protein oxidation, which results in enzyme inactivation and the formation of protease-resistant cross-linked aggregates **(Berlett and Stadtman, 1997; Sairam *et al.*, 2005)**. ROS are scavenged by antioxidant enzymes, which serve as indicators of plant drought defense. According to this research, drought stress causes an increase in the levels of the antioxidant enzymes CAT and GPX. Catalase and oxidase synthesis have been shown to help sustain metabolism and prevent membrane damage by lowering H_2O_2 **(Lismont *et al.*, 2019)**. As a result, seedlings inoculated with bacterial endophytes showed a considerable decrease in antioxidant enzyme activity under drought stress. An increase in antioxidants during low to moderate drought indicates strengthened drought tolerance machinery. However, during severe drought, antioxidant enzyme activity can no longer balance tolerance or sensitivity to drought, leading to a plethora of cellular damage **(Benešová *et al.*, 2012)**. However, the decrease in antioxidant activity mediated by the BCAs might be associated with decreased plant drought severity. It is well documented that nonenzymatic antioxidants such as phenols and flavonoids can repair membrane lipid peroxidation and scavenge excess ROS under stressful conditions. Under drought stress, increased levels of phenols and flavonoids are indicative of the plant's adaptation to the stress **(Liu *et al.*, 2011; Yanlin *et al.*, 2019)**. This is in line with our findings, whereby seed bacterization with the strains *Bacillus megaterium* RR13 and *unidentified* LPR1+ improved the total phenol and flavonoid contents under drought conditions, revealing a drought stress adaptation mechanism in maize. According to the findings of this study, *Bacillus megaterium* RR13 and unidentified LPR1+ exhibited the greatest potential for inducing drought tolerance in young maize plants. These compounds may therefore be promising agents for addressing the concurrent effects of *F. verticillioides* and drought in maize.

Although drought is a severe hazard to plant development, the harm is amplified when paired with disease assault. The former increases plant sensitivity to pathogenic fungi, changes plant-

fungi interactions and affects the incidence and spread of diseases **(Sinha *et al.*, 2019)**. However, existing control measures focus on individual stress and often overlook the combined effects of these stressors. However, finding a broad-spectrum BCA capable of addressing both infections and drought is currently limited **(Eke *et al.*, 2023a)**. To address this issue, we studied the capacity of BCA to protect maize against the single and combined impacts of *F. verticillioides* and drought stress. Overall, the combination of stressors accentuated the individual impacts, but an additive effect was not always noted, depending on the parameter examined, which confirms many authors' hypotheses **(Atkinson *et al.*, 2013; Pandey *et al.*, 2015; Ramu *et al.*, 2016)**. Our research revealed that combined stress had a cumulative negative effect on maize growth indicators, such as root and shoot growth, as well as on fresh biomass. Drought significantly affects maize growth parameters, and the detrimental impact of *F. verticillioides* was even more pronounced when paired with drought. This finding is supported by the findings of **Pandey *et al.* (2017a)**, who reported that drought and fungal infection have an additive impact on plant development. Indeed, depending on the pathosystem, drought may increase or decrease plant sensitivity to a certain pathogen. Drought, for instance, causes an increase in the production of root exudates, which include amino acids and carbohydrates, providing essential nutrients for the growth of soilborne pathogens and exacerbating preexisting infections **(Schroth and Hildebrand, 1964; Duniway, 1977)**. Furthermore, the combination of drought and root-infecting diseases exacerbates damage to the root system, leading to greater overall plant damage **(Pandey *et al.*, 2017b)**. These root-damaging organisms impair the ability of roots to absorb water under drought conditions, compromising their access to water in deeper soil profiles **(Chilakala *et al.*, 2022)**. However, the use of bacterial agents significantly improved the affected parameters.

As previously stated, leaf rolling and stomatal closure are plant-mediated processes that limit water loss by transpiration and are consequently employed for drought tolerance, resulting in a reduction in leaf area and RWC. According to **Qi *et al.*, (2018)**, pathogen infections may jeopardize a plant's ability to withstand drought by producing toxins that restrict stomatal closure and emphasize the effects of drought stress on the host plant. Furthermore, plant xylem is invaded by systemic infections such as *F. verticillioides*, which obstruct the flow of nutrients and water to the leaves, causing plant stunting as well as an increase in leaf rolling and chlorosis. The greatest levels of leaf chlorosis, rolling, and decreased leaf area observed in plants subjected to combined stress as opposed to individual drought and Fv rot during this investigation may be explained by these processes. Drought, Fv rot, and their interactions are

all detrimental factors that impair plant growth and production not only by disrupting the water regime or photosynthetic activity but also by causing an oxidative explosion **(Pandey *et al.*, 2015)**. Antioxidants such as catalase and GPX are produced to minimize ROS damage by scavenging free H_2O_2. In the present study, the co-occurrence of drought and Fv rot did not affect the activities of GPX and catalase compared to those in the normal control, despite each stress increasing the activity of one or both of these antioxidant enzymes. These data support the hypothesis that the combination of drought and Fv rot reduces/inhibits plant defense mechanisms against oxidative damage. However, bacterization, specifically by the strain unidentified LPR1+, greatly enhanced the antioxidant enzyme activities. Elsewhere, the reduction in cell water content by drought induces osmotic stress, and to overcome this stress, plants produce low molecular weight compounds with osmo-protective properties such as amino acids, sugars, and betaines. Proline is one of the most significant and widely distributed osmoprotectants in plants and plays various roles **(Kaur and Asthir, 2015)**. It functions as an osmoprotectant, a low molecular weight chaperone regulating enzyme activities and protecting protein integrity, an antioxidant with ROS scavenging abilities, and a biomarker of drought tolerance in plants **(Suprasanna *et al.*, 2016)**. There was a very high synthesis of proline under drought conditions and when associated with *Fusarium* rot. As a result, the use of the bacterial strains reduced the proline content to an unstressed level. This finding supports numerous studies that have shown that proline is highly produced under multiple stress conditions and is essential for plant survival in harsh environments. **Verbruggen and Hermans, (2008)** indicated that in stressful situations, proline concentrations might be 100 times greater than those in normal controls. The glutamate proline biosynthetic pathway is thought to be the most important source of proline accumulation, while the ornithine pathway is activated in chloroplasts or the cytoplasm under nitrogen-limiting or osmotic stress conditions **(Delauney *et al.*, 1993; Dar *et al.*, 2016)**. The reduction in proline content in stressed and bacterial-treated plants could be partially explained by stress reduction and in the other part by catabolism of synthesized proline. This catabolism improves plant growth and development by participating in oxidative respiration, producing energy, or acting as a metabolic signal for metabolite pool stabilization **(Verbruggen and Hermans, 2008; Kaur and Asthir, 2015)**. All of these findings support the ability of bacterial agents to confer resistance to stressors, notably combined drought and Fv rot, in maize plants and to synthesize chemicals involved in plant defense against combined stress. According to these findings, drought exacerbates the damage caused

by *Fusarium verticillioides* on young maize plants, and the inoculation of bacterial endophytes from desert plants represents a potential and critical solution to this problem.

CONCLUSION AND PERSPECTIVES

CONCLUSION

The present study aimed to evaluate the potential of bacterial endophytes from desert spurge (*Euphorbia antiquorum* L.) against the individual and combined effects of drought and *Fusarium verticillioides* rot on young maize plants. The following conclusions were drawn:

1. The *in vitro* antagonistic performance and plant growth-promoting profiles of the bacterial strains guided the selection of ten strains: *B. megaterium* RR13, *B. amyloliquefaciens* RA37 and BFL1, unidentified LPR1+ and LPR3, *C. indologenes* LPR17, *K. pneumoniae* RR19, *L. minipressuralis* LGR12, *B. velezensis* BE1, and *S. maltophilia* LPR6+. These ten bacterial strains reduce the severity of maize rot caused by *F. verticillioides*, with disease severity reductions (RDS) ranging from 22.5% to 45%. They also demonstrated capacities to increase root length by 13.5% to 79.8%. Although the *C. indologenes* LPR17 treatment showed the highest tolerance to *F. verticillioides* infection, all ten strains enhance the disease resistance in maize plants through root and shoot development or by boosting the plant defense system (GPX and PAL).

2. These results confirm that drought stress reduces plant biomass and photosynthetic parameters in maize. However, the application of antagonistic bacteria improved morphological, physiological, and biochemical parameters of plant development under drought conditions. The bacterial agents *Bacillus megaterium* RR13 and unidentified LPR1+ exhibited the most significant performance in inducing drought tolerance in maize, with an increase in root length of up to 85.3%, 141.8% in relative water content (RWC), and 106.9% in total phenols compared to the drought control. These two agents were further assessed for their capacity to protect maize against the combined impact of *Fusarium* rot and drought stress.

3. The combined stress of *Fusarium* rot and drought significantly aggravated the impact of each stress. The most significant reductions were observed in plant growth, chlorophyll content, leaf area, and antioxidant enzymes (catalase and guaiacol peroxidase). Additionally, there was a notable increase in leaf rolling, chlorosis, electrolyte leakage, proline, and PAL contents. However, the application of the two bacterial agents significantly improved various parameters, enhancing plant growth and

ameliorating the physiological and biochemical impacts of the combined stresses. The strain unidentified LPR1+ demonstrated the best overall performance.

PERSPECTIVES

Based on these results, there is an urgent need for further investigation into these promising agents to address the escalating challenge of combined drought and *Fusarium* rot affecting maize cultivation. To this end, we intend to:

- Identify and optimize the best culture conditions (temperature, time, medium, pH) for the growth and antifungal activity of unidentified LPR1+.
- Evaluate the stability and efficacy of different substrate-based biofungicides with unidentified LPR1+.
- Conduct a field study with the efficient biopesticide over two consecutive years (during dry periods) and study the impact of biofungicide application on soil composition.

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APPENDICES

APPENDICES

Appendix I: *F. verticillioides* mycelial growth inhibition by direct confrontation and through volatile metabolites

Bacterial strains	Inhibition by dual culture (%)	Inhibition by volatile metabolites (%)
***P. peneri RA*24**	7.5±1.5 ^{ghi}	0.0±0.0^{i}
***B. megaterium* RR13**	58.9±0.0 ^{bc}	50.0±1.3 ^{bc}
***E. cloacae* LGR26**	49.4±2.7 ^{de}	40.6±4.0 ^{cdef}
***B. amyloliquefaciens* BFR2**	14.1±0.0 ^{fg}	33.0±1.3 ^{ef}
***B. amyloliquefaciens* RA37**	68.6±0.9 ^{a}	17.9±4.0 ^{gh}
***Paracoccus* LPR20**	20.2±1.5 ^{f}	29.2±6.7 ^{fg}
Unidentified LPR1+	53.2±2.7 ^{cd}	36.8±9.3 ^{def}
B. amyloliquefaciens BFL1	19.1±0.0 ^{f}	71.7±0.0 ^{a}
***L. fusiformis* LPR19**	0.0±0.0 ^{i}	15.1±0.0 ^{h}
***L. minipressuralis* PA2+**	45.5±0.9 ^{de}	15.1±0.0 ^{h}
***P. aeruginosas* RR4**	42.30±0.0 ^{e}	49.1±0.0 ^{c}
C. indologenes LPR17	48.7±0.0 ^{de}	66.0±1.3 ^{a}
***K. pneumoneae* RR19**	48.1±0.9 ^{de}	61.3±1.3 ^{ab}
L. minipressuralis LGR12	53.8±0.0 ^{cd}	8.5±1.3 ^{hi}
***unidentied* LPR3+**	4.23±0.0 ^{hi}	40.6±4.0 ^{cdef}
***B. velezensis* BE1**	65.4±1.8 ^{ab}	48.1±1.3 ^{cd}
B. xiamenensis RA4	11.7±7.5 ^{fgh}	61.3±1.3 ^{ab}
Unidentified LPR3	8.5±0.0 ^{ghi}	67.0±1.3 ^{a}
***P. putida* RR20**	12.8±0.0 ^{fgh}	17.9±4.0 ^{gh}
***C. indologenes* LPR11+**	8.5±0.0 ^{ghi}	61.3±1.3 ^{ab}
S. maltophilia LPR6+	6.4±3.0 ^{ghi}	62.3±0.0 ^{a}
***P. putida* PA3+**	19.2±5.4 ^{f}	12.3±1.3 ^{h}
***C. fruendii* LPR4+**	5.3±1.5 ^{ghi}	43.4±0.0 ^{cde}
***L. minipressuralis* LPR12+**	7.4±1.5^{ghi}	43.4±0.0 ^{cde}
***S. maltophilia* RA33**	0.0±0.0^{i}	41.5±2.7 ^{cde}

The activities are expressed as means of values ±SD and values in each column with different letters are significantly different with respect to Tukey's multiple range test at $P< 0.05$

Appendix II: Effect of bacterial treatments and F.v inoculation on some morphological and biochemical parameters of young maize plants

Bacterial strains	Shoot length (cm)	Root length (cm)	Root dry weight (g)	Shoot dry weight (g)	GPX activities (OD/min/g FM)	PAL activities (OD/min/g FM)
Control	81.3 ± 1.8 ^{a}	25.0 ± 2.6 ^{abc}	0.2 ± 0.0 ^{a}	0.7 ± 0.0 ^{a}	604.9 ± 119.0 ^{d}	20.6 ± 3.5 ^{b}
F. verticillioides	60.3 ± 5.5 ^{cde}	14.8 ± 5.2 ^{c}	0.1 ± 0.0 ^{b}	0.4 ± 0.0 ^{bc}	1263.8 ± 354.0 ^{cd}	20.3 ± 2.0 ^{b}
B. megaterium RR13	60.6 ± 4.3 ^{de}	22.3 ± 5.5 ^{bc}	0.1 ± 0.0 ^{b}	0.2 ± 0.1 ^{c}	1132.4 ± 83.0 ^{cd}	28.1 ± 0.9 ^{a}
B. Amyloliquefaciens RA37	70.3 ± 4.6 ^{abcd}	30.5 ± 6.1 ^{ab}	0.2 ± 0.0 ^{ab}	0.5 ± 0.0 ^{abc}	1121.9 ± 112.0 ^{cd}	13.1 ± 0.5 ^{c}
Unidentified *LPR1+*	66.0 ± 1.0 ^{bcde}	26.4 ± 2.5 ^{ab}	0.1 ± 0.0 ^{b}	0.3 ± 0.0 ^{bc}	1056.2 ± 135.0 ^{cd}	16.0 ± 0.9 ^{bc}
B. amyloliquefaciens BFL1	52.5 ± 4.5 ^{e}	23.3 ± 2.0 ^{abc}	0.1 ± 0.0 ^{b}	0.2 ± 0.0 ^{bc}	912.4 ± 78.0 ^{cd}	28.5 ± 1.5 ^{a}
C. indologenes LPR17	72.9 ± 1.9 ^{abc}	32.0 ± 4.6 ^{ab}	0.1 ± 0.0 ^{b}	0.6 ± 0.2 ^{ab}	1539.1 ± 64.0 ^{bc}	31.1 ± 1.1 ^{a}
K. pneumoneae RR19	69.1 ± 1.4 ^{abcd}	33.8 ± 4.5 ^{a}	0.2 ± 0.0 ^{ab}	0.6 ± 0.0 ^{abc}	3115.4 ± 350.0 ^{a}	21.6 ± 2.3 ^{b}
L. minipressuralis LGR12	70.9 ± 8.2 ^{abcd}	28.8 ± 2.5 ^{ab}	0.1 ± 0.0 ^{b}	0.3 ± 0.1 ^{bc}	930.3 ± 4.0 ^{cd}	27.4 ± 1.0 ^{a}
B. velezensis BE1	75.8 ± 7.9 ^{ab}	28.3 ± 4.0 ^{ab}	0.1 ± 0.0 ^{b}	0.4 ± 0.1 ^{abc}	628.2 ± 13.0 ^{d}	20.6 ± 0.8 ^{b}
Unidentified *LPR3*	79.3 ± 2.9 ^{a}	27.5 ± 3.7 ^{ab}	0.1 ± 0.0 ^{b}	0.4 ± 0.0 ^{abc}	1458.6 ± 357.0 ^{bc}	16.8 ± 3.0 ^{bc}
S. maltophilia LPR6+	61.5 ± 2.9 ^{cde}	30.7 ± 1.5 ^{ehi}	0.1 ± 0.0 ^{b}	0.3 ± 0.0 ^{bc}	2066.2 ± 323.0 ^{b}	31.5 ± 2.2 ^{a}

The results are expressed as means of values ±SD and values in each column with different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$

Appendix III: Effect of bacterial treatments on drought related morphological, physiological and biochemical parameters in maize plants

Parameters	Control	*Drought*	*B. megaterium RR13*	Unidentified *LPR1+*	*B. amyloliquefaciens BFL1*	*C. indologenes LPR17*	*L. minipressuralis LGR12*	*B. velezensis BE1*	Unidentified *LPR3*	*S. maltophilia LPR6+*
Shoot length (Cm)	41.5 ± 0.6 ^{a}	30.5 ± 3.1 ^{bc}	39.0 ± 3.7 ^{ab}	34.5 ± 3.1 ^{abc}	31.4 ± 3.2 ^{bc}	29.0 ± 3.7 ^{c}	29.4 ± 3.8 ^{c}	30.1 ± 3.4 ^{bc}	31.5 ± 6.0 ^{bc}	30.5 ± 4.7 ^{bc}
Root length (Cm)	30.3 ± 7.2 ^{b}	24.8 ± 4.9 ^{b}	42.0 ± 4.2 ^{a}	28.3 ± 4.3 ^{b}	21.5 ± 4.7 ^{b}	30.5 ± 5.3 ^{b}	27.0 ± 6.7 ^{b}	24.0 ± 1.4 ^{b}	25.3 ± 2.9 ^{b}	31.7 ± 7.3 ^{ab}
Shoot fresh weight (g)	1.9 ± 0.0 ^{a}	1.0 ± 0.3 ^{bcd}	1.6 ± 0.2 ^{abc}	1.7 ± 0.4 ^{ab}	0.7 ± 0.0 ^{d}	1.0 ± 0.2 ^{bcd}	0.8 ± 0.3 ^{cd}	0.9 ± 0.1 ^{bcd}	0.9 ± 0.2 ^{bcd}	0.9 ± 0.1 ^{bcd}
Root fresh weight (g)	5.0 ± 0.9 ^{a}	2.6 ± 0.3 ^{bc}	1.8 ± 0.5 ^{cd}	3.0 ± 0.4 ^{b}	2.0 ± 0.4 ^{cd}	1.5 ± 0.4 ^{cd}	1.5 ± 0.4 ^{d}	2.0 ± 0.3 ^{cd}	1.4 ± 0.3 ^{d}	1.3 ± 0.4 ^{d}
Chlorophyll a (mg mL^{-1})	0.20 ± 0.01 ^{a}	0.06 ± 0.00^{ef}	0.19 ± 0.04 ^{ab}	0.13 ± 0.00 ^{bcd}	0.12 ± 0.02 ^{cdef}	0.12 ± 0.03 ^{cde}	0.08 ± 0.02 ^{def}	0.05 ± 0.01 ^{f}	0.10 ± 0.01^{def}	0.18 ± 0.04 ^{abc}
Chlorophyll b (mg mL^{-1})	0.07 ± 0.00 ^{a}	0.04 ± 0.00 ^{bcd}	0.05 ± 0.02 ^{ab}	0.04 ± 0.0 ^{bcd}	0.04 ± 0.01 ^{bcd}	0.04 ± 0.0 ^{bcd}	0.03 ± 0.00 ^{cd}	0.02 ± 0.0 ^{d}	0.03 ± 0.0 ^{bcd}	0.05 ± 0.01 ^{bc}
Total Chlorophyll (mg g^{-1})	309.1 ± 15.4 ^{a}	113.0 ± 5.6 ^{cd}	239.7 ± 22.8 ^{ab}	193.3 ± 10.5 ^{bc}	187.9 ± 32.7 ^{bcd}	182.2 ± 33.0 ^{bcd}	129.1 ± 27.6 ^{cd}	89.6 ± 9.4 ^{d}	147.6 ± 21.2^{cd}	297.9 ± 7.4 ^{ab}
Carotenoids (mg mL^{-1})	0.13 ± 0.01 ^{a}	0.07 ± 0.0 ^{b}	0.08 ± 0.02 ^{b}	0.07 ± 0.0 ^{b}	0.09 ± 0.02 ^{ab}	0.07 ± 0.01 ^{b}	0.07 ± 0.0 ^{b}	0.06 ± 0.0 ^{b}	0.08 ± 0.0 ^{b}	0.08 ± 0.02 ^{b}
Rolled leaves (%)	0.0 ± 0.0 ^{c}	100.0 ± 0.0 ^{a}	100.0 ± 0.0 ^{a}	75.0 ± 28.9 ^{b}	100.0 ± 0.0 ^{a}	100.0 ± 0.0 ^{a}	100.0 ± 0.0 ^{a}	100.0 ± 0.0 ^{a}	93.8 ± 12.5 ^{ab}	100.0 ± 0.0 ^{a}
Leaves chlorosis (%)	0.0 ± 0.0 ^{b}	50.0 ± 11.5 ^{a}	40.0 ± 0.0 ^{a}	55.0 ± 10.0 ^{a}	40.0 ± 0.0 ^{a}	50.0 ± 11.5 ^{a}	35.0 ± 10.0 ^{a}	45.0 ± 10.0 ^{a}	55.0 ± 10.0 ^{a}	50.0 ± 11.5 ^{a}
Leaf area (Cm²)	40.5 ± 6.5 ^{a}	11.4 ± 0.8 ^{c}	11.3 ± 0.9 ^{c}	25.9 ± 0.9 ^{b}	8.5 ± 1.0 ^{c}	11.1 ± 1.0 ^{c}	10.7 ± 2.4 ^{c}	9.3 ± 1.2 ^{c}	11.7 ± 2.3 ^{c}	11.0 ± 1.4 ^{c}
Relative water content (%)	89.4 ± 5.8 ^{a}	35.1 ± 0.0 ^{cd}	57.3 ± 0.0 ^{b}	30.6 ± 0.0 ^{bcd}	19.8 ± 0.0 ^{cd}	19.5 ± 0.0 ^{cd}	45.2 ± 15.1 ^{bc}	26.9 ± 6.0 ^{cd}	13.3 ± 0.0 ^{d}	6.9 ± 1.8 ^{d}
Total phenol (OD µg Eq gallic acid/mg FM)	498.7 ± 25.2^{cd}	317.5 ± 24.4 ^{g}	656.8 ± 33.4 ^{a}	564.2 ± 25.2 ^{bc}	400.9 ± 3.1 ^{ef}	379.9 ± 27.4 ^{fg}	374.7 ± 8.7 ^{fg}	458.6 ± 16.0^{de}	584.1 ± 3.8 ^{ab}	445.4 ± 3.5 ^{def}
Flavonoid (OD µg Eq gallic acid/mg FM)	273.1 ± 17.8^{ab}	162.8 ± 11.6 ^{d}	283.6 ± 10.2 ^{a}	263.2 ± 35.1 ^{ab}	195.7 ± 3.6 ^{cd}	199.2 ± 9.9 ^{cd}	206.4 ± 8.2 ^{cd}	214.4 ± 6.6 ^{bcd}	248.0±19.5^{abc}	187.7 ± 7.9 ^{d}
Catalase (OD/min/mg FM)	45.3 ± 1.8 ^{d}	82.4 ± 0.0 ^{ab}	76.1 ± 4.4 ^{b}	62.9 ± 2.9 ^{c}	85.0 ± 0.0 ^{a}	42.5 ± 1.5 ^{d}	65.8 ± 2.9 ^{c}	34.7 ± 0.04 ^{e}	46.5 ± 1.2 ^{d}	83.3 ± 2.0 ^{ab}
Gaiacol peroxidase (OD/min/mg FM)	231.9 ± 28.1 ^{a}	205.5 ± 107 ^{a}	208.3 ± 67.5 ^{a}	281.3 ± 0.0 ^{a}	264.5 ± 49.8 ^{a}	246.5 ± 10.5 ^{a}	249.2 ± 9.5 ^{a}	197.2 ± 36.2 ^{a}	225.3 ± 66.7 ^{a}	240.9 ± 68.1 ^{a}

The results are expressed as means of values ±SD and values in each column with different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$

Appendix IV: Effect of bacterial treatments on morphological, physiological and biochemical parameters of maize plants under cooccurrence of drought and *F. verticillioides* rot

Parameters	Control	Drought	*F. verticillioides*	Drought + *F. verticillioides*	D + *Fv* + unidentified LPR1+	D+*Fv*+*B. megaterium* RR13
Shoot lenght (Cm)	83.3 ± 17.1 ^{a}	71.3 ± 14.6 ^{ab}	61.5 ± 13.7 ^{abc}	47.6 ± 7.0 ^{c}	56.4 ± 2.6 ^{bc}	45.6 ± 8.5 ^{c}
Root lenght (Cm)	24.3 ± 8.1 ^{a}	20.3 ± 5.6 ^{ab}	10.0 ± 1.7 ^{c}	10.1 ± 1.0 ^{c}	18.5 ± 1.3 ^{abc}	13.4 ± 4.4 ^{bc}
Shoot fresh weight (g)	8.6 ± 3.3 ^{a}	4.2 ± 1.1 ^{b}	2.9 ± 1.4 ^{bc}	1.2 ± 0.5 ^{c}	2.9 ± 0.5 ^{bc}	1.3 ± 0.4 ^{bc}
Root fresh weight (g)	1.5 ± 0.9 ^{a}	0.8 ± 0.4 ^{b}	0.7 ± 0.2 ^{b}	0.4 ± 0.2 ^{b}	0.7 ± 0.1 ^{b}	0.4 ± 0.1 ^{b}
Leaves chlorosis (%)	0.0 ± 0.0 ^{c}	77.1 ± 24.3 ^{ab}	54.3 ± 25.1 ^{b}	94.3 ± 9.8 ^{a}	77.1 ± 18.0 ^{ab}	65.0 ± 25.2 ^{ab}
Rolled leaves (%)	0.0 ± 0.0 ^{b}	56.7 ± 23.4 ^{a}	52.0 ± 26.8 ^{a}	82.9 ± 18.0 ^{a}	66.7 ± 24.2 ^{a}	65.0 ± 30.0 ^{a}
Leaf area (Cm²)	87.0 ± 18.2 ^{a}	48.4 ± 16.2 ^{b}	36.7 ± 10.9 ^{bc}	22.2 ± 3.9 ^{c}	37.7 ± 8.5 ^{bc}	24.1 ± 11.6 ^{bc}
Relative water content (%)	82.0 ± 3.9 ^{a}	64.1 ± 7.7 ^{a}	69.5 ± 13.5 ^{a}	74.6 ± 0.1 ^{a}	81.7 ± 2.2 ^{a}	74.7 ± 2.3 ^{a}
Chlorophyll a (mg mL^{-1})	0.6 ± 0.0 ^{a}	0.5 ± 0.0 ^{ab}	0.6 ± 0.2 ^{ab}	0.3 ± 0.1 ^{b}	0.4 ± 0.0 ^{ab}	0.5 ± 0.0 ^{ab}
Chlorophyll b (mg mL^{-1})	0.17 ± 0.0 ^{a}	0.2 ± 0.0 ^{a}	0.1 ± 0.0 ^{a}	0.1 ± 0.0 ^{a}	0.1 ± 0.0 ^{a}	0.2 ± 0.0 ^{a}
Total chlorophyll (mg g^{-1})	882.6 ± 61.8 ^{a}	794.9 ± 4.3 ^{ab}	667.9 ± 0.0 ^{bc}	537.3 ± 77.1 ^{c}	588.1 ± 12.8 ^{c}	753.3 ± 11.5 ^{b}
Carotenoids (mg mL^{-1})	0.1 ± 0.0 ^{a}	0.1 ± 0.0 ^{a}	0.09 ± 0.0 ^{b}	0.05 ± 0.0 ^{c}	0.09 ± 0.0 ^{b}	0.1 ± 0.0 ^{a}
Electrolyte leackage (%)	51.2 ± 3.6 ^{bc}	83.6 ± 1.7 ^{a}	66.6 ± 7.3 ^{ab}	90.9 ± 17.9 ^{a}	32.8 ± 6.6 ^{c}	32.1 ± 5.0 ^{c}
Catalase (OD/min/mg FM)	46.5 ± 5.3 ^{c}	73.8 ± 0.0 ^{a}	56.8 ± 3.3 ^{bc}	43.7 ± 6.2 ^{c}	67.5 ± 6.5 ^{ab}	56.3 ± 5.9 ^{bc}
Gaiacol peroxidase (OD/min/mg FM)	556.0 ± 59.4 ^{b}	636.5 ± 24.2 ^{ab}	743.1 ± 59.0 ^{a}	613.8 ± 20.1 ^{ab}	737.6 ± 75.4 ^{a}	678.9 ± 19.6 ^{ab}
Proline (mg/g FM)	0.1 ± 0.0 ^{de}	0.7 ± 0.0 ^{b}	0.1 ± 0.0 ^{e}	1.0 ± 0.0 ^{a}	0.2 ± 0.0 ^{d}	0.3 ± 0.0 ^{c}
PAL activity (OD/min/mg FM)	14.6 ± 0.7 ^{d}	16.5 ± 1.1 ^{bc}	15.8 ± 0.7 ^{cd}	18.0 ± 0.6 ^{b}	15.3 ± 1.3 ^{cd}	21.2 ± 0.7 ^{a}

The results are expressed as means of values ±SD and values in each column with different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$

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Endophytic bacteria derived from the desert-spurge (*Euphorbia antiquorum* L.) suppress *Fusarium verticillioides,* the causative agent of maize ear and root rot

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ABSTRACT

Fungal plant pathology is a huge concern to the agricultural industry across the globe. *Fusarium verticillioides* is recognized as the most widespread and severe pathogenic fungus associated with maize production. This study aimed to investigate the antagonistic potential of twenty-seven (27) endophytic bacteria from the Desert spurge (*Euphorbia antiquorum* L.) against *Fusarium verticillioides* causing ear and root rot in maize. The *in vitro* screening revealed that six (6) bacteria restricted *Fusarium verticillioides* mycelial growth upon direct confrontation, resulting in a 68.59% reduction and nine (9) inhibited via the production of VOMs (71.7% mycelial growth reduction). *In vitro*, all the tested bacteria produced several extracellular enzymes and various plant growth-promoting factors, such as indole acetic acid (IAA), ammonia, siderophores and solubilized phosphate. In the greenhouse experiment, potent endophytic bacteria significantly lowered the severity of ear and root rot disease caused by *F. verticillioides* in maize seedlings, with *Citrobacter fruendii* LPR4^{+} being the most effective. Moreover, the protective effect was strongly associated with PGP aptitudes, which was manifested by increases in root and shoot development by up to 79 and 28%, respectively, as well as guaiacol peroxidase and phenylalanine ammonialyase specific activities. The current data constitute the starting point toward an ecological solution to control ear and root rot disease in maize.

1. Introduction

Pathogenic *Fusarium* species hamper crop productivity worldwide (Nguvo and Gao, 2019). Species such as *F. graminearum*, *F. asiaticum*, *F. culmorum*, *F. solani*, *F. avenaceum*, *F. proliferatum*, *F. oxysporum, F. subglutinans,* and *F. verticillioides* have frequently been reported to cause crown rot, head blight (scab), root rot, and ear rot in over 81 plant species on earth, including cereals such as wheat, barley, rice and maize, to which they cause significant yield decline (Leslie and Summerell, 2006; Dean et al., 2012, 2016; Chetouhi et al., 2016; Coleman, 2016). *F. verticillioides* is the most important and the most commonly cited fungal pathogen of maize. The fungus thrives in hot and dry conditions, taking advantage of the weakened crop defense system (Hernández-Rodríguez et al., 2008; Pfordt et al., 2020). *F. verticillioides* enters corn systemically from seeds and silks or through wounds created by insects (Pfordt et al., 2020). Disease propagules are thereafter transmitted from seeds to kernels in four major steps, including (i) seeds to seedlings, (ii) colonization of the stalk, (iii) movement into the ear, and (iv) spread within the ear, resulting in heavy economic costs culminating at as high as 30–50% grain yield and contaminated planting material (Munkvold and Carlton, 1997; Oren et al., 2003; Horst, 2013).

Maize (*Zea mays* L.) is the most cultivated and consumed grain worldwide (Shahbandeh, 2022). It is a diversified crop with more than 600 derived products with various applications in human nutrition, farming (livestock and poultry) and the energy industry (Ntsama et al., 2019). According to the Food and Agricultural Organization (FAO), the

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global target of 2.4% production yield improvement per year will not be achieved, as estimates forecast a 7% yield decline per annum due to biotic and abiotic factors (Alexandratos and Bruinsma, 2012; Ray et al., 2013; Admin, 2022), among which diseases caused by *F. verticillioides* are of great significance and deserve serious attention. For instance, outbreaks of *F. verticillioides* causing up to 70% yield losses in severe cases were reported in several maize production hotspots in Central Africa (Ngoko et al., 2001).

To control *F. verticillioides* infection in maize farms, conventional chemical fungicides and fumigants, including azoxystrobin, cyproconazole, methyl thiophanate, Apron Star, benlate/benomyl and carbofuran, are commonly used for seed and soil treatment (Lanza et al., 2017; Guimarães, 2018). However, the drawbacks resulting from agrochemicals are steadily redirecting the quest toward novel ecological approaches (Eke et al., 2016, 2021). In this sense, biocontrol agents (BCAs) are gaining wide acceptance as eco-friendly and cost-effective fungicides against a vast array of agriculturally destructive phytopathogens (Medeiros et al., 2012; Panth et al., 2020).

Extensive field and laboratory attempts have indeed demonstrated the bioefficiency of endophytic bacteria in promoting growth and repressing many plant diseases (Eke et al., 2016; Mowafy et al., 2021; Rios-Galicia et al., 2021). In our previous investigation, endophytic bacteria from desert spurge (*E. antiquorum* L.) was found to confer drought tolerance and induce growth promotion in tomato (*Solanum lycopersicum* L.) (Eke et al., 2019, 2021). Thus, we anticipated that these endospheric bacteria could possess special biocontrol attributes. Herein, we investigated the biocontrol potential of twenty-seven desert spurge-derived endospheric bacteria against maize root and ear rot caused by *F. verticillioides* under laboratory and greenhouse conditions.

2. Materials and methods

2.1. Bacterial strains

The bacterial (27) strains used in this work (Table 1) were obtained from the core collection of Biocontrol Agent Sub-Unit of the Antimicrobial and Biocontrol Agents Unit, Faculty of Sciences, University of Yaoundé I, Cameroon. They were previously isolated from healthy roots, seeds, and spines of *E. antiquorum* naturally grown in Kousséri, located in the hot Far North Region of Cameroon (Latitude: 12°5′13.4988″N; longitude 15° 0′53.3952″E), bordering the Sahara Desert. The host plant was harvested, cleaned with distilled water, cut into small pieces and ground in NaCl (0.9%). The solution was incubated at 28 °C for 2 h for the release of endophytes. Thereafter, a serial dilution was made in Muller Hinton agar medium supplemented with benomyl, and the grown colonies were streaked on new medium. Pure cultures were stored at −20 °C and −80 °C for further use. Data pertaining to their identity (16S-rDNA gene sequencing) can be found in (Eke et al., 2021). All bacteria used in the present study were selected based on their abilities to enhance growth and induce systemic resistance in tomatoes against the bacterial wilt pathogen *R. solanacearum* (data not shown) (Table 1).

Table 1
Bacterial strains and GenBank accessions.

S/N	Strain code	Identity	Accession code (GenBank)
1	BFL1	*Bacillus Amyloliquefaciens*	MH788971
2	RA37	*Bacillus Amyloliquefaciens*	MH788970
3	BFR2	*Bacillus Amyloliquefaciens*	MH788971
4	RR13	*Bacillus megaterium*	MH788974
5	RR10	*Bacillus megaterium*	MH788974
6	BE1	*Bacillus velezensis*	MH788975
7	RA4	*Bacillus xiamenensis*	MH788976
8	LPR17	*Chryseobacterium indologenes*	MH788978
9	LPR11^{+}	*Chryseobacterium indologenes*	MH788978
10	LPR4^{+}	*Citrobacter fruendii*	MH788979
11	LGR26	*Enterobacter cloacae*	MH788981
12	LPR2^{+}	*Enterobacter cloacae*	MH788982
13	RR19	*Klebsiella pneumoneae*	MH788983
14	PA2^{+}	*Lelliottia minipressuralis*	MH788984
15	LGR12^{+}	*Lelliottia minipressuralis*	MH788984
16	LGR12	*Lelliottia minipressuralis*	MH788984
17	LPR19	*Lynsinibacillus fusiformis*	MH788987
18	LPR20	*Paracoccus* sp.	MH788988
19	RA24	*Proteus peneri*	MH788991
20	PA3^{+}	*Pseudomonas putida*	MH788990
21	RR20	*Pseudomonas putida*	MH788990
22	RR4	*Pseudomonas aeruginosas*	MH788989
23	RA33	*Stenotrophomonas maltophila*	MH788993
24	LPR6	*Stenotrophomonas maltophilia*	MH788995
25	LPR1^{+}	*Unidentified*	/
26	LPR3	*Unidentified*	/
27	LPR3^{+}	*Unidentified*	/

Unidentified: Isolates not identified in the nucleotide databases used by Eke et al. (2021).

2.2. Isolation of the phytopathogen

F. verticillioides was isolated from maize stems exhibiting symptoms of ear and root rot obtained from a commercial farm in the Centre Region of Cameroon (4° 45' 0" N, 12° 0' 0" E). Symptomatic parts were cut into fragments (0.5–1 cm lengths), successively washed with tap water, disinfected with 70% ethanol (2 min) and 2% sodium hypochlorite (5 min), and thoroughly rinsed with sterile distilled water. Disinfected fragments were dried on blotted paper in a laminar flow cabinet and placed on PDA (potato dextrose agar) plates supplemented with chloramphenicol (500 mg/L). The plates were sealed with parafilm tape and incubated in the dark at 25 ± 2 °C. After three days of incubation, mycelial tips emerging from the explants were subcultured on fresh PDA medium until pure cultures were obtained.

2.3. Pathogenicity test

The pathogenicity of the obtained isolates was assessed according to Grisham and Anderson (1983). Briefly, to obtain fungal inoculum, 200 g of autoclaved (121 °C, 30 min) cornmeal sand introduced into flasks was inoculated with small mycelial plugs 4 cm in diameter from a seven-day-old *F. verticillioides* culture on PDA medium. The flasks were incubated at 25 ± 2 °C for four weeks in the dark and shaken every two days for homogenous fungal growth. Upon incubation, the slurry was air-dried and ground to a powder. The resulting inoculum (colonized cornmeal) was thoroughly mixed with sterilized garden soil (121 °C, 30 min) at rates of 5% and 10% (w/w), as described by Sumner and Minton (1989) with slight modifications. The potting mix was then filled in 1 kg plastic pots alongside the control set, consisting of noninfested autoclaved cornmeal-sand and garden soil. Maize seeds (CMS-8704 composite) were surface sterilized with 5% sodium hypochlorite (5 min), rinsed three times with sterile distilled water and sown (four seeds per pot, 10 pots per treatment) at 4 cm deep. The pots were then arranged in the greenhouse following a simple block design, watered every two days and maintained at 25 ± 2 °C under a 12 h light/12 h dark photoperiod, RH 73%. The germination percentage was recorded per concentration at pre- and postemergence stages from 3 to 21 days after sowing (das). The disease parameters were calculated according to the following formula (1):

$$\text{Incidence} = (\text{Number of infected plants/Total number of plants}) \times 100 \quad (1)$$

The degree of pathogenicity of the isolates was evaluated based on the percentage of pre- and postemergence damping-off as well as severity using a 0 to 5 disease rating scale described by (Mańka, 1989) (Table 2).

Table 2
Scale for estimation of the pathogenicity of the Fusarium isolates.

S/N	Rating scale	Degree of pathogenicity
0	0–10%	Not Pathogenic
1	11–20%	Very lowly Pathogenic
2	21–40%	Lowly Pathogenic
3	41–60%	Moderately Pathogenic
4	61–80%	Highly Pathogenic
5	81–100%	Very Highly Pathogenic

2.4. Molecular characterization of the fungal pathogen

The identification of this strain was performed based on a phylogenetic analysis of the sequences of two genes, the *internal transcribed spacer* (*ITS*) region of the 5,8S nuclear ribosomal gene and the *translation elongation factor 1-α gene* (*TEF1*). The *F. verticillioides* isolate was cultured in potato dextrose broth (PDB) medium under constant shaking (160 rpm; 3 days), and the mycelial mats were collected by filtration. The mycelium was cultured with RNase and buffer to allow RNA degradation, and DNA was extracted using the commercial Red Extract-N-Amp Plant PCR kit (Sigma Aldrich) as recommended by the manufacturer. The *internal transcribed spacer* (ITS) of nuclear ribosomal DNA was used, and the ITS1-5.8S rRNA-ITS2 region was amplified by PCR using primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al., 1990). The primers Fusa-Tef1 (5′-ATGGGTAAGGARGACAAGAC-3′) and Fusa-Tef2 (5′-GGARGTACCAGTSATCATGTT-3′) were utilized to amplify part of the *translation elongation factor 1-α* (*TEF1*). The amplification conditions were a start step of 2 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 1 min at 58 °C for *ITS*, or 55 °C for *TEF1* and 1 min at 72 °C, followed by a finishing step of 3 min at 72 °C.

PCR amplicons were sequenced at the DNA sequencing service of the University of Salamanca (Spain). Sequences generated were compared with reference sequences in the GenBank nucleotide database to determine the closest relatives for the phylogenetic studies. For each of the two loci (ITS and TEF1), the DNA sequences obtained in this study together with those retrieved from GenBank were aligned using the ClustalW algorithm included in the MEGA X software package. For the maximum likelihood phylogenetic analysis, the best evolutionary model for each partition was determined using MEGA X and incorporated into the analyses. The maximum likelihood phylogenetic tree searches were performed in MEGA X with the tree Bisection and reconnection (TBR) algorithm, where gaps were treated as missing data. The robustness of the topology was evaluated by 1000 bootstrap replications.

2.5. Antagonism potential of BCAs against F. verticillioides

2.5.1. Direct antagonism assay

The ability of the bacterial endophytes to inhibit *F. verticillioides* growth in dual culture was assessed *in vitro* as described by Karimi et al., (2016) with slight modifications. Briefly, a 5 mm diameter agar plug from a 3-day-old *F. verticillioides* culture was placed at the center of Petri dishes containing a mixture of 50% PDA and 50% Luria Bertani agar (LBA). Thereafter, 5 mm discs of Whatman (No. 1) filter paper were placed at the four peripheral sides of the fungal plug and inoculated with 5 μL of physiological water and bacterial suspensions (1.5×10^8 CFU/mL) for control and test plates, respectively. Upon 5 days of incubation, mycelial growth inhibition was calculated as given by formula (2).

$$I\ (\%) = [(C\text{-}T)/C] \times 100 \quad (2)$$

where I (%) = the percentage of inhibition; C = the mycelial growth of *F. verticillioides* in control plates and T = fungal growth in the presence of BCAs.

2.5.2. Antifungal potential of volatile organic metabolites

The inhibitory effect of volatile organic metabolites (VOMs) emitted by the endophytic bacteria was assessed *in vitro* (Jayaswal et al., 1993). Briefly, bacterial strains were streaked onto LBA medium for 24 h. Then, mycelial plugs (5 mmϴ) taken from the growth edge of three-day-old *F. verticillioides* colonies were centrally inoculated onto separated PDA plates. The plates bearing the BCAs and the pathogen were superposed to place the organisms face to face and sealed with parafilm tape. The control plates were made up of superposition of *F. verticillioides* PDA plates over uninoculated LBA medium. The plates were incubated at 25 ± 2 °C for 7 days, and mycelial growth inhibition was calculated using formula (2) above.

2.6. Production of hydrolytic enzymes by bacterial strains

The biological control potential of bacterial strains is closely related to the spectrum of hydrolytic enzymes (HE) released. These enzymes are also used by endophytes to facilitate host internal tissue colonization and deactivation of pathogen virulence enzymes. Hence, the bacterial strains were all screened for the production of protease, amylase, and cellulase on specific media.

2.6.1. Protease production

The culture medium was composed of a mixture of 10 g of agar dissolved in 100 mL of distilled water and 300 mL of 0.1 M citrate phosphate at pH 6.0. After autoclaving the mixture, 10 g of skim milk was aseptically added when the medium was still hot (50 °C) to avoid coagulation and charring of milk. The medium was then poured on Petri plates and allowed to solidify. The bacterial suspensions, set at 0.5 MacFarland standards were spotted (5 μL) and inoculated onto skim milk agar medium. Plates were incubated for 4 days at 37 °C, then 10% tannic acid was flooded on the medium, and the appearance of a clear zone around the colonies indicated protease production (Saran et al., 2007). The diameters of clear halos were measured, and the enzyme activity (EA) was calculated according to the following formula (3):

$$EA = (HD\text{-}CD)/CD \quad (3)$$

where HD = diameter of the clear halo and CD = diameter of the clear BCA's colony.

2.6.2. Cellulase production

Yeast peptone agar medium (yeast extract (1 g), peptone (0.5 g), agar–agar (16 g), 1000 mL distilled water) supplemented with 0.5% Na-carboxymethyl cellulose (CMC) was used for cellulase detection (Gupta et al., 2012). The bacterial strain was inoculated on plates containing the medium for 4 days at 28 °C, and the plates were then flooded with 0.1% aqueous Congo red solution. After 15 min of incubation, the diameters of clear halos around colonies were measured, and the enzyme activity (EA) was calculated according to formula (3) above.

2.6.3. Amylase production

Glucose Yeast Peptone Agar constituted by 1 g of glucose, 1 g of yeast extract, 0.5 g of peptone and 16 g of agar dissolved in 1000 mL of distilled water was used for bacterial culture. Starch (2%) was added to the medium as a substrate for enzyme activity (Liu and Xu, 2008). The plates were incubated for 4 days, after which they were iodine-flooded (1%), and the diameters of clear zones around colonies were measured to calculate the enzyme activities as previously described (formula (3)).

2.7. Plant growth-promoting ability of the endophytic bacteria

The capacity of the bacterial endophytes to produce IAA, siderophores, ammonia, and solubilize phosphate, which are essential elements for plant growth, was assessed.

2.7.1. Phosphate solubilization

The solubilization of complexed phosphate (tri-calcium phosphate) was evaluated on Pikovskaya agar medium (MgSO4–7H2O (0.1 g), dextrose (10 g), yeast extract (0.5 g), glucose (13 g), (NH4)2SO4 (0.5 g), KCl (0.2 g), CaHPO4 (2.5 g), MnSO4 (0.0002 g), NaCl (0.2 g), FeSO4–7H2O (0.0002 g), agar (15 g) in 1000 mL of distilled water). The development of a clear halo around BCA colonies after 5 days of incubation indicated the solubilization of phosphate (Onyia et al., 2015). The phosphate solubilization activity (clean halo) was measured and expressed in millimeters (mm). The experiment was performed in triplicate and repeated twice.

2.7.2. Ammonia production

For ammonia production, 2 μL bacterial suspensions at 1.5×10^8 cells/mL were placed in 300 μL peptone water (10%) for 72 h, and 1 mL Nessler reagent was added to 200 μL of culture supernatant. The optical density (OD) of the brown to yellow complex developed was read at 450 nm (Ogbo and Okonkwo, 2012) using a microplate reader spectrophotometer (infinite M200 TECAN). The ammonia concentration was determined using a standard curve of $(NH4)_2SO_4$ at concentrations ranging from 1 to 10 μmol/mL ($R^2 = 0.96$). The experiment was performed in triplicate and repeated twice.

2.7.3. IAA production

The (Goswami et al., 2013) protocol was used to reveal IAA production. The bacteria were cultured in LB medium supplemented with L-tryptophan (200 μg/mL) and NaCl (2%) for 72 h. Afterward, 100 μL of Salkowski reagent (12.5 mL perchloric acid (70%); 0.5 mL $FeCl_3$ (0.5 M) and 12.25 mL distilled water) were added to 150 μL of culture supernatant. The pink coloration developed was measured at 530 nm (Infinite M200, TECAN), and IAA was quantified by extrapolation on the IAA standard curve prepared at concentrations ranging from 10 to 100 μg/mL.

2.7.4. Siderophore production

The capacity of bacterial strains to produce siderophores was assessed. The supernatant of a 24-day-old bacterial broth culture (LB medium) was mixed with 2% ferric chloride and 1 mL $CuSO_4$ (250 μM) prepared in 2 mL acetate buffer. The color change from yellow to red marking the production of siderophores was measured at 450 nm, 495 nm, and 280 nm for hydroxamate, catecholate, and carboxylate siderophores, respectively. Then, siderophore production was determined using the following formula from (Payne, 1994):

$$\% \text{ Siderophore units} = (A_{ref}\text{-}As/A_{ref}) \times 100$$

where A_{ref} is the absorbance of the reference and As is the sample absorbance.

2.8. In planta ear and root rot suppression

2.8.1. Seed bacterization

Maize seeds (genotype CMS-8704) were surface disinfected using 5% sodium hypochlorite (5 min), rinsed three times with sterile distilled water and pregerminated for two days in Petri dishes provided by three layers of humidified sterile filter paper and placed on a greenhouse bench (25 ± 2 °C, photoperiod of 12 h light/12 h dark, RH 73%). Pregerminated seeds were then soaked for 6 h at room temperature in either bacterial suspensions (3×10^8 CFU/mL) prepared in sterile saline water for test samples or in sterile saline water for the negative control.

2.8.2. Substrate infestation, sowing, and experimental layout

The *F. verticillioides* inoculum was prepared as described by Sumner and Minton (1989) with slight modifications. Cornmeal sand was inoculated with fragments of mycelial culture of the strain (as described in the pathogenicity section) and mixed with garden soil at a rate of 10% (w/w). Uninoculated cornmeal mixed with garden soil served as controls. Inoculated and noninoculated substrates were filled in 1 kg plastic pots, two-thirds full. Two maize seeds were sown in each pot. The treatments were disposed of on greenhouse benches in a completely randomized block design with 29 treatments made up of uninoculated controls, the pathogen only (negative control), and plants infected by *F. verticillioides* and each of the 27 endophytic bacteria investigated. Ten replicates were prepared per treatment for a total of 290 pots. The pots were watered every two days for 35 days, after which plants were harvested and data were recorded.

2.8.3. Disease and agro-morphological parameter recording

Eleven (11), 15, 19, 23, 27, 31, and 35 days after sowing, plants were delicately uprooted and rinsed with tap water. The disease severity (DS) was investigated on shoots as described by Ferniah et al. (2014) as follows: 0 = no symptoms, 1 = lower height compared to control, 2 = lower height and chlorosis, 3 = 10% chlorosis and/or 10% wilting, 4 = 11–25% wilting, 5 = 26–50% wilting and shrinkage of stalk, 6 = 51–100% wilting and dead. Moreover, symptoms of root rot were also determined as described by du Toit et al. (1997) according to the following criteria: 0 = 0%; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100% root discoloration/rotting; 5 = root system dead and fragmented; and 6 = whole seedling dead.

Thereafter, the reduction in disease severity (RDS) was calculated using the following formula (4):

$$RDS = [(DS_{control} - DS_{treatment}) / DS_{control}] \times 100 \quad (4)$$

Morphological parameters such as plant root and shoot lengths (Cm) and dry weights were recorded 35 days after sowing.

2.8.4. Assessment of some disease-related biochemical markers

2.8.4.1. Phenylalanine ammonia lyase activity (EC 4.3.1.24). Phenylalanine ammonia-lyase (PAL) is an important enzyme located at the interface of primary and secondary metabolism. Its activity is triggered during infection by plant pathogens. One gram of fresh root and shoot parts was crushed in 50 mM potassium phosphate buffer (pH 7.2) and centrifuged (5000 rpm at 4 °C). The supernatant was used for PAL activity determination (Whetten and Sederoff, 1992). Fifty microliters (50 μL) of enzymatic extract was mixed with 150 μL of phosphate buffer (50 mM; pH 7.2) and 50 μL of phenylalanine (50 mM) and incubated for 1 h at room temperature (25 °C). The reaction was stopped by the addition of 2 N HCl, and the optical density (OD) readout was made at 290 nm. The PAL specific activity was expressed in terms of the increase in OD (ΔOD)/minute/gram fresh weight ($\Delta OD.min^{-1}. g^{-1}$ fresh weight).

2.8.4.2. Guaiacol peroxidase activity (EC 1.11.1.7). The peroxidase-specific activity was evaluated to assess the ability of bacteria to fight against stress induced by *F. verticillioides* ingress in plant cells (Paglia and Valentine, 1967). Briefly, 143 μL guaiacol buffer (50 mM phosphate buffer (pH 7), 9 mM guaiacol) was mixed with 2.5 μL H_2O_2 and 10 μL enzymatic extract. After that, the OD of the tetraguaiacol formed was read at 470 nm. The GPX activity was then expressed as $\Delta OD.\ Min^{-1}\ g^{-1}$ fresh weight.

2.9. Statistical analysis

The data obtained from the experiments were explored and subjected to ANOVA using GraphPad Prism version 8.0 statistical software. The mean values generated were compared using the Tukey multiple range comparison post hoc test at $P \leq 0.05$. The results are expressed as the mean ± standard deviation (mean ± SD).

3. Results

3.1. Pathogenicity test

The pathogenicity of *F. verticillioides* was investigated (Fig. 1). Fusarium ear and root rot symptoms included delayed seed germination (Fig. 1a), the postemergence sudden death of seedlings (Fig. 1b), stunted growth, yellowing leaves (Fig. 1c), and a shrunken root system. Following a final count at 35 das, the isolate with extremely high pathogenicity (85%), with 100% mean disease incidence, and a severity score of four was selected for additional investigation (Fig. 1).

3.2. Pathogen identification based on ITS and TEF-1α gene sequencing

The ITS rDNA region and the translation elongation factor 1-alpha (TEF-1α) of the pathogenic isolate were sequenced, and the identification was performed by comparison with published sequences in GenBank. The results from the BLAST search revealed that the obtained ITS sequence displayed 98% similarity with sequences from *F. verticillioides*, *Fusarium proliferatum*, and *F. oxysporum* in the NCBI database. However, the results from TEF-1α showed similarity with only sequences of *F. verticillioides* species. To infer the evolutionary history of our isolate, the maximum likelihood method was used for the phylogenetic analysis. The Jukes-Cantor model with discrete gamma-distributed rates was implemented for the ITS analysis (Fig. 2), and the Kimura 2-parameter model with discrete gamma distribution rates was implemented for TEF1 analysis (Fig. 2). From the results, the delimitation power of the ITS gene was lower. Our isolate was found to be closely related to *F. verticillioides, F. proliferatum, F. mangifera* and *F. circinatum* belonging to the *F. fujikuroi* species complex. However, the TEF-1-based phylogenetic analysis showed that our pathogenic isolate was very distinct from *F. proliferatum, F. mangifera* and *F. circinatum* but closely related to *F. verticillioides* species in a clade well-supported clade of 97 bootstrap support. Therefore, our isolate was confirmed to be *F. verticillioides* FV (see Fig. 3).

3.3. In vitro antagonism assays

3.3.1. Bioantagonistic effects of bacterial endophytes against *F. verticillioides*

F. verticillioides growth inhibition ranging from 4.26 to 68.6% was recorded. Six out of 27 bacteria (Fig. 4), including *B. amyloliquefaciens* RA37 (68.6%), *B. velezensis* BE1 (65.4%), *B. megaterium* RR13 (59.0%), *E. cloacae* LPR2^{+} (50.0%), *L. minipressuralis* LGR12 (53.9%) and unidentified LPR1^{+} (53.2%), displayed remarkable inhibitory activity against *F. verticillioides*. The most effective strains were

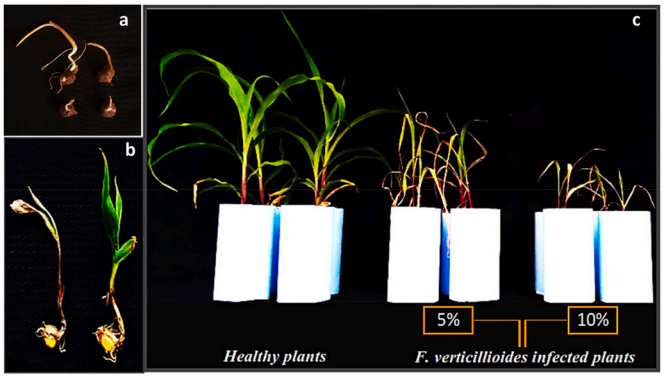

Fig. 1. Virulence of *F. verticillioides* on maize germination and vegetative growth. (a) *F. verticillioides*-infested seeds with inhibited germination. (b) Pre-emergence damping-off at 7 das, (c) stunted growth, leaf chlorosis and ear rot at 5% and 10% inoculum loads at 21 das.

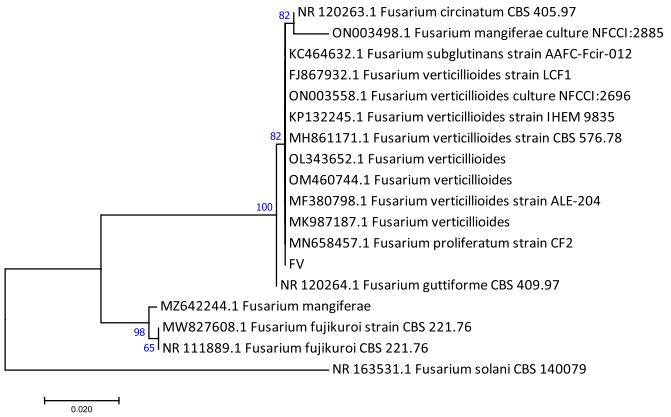

Fig. 2. Molecular phylogenetic analysis of *F. verticillioides* F.V. based on the ITS gene region. The evolutionary history was inferred by using the maximum likelihood method based on the Jukes-Cantor model [1]. The tree with the highest log likelihood (- 972.93) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the maximum parsimony method. A discrete gamma distribution was used to model evolutionary rate differences among sites (2 categories (+*G*, parameter = 0.0500)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 437 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

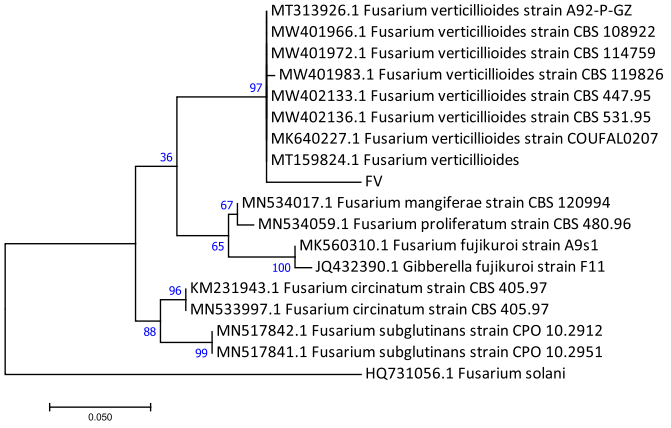

Fig. 3. Molecular phylogenetic analysis of *F. verticillioides* F.V. based on the TEF 1α gene region. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model [1]. The tree with the highest log likelihood (−798.84) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree (s) for the heuristic search were obtained automatically by applying the maximum parsimony method. A discrete gamma distribution was used to model evolutionary rate differences among sites (2 categories (+*G*, parameter = 0.1845)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 252 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [2].

B. amyloliquefaciens RA37 and *B. velezensis* BE1, with inhibition percentages of 68.6% and 65.4%, respectively.

3.3.2. Volatile organic compounds from bacteria inhibit *F. verticillioides* growth

The volatile organic compounds (VOCs) released by endophytic bacteria differentially suppressed the mycelial growth of *F. verticillioides,*

with inhibition percentages ranging from 0.0 to 71.70%. Out of the 27 strains (Fig. 5), nine (18.5%), notably *B. amyloliquefaciens* BFL1 (71.7%); *C. indologenes* LPR17, unidentified LPR3 (66.9%); *S. maltophilia* LPR6^{+} (62.3%); *C. indologenes* LPR11^{+} (61.3%); *K. pneumoneae* RR19 (61.3%); *B. xiamenensis* RA4 (61.3%); *B. megaterium* RR13 (50.0%); and *E. cloacae* LPR2^{+} (50.0%), recorded inhibition percentages greater than or equal to 50%. Subsequently, *B. amyloliquefaciens* BFL1, unidentified LPR3 and *C. indologenes* LPR17, with inhibition percentages of 71.7%, 66.9%, and 66.9%, respectively, emitted the most potent antifungal VOMs.

3.4. Extracellular enzyme profiling

The aptitude of bacterial endophytes to produce specific hydrolytic enzymes such as protease, cellulase, and amylase was investigated (Table 3). At least two of the three hydrolytic enzymes examined were synthesized by each of the tested BCAs (Fig. 6).

As a result, all 27 bacteria released cellulase (100%) with activity ranging from 0.1 to 1.5 UI. *L. minipressuralis* PA2^{+} (1.5 UI), *B. megaterium* RR10 (1.3 UI), and *L. fusiformis* LPR19 (1.3 UI) were the most effective agents. Furthermore, 24 bacterial strains (88.8%) produced and released protease with a distinct halo on skim milk agar, with activity ranging from 0.23 to 1.14 UI. *Bacterium* LPR1^{+} (1.13 UI), *S. maltophilia* LPR6^{+} (1.13 UI), and *P. peneri* RA24 (1.01 UI) produced the most protease. Twenty-two (85.18%) of the bacterial strains tested positive for amylase production, with enzyme activity ranging from 0.31 to 1.72 UI. With activities of 1.7 and 1.6 UI, respectively, *B. velezensis* BE1 and *S. maltophilia* RA33 performed well.

3.5. Plant growth-promoting traits

All the endophytic bacterial strains displayed a great ability to produce multiple PGP factors, such as IAA, siderophores, and phosphate solubilization factors, at various intensities (Table 4). For instance, all 27 (100%) bacteria produced significant amounts of ammonia with concentrations ranging from 0.7 to 6.4 g/mL. Turkey's multiple range comparisons demonstrated considerably greater ammonia production by strains *L. fusiformis* LPR19 (6.4 g/mL) and *B. velezensis* BE1 (6.3 g/mL). Similarly, IAA was produced by all of the BCAs. The strains *B. megaterium* RR10 (55.7 g/mL), RR13 (49 g/mL), and unidentified LPR3 (57.4 g/mL) were the best producers. All bacterial strains tested positive for all three types of siderophores investigated, with a preferential synthesis of catecholate siderophores followed by hydroxamate siderophores. There was no significant difference between the bacteria regardless of the siderophores. In contrast to other PGP attributes, only 14 of the 27 bacteria could solubilize phosphate with halo diameters ranging from 0.5 to 6 mm, with the most active strain being *B. velezensis* BE1 (6 mm) (Table 4).

3.6. In planta ear rot suppression by bacterial endophytes

3.6.1. Reduced ear rot incidence and severity by bacteria

Under greenhouse conditions, all 27 bacteria investigated *in vitro* for biological potential were further evaluated for their ability to safeguard maize against *F. verticillioides* (Table 5). According to the findings, nearly all of the bioagents exhibited a certain degree of inhibition against *F. verticillioides* in greenhouse, as exemplified by reduced seed degradation before germination and reduction of ear rot severity (RDS). Overall, while the Fusarium-infected plantlets recorded the highest number of preemergence seed decay (40%), significantly fewer decayed seeds were obtained in bacterized seeds ($p < 0.05$), culminating at as high as 90% protection rates. *P. peneri* RA24, *B. megaterium* RR10, RR13, *B. amyloliquefaciens* BFL1, BFR2, unidentified LPR3^{+}, and unidentified LPR1^{+} recorded dramatic RDS in bacterized seedlings compared to negative controls (water control) at 35 days postplanting. *C. fruendii* LPR4+, *P. putida* PA3+, and *B. xiamenensis* RA4 were the most efficient bacterial strains, with RDS values of 55%, 47.5% and 47.5%, respectively.

3.6.2. Endophytic bacteria alter maize plant growth under F. verticillioides challenge

Almost all bacterial agents maintained or significantly increased

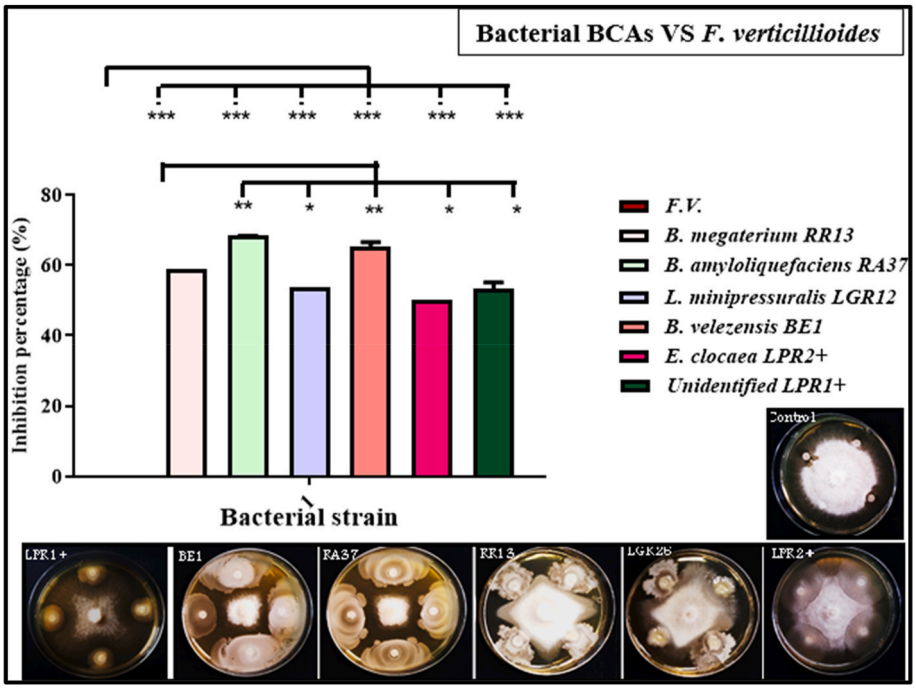

Fig. 4. Inhibitory profile of the most active endophytic bacteria in dual culture against *F. verticillioides*. *, ** and *** indicate significant differences at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. (LPR1^{+}): *Unidentified* LPR1^{+}; (BE1): *B. velezensis* BE1; (RA37): *B. amyloliquefaciens* RA37; (RR13): *B. megaterium* RR13; (LGR26): *E. cloacae* LGR26; (LPR2^{+}): *E. cloacae* LPR2^{+}.

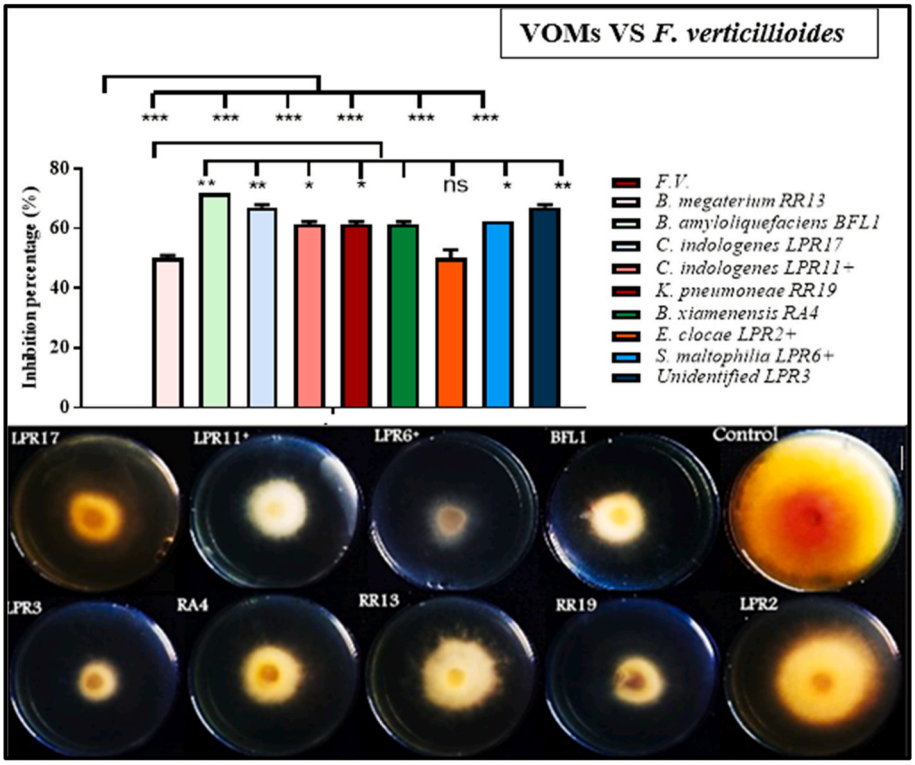

Fig. 5. Inhibitory effects of VOMs from the best bacteria against *F. verticillioides* F.V. *, ** and *** indicate significant differences at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively. "ns" indicates a nonsignificant difference compared to other treatments. (LPR17): *C. indologenes* LPR17; (LPR11^{+}): *C. indologenes* LPR11^{+}; (PLR6^{+}): *B. amyloliquefaciens* BFL1; (LPR3): *unidentified* LPR3; (RA4): *B. xiamenensis* RA4; (RR13): *B. megaterium* RR13; (RR19): *K. pneumoneae* RR19; (LPR2^{+}): *E. cloacae* LPR2^{+}.

Table 3
Hydrolytic enzyme (protease, amylase, and cellulase) profile of the tested bacteria.

S/N	Bacterial strain	Enzyme activity (UI)		
		Protease	Amylase	Cellulase
1.	*P. peneri* RA24	1.0 ± 0.0^{ijk}	0.0 ± 0.0^{a}	0.6 ± 0.1^{bef}
2.	*B. megaterium* RR13	0.5 ± 0.0^{de}	1.3 ± 0.3^{hi}	0.7 ± 0.1^{dgh}
3.	*E. cloacae* LPR26	0.9 ± 0.1^{hij}	0.8 ± 0.2^{def}	0.6 ± 0.0^{cfg}
4.	*B. Amyloliquefaciens* BFR2	0.7 ± 0.1^{egh}	0.9 ± 0.2^{def}	0.5 ± 0.0^{bde}
5.	*B. Amyloliquefaciens* RA37	0.0 ± 0.0^{a}	0.6 ± 0.1^{bde}	0.7 ± 0.1^{egh}
6.	*Paracoccus* sp LPR20	0.4 ± 0.0^{cd}	0.8 ± 0.0^{cde}	0.5 ± 0.1^{bef}
7.	*Unidentified* LPR1+	1.1 ± 0.2^{k}	1.4 ± 0.5^{hij}	0.8 ± 0.1^{egh}
8.	*B. amyloliquefaciens* BFL1	0.2 ± 0.0^{bc}	0.3 ± 0.1^{ab}	0.3 ± 0.0^{abc}
9.	*L. fusiformis* LPR19	0.9 ± 0.2^{gij}	0.4 ± 0.0^{ab}	1.3 ± 0.1^{jk}
10.	*L. minipressuralis* PA2+	0.7 ± 0.1^{egh}	0.9 ± 0.1^{efg}	1.5 ± 0.4^{k}
11.	*P. aeruginosas* RR4	0.6 ± 0.1^{dfg}	1.2 ± 0.0^{fgh}	1.2 ± 0.0^{ijk}
12.	*C. indologenes* LPR17	0.6 ± 0.0^{def}	0.0 ± 0.0^{a}	0.9 ± 0.1^{ghi}
13.	*K. pneumoneae* RR19	0.3 ± 0.0^{bc}	0.9 ± 0.1^{efg}	0.8 ± 0.1^{egh}
14.	*L. minipressuralis* LGR12	0.8 ± 0.1^{gij}	0.4 ± 0.0^{bc}	1.0 ± 0.4^{hij}
15.	*Unidentified* LPR3+	0.4 ± 0.0^{bcd}	0.6 ± 0.1^{bcd}	0.7 ± 0.0^{dgh}
16.	*B. velezensis* BE1	0.2 ± 0.0^{b}	1.7 ± 0.4^{j}	0.8 ± 0.1^{egh}
17.	*B. xiamenensis* RA4	0.7 ± 0.3^{egh}	0.5 ± 0.1^{bc}	1.3 ± 0.4^{ijk}
18.	*Unidentified* LPR3	0.6 ± 0.0^{def}	0.0 ± 0.0^{a}	0.1 ± 0.0^{a}
19.	*P. putida* RR20	0.9 ± 0.1^{hij}	1.0 ± 0.1^{egh}	0.2 ± 0.0^{ab}
20.	*C. indologenes* LPR11+	0.7 ± 0.0^{egh}	1.3 ± 0.0^{fgh}	0.5 ± 0.0^{bde}
21.	*E. cloacae* LPR2+	1.0 ± 0.1^{ijk}	0.5 ± 0.1^{bc}	1.0 ± 0.3^{hij}
22.	*S. maltophilia* LPR6+	1.1 ± 0.0^{k}	0.0 ± 0.0^{a}	0.5 ± 0.0^{bde}
23.	*P. putida* PA3+	0.0 ± 0.0^{a}	0.9 ± 0.1^{defg}	0.9 ± 0.1^{fhi}
24.	*C. fruendii* LPR4+	0.3 ± 0.1^{bc}	1.2 ± 0.1^{gh}	0.3 ± 0.1^{abc}
25.	*B. megaterium* RR10	0.0 ± 0.0^{a}	0.4 ± 0.0^{ab}	1.3 ± 0.4^{jk}
26.	*L. minipressuralis* LGR12+	0.8 ± 0.0^{fhi}	1.2 ± 0.2^{fgh}	0.4 ± 0.0^{bde}
27.	*S. maltophila* RA33	1.0 ± 0.2^{ijk}	1.6 ± 0.3^{ij}	0.7 ± 0.0^{dgh}

Mean values ± SD (standard deviation) superscripted with different letters are significantly different with respect to Tukey's multiple range test at $P < 0.05$.

shoot and root development of maize compared to only the infected control, except for treatments with *B. amyloliquefaciens* BFL1 and *L. fusiformis* LPR19, which displayed repressed shoot development compared to negative control plants (Table 6). When compared to the normal (uninfected) control, the treatment with unidentified LPR3 increased shoot development to a value higher than the normal growth of the plant under uninfected conditions. However, root growth was significantly increased by all the bacterial treatments compared to the infected control and by almost all of them compared to the uninfected control. Regarding the dry weights, most of the treatments did not significantly impact the root dry masses compared to the negative control. Nevertheless, some treatments (6) reached the normal control root dry weight. The shoot dry masses of the plants were significantly reduced by some bacterial treatments compared to the negative control. However, three treatments (*P. aeruginosa RR4*; *L. minipressuralis LGR12*+ and *C. fruendii LPR4*+) reached the dry weight of the normal control.

3.6.3. Altered PAL and GPX enzyme activities upon maize seedling bacterization

Guaiacol peroxidase (GPX) and phenylalanine ammonia lyase (PAL) are important enzymes implicated in the plant defense response against pathogen attacks. The ability of a given bacterium to trigger its activity was evaluated (Table 7). The results demonstrated that the *Fusarium* treatment significantly increased GPX activity compared to the normal control, and ten of the bacterial treatments significantly increased the enzyme activity in the presence of *F. verticillioides* compared to the pathogen treatment, with an activity range of 604.9 OD/min/g FM in the uninoculated control to 2867.8 OD/min/g FM for the pathogen inoculation alone. Specific activities of 2867.8, 2066.2, 2035.1 and 1775.8 OD/min/g FM were recorded when seeds were primed with the BCAs K. *pneumonia* RR19, *S. maltophila* LPR6^{+}, RA33, and *C. frundii* LPR4^{+}.

For PAL activity, the single inoculation with *F. verticillioides* had no significant impact on the enzyme activity compared to the uninoculated control. Fifteen (15) bacteria significantly increased PAL activity compared to *F. verticillioides* and the uninoculated control, indicating a redirection of the plant's innate immune system. Among these bacterial agents, *S. maltophila* RA33 (44.5 OD/min/g FM) and LPR6^{+} (31.5 OD/min/g FM) exhibited the most important activity. The increase in GPX and PAL activities observed in the present study reveals the ability of the

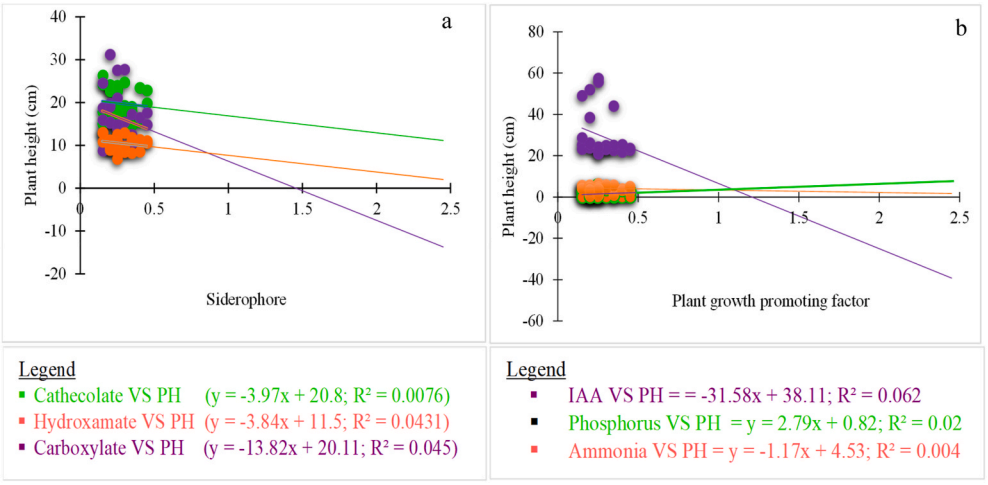

Fig. 6. Linear forecast regression model between maize plant growth performance (under greenhouse conditions) and some *in vitro* plant growth-promoting abilities (PH) displayed by the endophytic bacteria investigated. (a): For the siderophore versus growth parameter, green, orange and purple lines represent the forecasted bacteria-induced growth response (PH) of maize with respect to their *in vitro* abilities to produce catecholate-, hydroxamate- and carboxylate-type siderophores, respectively. (b): Green, orange, and purple lines represent the forecasted BCA-induced growth response (PH) of maize with respect to their *in vitro* abilities to produce phosphate solubilizing agents and to produce IAA and ammonia, respectively.

Table 4
Recap of the plant growth-promoting potentials (ammonia, indole acetic acid, phosphate, and siderophores) by the twenty-seven bacterial agents. Mean values ± SD (standard deviation) per column, superscribed with different alphabet letter(s), are significantly different from each other as given by Duncan's multiple comparison test ($p < 0.05$).

S/N	Bacterial strains	Phosphate Solubilization	Ammonia Production	IAA Production	Siderophore catecholate	Siderophore hydroxamate	Siderophore carboxylate
1.	*P. peneri* RA24	0.0 ± 0.0^{a}	2.7 ± 0.6^{bc}	23.8 ± 0.7^{bef}	24.1 ± 0.6^{gh}	15.4 ± 2.9^{egh}	11.2 ± 2.2^{bfg}
2.	*B. megaterium* RR13	0.0 ± 0.0^{a}	4.3 ± 0.4^{def}	49.0 ± 2.5^{m}	24.2 ± 0.0^{gh}	15.9 ± 1.1^{ehi}	13.0 ± 0.0^{g}
3.	*E. cloacae* LGR26	2.5 ± 0.7^{abc}	0.9 ± 0.6^{a}	26.2 ± 1.6^{i}	22.6 ± 2.2^{dgh}	15.2 ± 1.0^{egh}	8.9 ± 1.9^{acd}
4.	*B. amyloliquefaciens* BFR2	3.0 ± 1.4^{abc}	5.5 ± 0.6^{gij}	25.3 ± 1.6^{hi}	17.8 ± 0.0^{bcd}	24.5 ± 0.0^{l}	12.9 ± 0.0^{g}
5.	*B. amyloliquefaciens* RA37	4.0 ± 1.4^{abc}	1.1 ± 0.3^{a}	44.0 ± 0.0^{l}	17.9 ± 3.4^{bcd}	12.5 ± 1.2^{bde}	11.8 ± 0.0^{dfg}
6.	*Paracoccus* LPR20	2.0 ± 0.0^{abc}	5.5 ± 0.5^{gij}	23.2 ± 1.4^{bde}	15.0 ± 2.6^{bc}	8.7 ± 3.4^{a}	10.8 ± 1.6^{bcfg}
7.	*Unidentified* LPR1^{+}	0.0 ± 0.0^{a}	4.1 ± 1.1^{de}	52.0 ± 2.6^{n}	15.7 ± 0.0^{bc}	10.9 ± 0.0^{acd}	9.5 ± 2.9^{aef}
8.	*B. amyloliquefaciens* BFL1	0.0 ± 0.0^{a}	4.2 ± 0.4^{def}	28.6 ± 3.0^{j}	26.3 ± 0.0^{h}	18.7 ± 0.0^{gjk}	12.9 ± 0.0^{g}
9.	*L. fusiformis* LPR19	0.0 ± 0.0^{a}	6.4 ± 0.6^{k}	20.9 ± 0.9^{ab}	23.9 ± 0.0^{fgh}	14.1 ± 1.2^{dfg}	10.7 ± 17^{aef}
10.	*L. minipressuralis* PA2	0.0 ± 0.0^{a}	5.8 ± 0.3^{jk}	22.6 ± 0.5^{bc}	14.7 ± 0.8^{b}	10.4 ± 0.9^{abc}	8.2 ± 0.0^{ab}
11.	*P. aeruginosas* RR4	0.0 ± 0.0^{a}	3.9 ± 0.5^{de}	21.9 ± 0.7^{ab}	15.4 ± 0.3^{bc}	10.6 ± 1.5^{acd}	11.3 ± 1.2^{efg}
12.	*C. indologenes* LPR17	0.5 ± 0.0^{a}	5.6 ± 0.0^{hij}	25.0 ± 0.8^{hi}	19.0 ± 5.1^{bde}	17.2 ± 0.0^{fjk}	11.1 ± 0.0^{bfg}
13.	*K. pneumoniae* RR19	3.0 ± 0.0^{abc}	3.3 ± 0.9^{cd}	25.4 ± 1.1^{ghi}	23.4 ± 3.1^{egh}	16.4 ± 2.0^{fij}	8.5 ± 0.9^{abc}
14.	*L. minipressuralis* LGR12	0.0 ± 0.0^{a}	5.8 ± 0.3^{jk}	38.5 ± 0.0^{k}	22.9 ± 1.1^{egh}	19.7 ± 1.6^{gjk}	9.7 ± 3.0^{aef}
15.	*Unidentied* LPR3^{+}	4.0 ± 0.0^{abc}	5.9 ± 1.0^{jk}	23.6 ± 0.7^{bcd}	15.3 ± 0.0^{bc}	27.7 ± 0.0^{lmn}	8.4 ± 0.4^{abc}
16.	*B. velezensis* BE1	6.0 ± 1.4^{c}	6.3 ± 0.1^{jk}	23.3 ± 0.7^{bef}	22.6 ± 3.7^{dgh}	21.0 ± 1.8^{k}	12.0 ± 1.0^{efg}
17.	*B. xiamenensis* RA4	0.0 ± 0.0^{a}	5.5 ± 0.4^{gij}	24.9 ± 1.2^{fhi}	19.2 ± 0.0^{bef}	15.4 ± 0.0^{egh}	12.9 ± 0.0^{g}
18.	*Unidentified* LPR3	3.0 ± 0.0^{abc}	4.9 ± 0.3^{egh}	57.4 ± 1.9^{o}	16.6 ± 1.5^{bc}	10.9 ± 1.5^{acd}	12.5 ± 0.0^{fg}
19.	*P. putida* RR20	0.0 ± 0.0^{a}	0.8 ± 0.5^{a}	21.4 ± 3.7^{a}	19.5 ± 0.1^{bfg}	13.8 ± 0.3^{cef}	6.8 ± 2.1^{a}
20.	*C. indologenes* LPR11^{+}	4.5 ± 2.1^{bc}	4.4 ± 0.4^{efg}	23.1 ± 0.1^{bcd}	17.0 ± 0.0^{bc}	9.0 ± 0.0^{ab}	9.1 ± 1.2^{ade}
21.	*E. cloacae* LPR2^{+}	3.5 ± 0.7^{abc}	5.8 ± 0.0^{ijk}	23.6 ± 1.0^{cfg}	24.7 ± 0.0^{h}	9.8 ± 1.9^{ab}	9.7 ± 1.5^{aef}
22.	*S. maltophilia* LPR6^{+}	2.0 ± 0.0^{abc}	4.7 ± 0.7^{egh}	24.3 ± 0.5^{cgh}	23.6 ± 0.0^{egh}	19.0 ± 3.7^{ijk}	11.2 ± 2.0^{bfg}
23.	*P. putida* PA3^{+}	0.0 ± 0.0^{a}	1.7 ± 0.8^{ab}	24.2 ± 1.2^{dfg}	23.4 ± 0.0^{egh}	27.5 ± 0.0^{lm}	8.3 ± 0.1^{abc}
24.	*C. fruendii* LPR4^{+}	0.0 ± 0.0^{a}	2.3 ± 0.3^{bc}	22.8 ± 1.5^{bef}	22.8 ± 0.0^{egh}	14.8 ± 3.2^{efg}	11.0 ± 2.6^{bfg}
25.	*B. megaterium* RR10	1.5 ± 0.7^{abc}	3.9 ± 0.2^{de}	55.7 ± 0.5^{o}	17.6 ± 1.3^{bc}	14.8 ± 1.4^{efg}	10.8 ± 1.0^{bfg}
26.	*L. minipressuralis* LGR12^{+}	3.0 ± 1.4^{abc}	5.1 ± 0.3^{ijk}	23.6 ± 1.0^{cfg}	19.8 ± 2.5^{cfg}	17.6 ± 0.0^{jk}	10.5 ± 0.6^{bfg}
27.	*S. maltophilia* RA33	0.0 ± 0.0^{a}	3.4 ± 0.8^{de}	25.7 ± 0.8^{ghi}	9.5 ± 0.0^{a}	31.2 ± 0.0^{n}	9.5 ± 2.3^{aef}

tested endophytic bacteria to boost the maize seedling defense system in response to *F. verticillioides* ingress.

4. Discussion

Biological control approaches involving bacterial agents have emerged as genuine tools for sustainable agriculture (Kant et al., 2011). Elsewhere, there is a profusion of evidence pointing out fluctuations in the environmental conditions in agro-ecosystems as the key limiting factor to the success of biocontrol agents and their widespread incorporation into modern agriculture (Goswami et al., 2014). Hence, the ability of biocontrol agents to thrive in harsh conditions is an added value that can discriminate the best performing bioagents. Accordingly, active endophytic bacteria from drought-resilient hosts are likely more prone to succeed as potential biological control agents. This study reports for the very first time the antifungal potential of bacterial endophytes from *Euphorbia antiquorum* L against *Fusarium verticillioides* in maize.

F. verticillioides was isolated from symptomatic maize plants and assessed for pathogenicity on a local commercial maize variety. The most aggressive isolate was identified as *F. verticillioides FV* based on the sequencing of the internal transcribed spacer and translation elongation factor 1α sequences. Indeed, *F. verticillioides* is one of the most prevalent fungal pathogens of maize prevailing in the main production basins of Cameroon (Ngoko et al., 2001) and worldwide.

The tested BCAs displayed the ability to suppress *F. verticillioides* mycelial growth with inhibition percentages of up to 68% and 71% in direct and indirect confrontation assays, respectively. Such inhibitory

Table 5
Effect of bacterial inoculation on the reduction of ear and root rot severity in young maize plants at various time intervals.

Bacterial strain	Disease severity reduction (%)						
	Days after sowing (das)						
	11	15	19	23	27	31	35
F.V (*F. verticillioides*)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F. V. + *P. peneri* RA24	77.5	72.5	65.0	57.5	45.0	25.0	25.0
F. V. + *B. megaterium* RR13	62.5	62.5	57.5	45.0	32.5	32.5	22.5
F. V. + *E. cloacae* LPR26	90.0	82.5	75.0	52.5	42.5	32.5	30.0
F. V. + *B. Amyloliquefaciens* BFR2	75.0	65.0	62.5	50.0	35.0	25.0	15.0
F. V. + *B. Amyloliquefaciens* RA37	92.5	87.5	80.0	62.5	60.0	40.0	37.5
F. V. + *Paracoccus* sp LPR20	95.0	90.0	87.5	70.0	57.5	52.5	45.0
F. V. + *Unidentified* LPR1+	87.5	75.0	65.0	57.5	45.0	40.0	40.0
F. V. + *B. amyloliquefaciens* BFL1	50.0	42.5	42.5	27.5	27.5	25.0	22.5
F. V. + *L. fusiformis* LPR19	70.0	62.5	57.5	37.5	32.5	25.0	15.0
F. V. + *L. minipressuralis* PA2+	92.5	82.5	75.0	65.0	40.0	42.5	37.5
F. V. + *P. aeruginosas* RR4	85.0	80.0	72.5	60.0	47.5	42.5	42.5
F. V. + *C. indologenes* LPR17	87.5	77.5	75.0	70.0	45.0	45.0	45.0
F. V. + *K. pneumoneae* RR19	87.5	82.5	75.0	70.0	47.5	45.0	45.0
F. V. + *L. minipressuralis* LGR12	90.0	77.5	72.5	65.0	42.5	37.5	37.5
F. V. + *Unidentified* LPR3+	65.0	52.5	42.5	55.0	35.0	27.5	25.0
F. V. + *B. velezensis* BE1	90.0	72.5	70.0	65.0	40.0	40.0	35.0
F. V. + *B. xiamenensis* RA4	95.0	82.5	82.5	70.0	55.0	50.0	47.5
F. V. + *Unidentified* LPR3	92.5	87.5	80.0	80.0	52.5	50.0	37.5
F. V. + *P. putida* RR20	95.0	80.0	80.0	60.0	50.0	45.0	37.5
F. V. + *C. indologenes* LPR11+	97.5	85.0	82.5	67.5	47.5	37.5	30.0
F. V. + *E. cloacae* LPR2+	95.0	90.0	85.0	70.0	55.0	45.0	42.5
F. V. + *S. maltophilia* LPR6+	70.0	57.5	52.5	52.5	35.0	35.0	35.0
F. V. + *P. putida* PA3+	92.5	87.5	80.0	72.5	50.0	47.5	47.5
F. V. + *C. fruendii* LPR4+	100.0	85.0	85.0	72.5	62.5	55.0	55.0
F. V. + *B. megaterium* RR10	85.0	77.5	77.5	60.0	45.0	45.0	45.0
F. V. + *L. minipressuralis* LGR12+	92.5	82.5	80.0	67.5	52.5	45.0	42.5
F. V. + *maltophila* RA33	95.0	87.5	82.5	67.5	47.5	40.0	37.5

Controls (uninoculated) and treatments consisting of plants infected by *F. verticillioides* and individual BCA were frequently watered for 35 days, after which plants were harvested and disease severity data were recorded.

responses have been demonstrated by species such as *Bacillus*, *Pantoea, Pseudomonas*, *Serratia*, *Stenotrophomonas*, *Streptomyces*, *Enterobacter, Klebsiella,* and many other biocontrol bacteria toward a wide variety of phytopathogens (Thomashow et al., 2008; Raaijmakers and Mazzola, 2012). In this study, members of the *Bacillus* genus demonstrated remarkable inhibitory effects compared to others. Presumably due to their ability to produce broad-spectrum antibiotics and/or to form endospores (Figueroa-López et al., 2016; Karimi et al., 2016b). The thus termed antibiosis, standing for the production of antimicrobial substances, has been touted as one of the most expected attributes of promising bacteria from the *Bacillus* and *Pseudomonas* genera (Caulier et al., 2018; Dimkić et al., 2022). Approximately 32 gene clusters are

Table 6
Overview of maize seedling agromorphological parameters as affected by bacterial inoculation challenged with *F. verticillioides* F.V. under greenhouse conditions.

Bioagents	Agro-morphological parameters			
	Shoot length (cm)	Root length (cm)	Root dry weight (g)	Shoot dry weight (g)
Control	71.9 ± 9.7^{jk}	24.0 ± 2.9^{cfg}	0.2 ± 0.0^{g}	0.7 ± 0.0^{ij}
F. verticiloides FV	57.7 ± 6.8^{bc}	17.8 ± 4.7^{a}	0.1 ± 0.0^{bef}	0.4 ± 0.0^{afg}
F. V. + *P. peneri RA24*	64.0 ± 5.3^{cgh}	31.0 ± 7.0^{fhi}	0.1 ± 0.0^{a}	0.3 ± 0.0^{acd}
F. V. + *B. megaterium RR13*	57.6 ± 1.5^{bcd}	20.2 ± 5.3^{bc}	0.1 ± 0.0^{ab}	0.2 ± 0.1^{ab}
F. V. + *E. cloacae LPR26*	58.5 ± 5.6^{bde}	20.1 ± 3.1^{bc}	0.1 ± 0.0^{acd}	0.3 ± 0.1^{ade}
F. V. + *B. Amyloliquefaciens BFR2*	52.6 ± 7.7^{abc}	18.6 ± 3.6^{ab}	0.1 ± 0.0^{acd}	0.2 ± 0.0a
F. V. + *B. Amyloliquefaciens RA37*	61.6 ± 4.9^{gij}	30.0 ± 0.0^{fhi}	0.2 ± 0.0^{ef}	0.5 ± 0.0^{eij}
F. V. + *Paracoccus* sp *LPR20*	66.7 ± 7.8^{chi}	25.5 ± 2.4^{dgh}	0.1 ± 0.0^{def}	0.2 ± 0.0^{a}
F. V. + *Unidentified LPR1+*	64.6 ± 2.0^{cfg}	22.5 ± 2.8^{cfg}	0.1 ± 0.0^{acd}	0.3 ± 0.0^{ade}
F. V. + *B. amyloliquefaciens BFL1*	49.0 ± 7.5^{a}	21.0 ± 0.0^{bde}	0.1 ± 0.0^{def}	0.2 ± 0.0^{abc}
F. V. + *L. fusiformis LPR19*	54.0 ± 1.4^{ab}	25.6 ± 4.7^{ehi}	0.1 ± 0.0^{def}	0.4 ± 0.1^{agh}
F. V. + *L. minipressuralis PA2+*	59.0 ± 1.4^{cef}	29.0 ± 5.9^{ghi}	0.2 ± 0.0^{fg}	0.5 ± 0.1^{dh}i
F. V. + *P. aeruginosas RR4*	70.0 ± 7.4^{gij}	29.6 ± 6.6^{fhi}	0.1 ± 0.0^{bef}	0.7 ± 0.1^{j}
F. V. + *C. indologenes LPR17*	68.2 ± 6.4^{hjk}	32.0 ± 4.6^{i}	0.1 ± 0.0^{def}	0.6 ± 0.2^{hij}
F. V. + *K. pneumoneae RR19*	64.0 ± 6.3^{ehi}	28.2 ± 5.9^{ghi}	0.2 ± 0.0^{efg}	0.6 ± 0.0^{gij}
F. V. + *L. minipressuralis LGR12*	47.2 ± 11.2^{dhi}	25.8 ± 4.9^{ehi}	0.1 ± 0.0^{cef}	0.3 ± 0.1^{afg}
F. V. + *Unidentified LPR3+*	59.2 ± 7.9^{cgh}	29.2 ± 4.9^{hi}	0.1 ± 0.0^{abc}	0.5 ± 0.0^{gij}
F. V. + *B. velezensis BE1*	71.3 ± 9.4^{ijk}	26.5 ± 4.9^{ehi}	0.1 ± 0.0^{aef}	0.4 ± 0.1^{bgh}
F. V. + *B. xiamenensis RA4*	70.0 ± 10.5^{ejk}	29.8 ± 4.7^{hi}	0.1 ± 0.0^{cef}	0.5 ± 0.0^{eij}
F. V. + *Unidentified LPR3*	74.1 ± 6.5^{k}	28.3 ± 4.9^{ehi}	0.1 ± 0.0^{cef}	0.4 ± 0.0^{agh}
F. V. + *P. putida RR20*	64.8 ± 8.4^{bde}	25.9 ± 4.1^{bcd}	0.1 ± 0.0^{ade}	0.4 ± 0.1^{afg}
F. V. + *C. indologenes LPR11+*	62.2 ± 4.0^{cfg}	23.0 ± 2.0^{bcd}	0.1 ± 0.0^{acd}	0.5 ± 0.0^{dhi}
F. V. + *E. cloacae LPR2+*	71.3 ± 7.8^{gjk}	27.8 ± 5.1^{ghi}	0.1 ± 0.0^{def}	0.5 ± 0.1^{fij}
F. V. + *S. maltophilia LPR6+*	60.4 ± 5.3^{bc}	25.1 ± 5.3^{ehi}	0.1 ± 0.0^{bef}	0.3 ± 0.0^{ade}
F. V. + *P. putida PA3+*	63.8 ± 7.9^{cgh}	28.6 ± 6.3^{i}	0.2 ± 0.0^{gh}	0.3 ± 0.1^{ade}
F. V. + *C. fruendii LPR4+*	65.3 ± 9.8^{gij}	26.6 ± 5.1^{ehi}	0.2 ± 0.1^{fg}	0.7 ± 0.1^{ij}
F. V. + *B. megaterium RR10*	64.6 ± 8.6^{gij}	28.2 ± 4.0^{fhi}	0.1 ± 0.0^{acd}	0.4 ± 0.1^{cgh}
F. V. + *L. minipressuralis LGR12+*	65.9 ± 8.7^{fij}	24.3 ± 3.3^{cef}	0.2 ± 0.0^{efg}	0.7 ± 0.1^{ij}
F. V. + *S. maltophila RA33*	62.8 ± 8.8^{gjk}	20.0 ± 2.9^{bc}	0.1 ± 0.0^{def}	0.3 ± 0.1^{aef}

Mean values ± SD (standard deviation) per column, superscribed with different alphabet letter(s), are significantly different from each other as given by Duncan's multiple comparison test ($p < 0.05$).

Table 7
Changes in the specific activities of maize guaiacol peroxidase and phenylalanine ammonia lyase as influenced by *F. verticillioides* and bacterial bioagent inoculation.

Bacterial strains	Defense-related enzymes activity (OD/min/g FM)	
	Guaiacol Peroxidase (GPX)	Phenylalanine Ammonia Lyase (PAL)
Control	604.9 ± 119.0^{abc}	20.6 ± 3.5^{dgh}
F. verticiloides FV	1263.8 ± 354.0^{dgh}	20.3 ± 2.0^{cgh}
F. V. + *P. peneri RA24*	1411.5 ± 73.0^{egh}	14.4 ± 3.1^{a}
F. V. + *B. megaterium RR13*	1132.4 ± 83.0^{cfg}	28.1 ± 0.9^{kmn}
F. V. + *E. cloacae LPR26*	445.1 ± 59.0^{a}	28.5 ± 5.0^{ikl}
F. V. + *B. Amyloliquefaciens BFR2*	488.7 ± 16.0^{ab}	23.8 ± 0.1^{hij}
F. V. + *B. Amyloliquefaciens RA37*	1121.9 ± 112.0^{cfg}	13.1 ± 0.5^{a}
F. V. + *Paracoccus* sp *LPR20*	753.4 ± 4.0^{acd}	19.8 ± 0.9^{bfg}
F. V. + *Unidentified LPR1+*	1056.2 ± 135.0^{bfg}	16.0 ± 0.9^{abc}
F. V. + *B. amyloliquefaciens BFL1*	912.4 ± 78.0^{aef}	28.5 ± 1.5^{kmn}
F. V. + *L. fusiformis LPR19*	1003.4 ± 213.0^{afg}	20.8 ± 1.3^{dgh}
F. V. + *L. minipressuralis PA2+*	936.5 ± 8.0^{aef}	23.6 ± 5.1^{gij}
F. V. + *P. aeruginosas RR4*	554.4 ± 33.0^{abc}	19.1 ± 0.3^{bef}
F. V. + *C. indologenes LPR17*	1539.1 ± 64.0^{gij}	31.1 ± 1.1^{mn}
F. V. + *K. pneumonia RR19*	3115.4 ± 350.0^{k}	21.6 ± 2.3^{ehi}
F. V. + *L. minipressuralis LGR12*	930.3 ± 4.0^{aef}	27.4 ± 1.0^{ilm}
F. V. + *Unidentified LPR3+*	987.8 ± 291.0^{afg}	15.7 ± 1.0^{ab}
F. V. + *B. velezensis BE1*	628.2 ± 13.0^{abc}	20.6 ± 0.8^{dgh}
F. V. + *B. xiamenensis RA4*	865.0 ± 0.0^{ade}	17.3 ± 3.1^{ade}
F. V. + *Unidentified LPR3*	1458.6 ± 357.0^{fhi}	16.8 ± 3.0^{acd}
F. V. + *P. putida RR20*	634.8 ± 48.0^{abc}	24.9 ± 6.9^{ijk}
F. V. + *C. indologenes LPR11+*	566.0 ± 153.0^{abc}	17.0 ± 0.4^{acd}
F. V. + *E. cloacae LPR2+*	1456.6 ± 570.0^{fhi}	21.9 ± 1.1^{fhi}
F. V. + *S. maltophilia LPR6+*	2066.2 ± 323.0^{j}	31.5 ± 2.2^{n}
F. V. + *P. putida PA3+*	1437.6 ± 163.0^{egh}	28.3 ± 3.1^{kmn}
F. V. + *C. fruendii LPR4+*	1432.2 ± 125.0^{hij}	29.7 ± 2.2^{lmn}
F. V. + *B. megaterium RR10*	451.3 ± 9.0^{a}	15.8 ± 1.0^{ab}
F. V. + *L. minipressuralis LGR12+*	1456.6 ± 570.0^{fhi}	21.9 ± 1.1^{fhi}
F. V. + *S. maltophila RA33*	2035.1 ± 104.0^{ij}	44.5 ± 1.5^{o}

Mean values ± SD (standard deviation) per column, superscribed with different alphabet letter(s), are significantly different from each other as given by Duncan's multiple comparison test ($p < 0.05$).

involved in the biosynthesis of antimicrobials such as cyclic lipopeptides, polyketides, and volatile compounds in the genome of *B. velezensis* LDO2 when challenged by the phytopathogen *A. flavus* (Caulier et al., 2019; Jacoby and Kopriva, 2019). In addition to antibiosis, the starvation of surrounding pathogens through molecular weight compounds such as siderophores capable of binding soil nutrients rendering them inaccessible by the pathogen are cited as another important attributes employed by the BCAs to suppress the growth of the pathogens (McNeely et al., 2017). Interestingly, the investigated endophytic bacteria exhibited a strong ability to produce proteases, amylases, and cellulases. Hence, the synthesis and release of these hydrolytic enzymes with the ability to lyse cell wall components causing fungal death have been extensively reported (Hasan and Anand, 2014). Fungi have a unique cell wall structure mainly composed of chitin, glucans, mannans, and glycoproteins, which are needed for nutrient transport, extracellular degradation of nonpermeable substrates, communication, and modifications of cell wall structures. Cell wall death might result in the degradation of signal molecules, receptors, and structural molecules that alter physio-pathological processes or cause direct cell death. The same phenomenon is reported with cellulase through the hydrolysis of 1, 4-β-D-glycosidic linkages in cellulose that make up many cell membranes (Jadhav et al., 2017).

Another well-documented mechanism employed by bacterial agents to counteract the effect of fungal pathogens is plant growth promotion. Phenomena such as compensation of damaged cells, provision of metabolic precursors for the main biochemical processes in the plant, and required energy (ATP) to fuel pathways implicated in plant defense responses have extensively been shown to link primary and secondary metabolism; hence, the plant defense system is primarily at key steps in plant development, such as seed germination and seedling development (Mengiste et al., 2003). There is a plethora of *in vitro* traits expected from promising BCAs, including but not limited to the ability to produce and release IAA, siderophores, ammonia, and solubilization of inorganic phosphate. Endophytic growth promoters solubilize inorganic phosphate (P) with the help of low molecular weight organic acids to make it available for the plant (Zaidi et al., 2009; Glick, 2012). This potential has widely been recorded with endophytic bacteria from the genera *Bacillus*, *Enterobacter*, *Klebsiella*, and many others (Khan et al., 2010, 2013). Our data further confirmed this assertion, as the linear model predicted a positive correlation between the phosphate solubilization abilities and plant growth under greenhouse conditions ($R^2 = 0.02$). In addition, iron is the major component for various vital functions of the plant, such as photosynthesis, synthesis of nucleosides, redox reagents, and amino acid enzyme cofactors present as Fe^{3+} inaccessible to both plants and microorganisms (Sah and Singh, 2015). Hence, bacteria produce low molecular weight chelators known as siderophores to acquire Fe^{3+} and reduce it to Fe^{2+} and then release them into cells, leading to plant growth improvement (Vansuyt et al., 2007; Ahmed and Holmström, 2014). Siderophores are classified based on coordinating groups that chelate the Fe^{3+} ion, and the most common coordinating groups are catecholate, hydroxamates, and carboxylates (Ali and Vidhale, 2013). All the tested bacteria demonstrated the capacity to produce each of these three types of siderophores with potentials varying from one strain to another. A further plant growth mechanism used by biocontrol agents is indole acetic acid (IAA) production. Auxin is implicated in almost all plant growth processes, such as plant cell division, seed germination, and vegetative growth. Endophytic bacteria, by producing IAA, increase root length and area, thereby increasing the accessibility of soil nutrients to plants (Ahemad and Kibret, 2014).

In this study, we deliberately attempted not to discard less-performing bacterial agents upon *in vitro* screening but to get them all involved in *in planta* evaluation. Interestingly, *B. velezensis* BE1 demonstrated the best performance *in vitro* but was contradictorily the least efficient *in planta*. In addition, the strain *C. fruendii* $LPR4^{+}$ with lower antagonistic and plant growth-promoting abilities was evidently the best in disease suppression *in planta*. As asserted by (Comby et al., 2017) and (Besset-Manzoni et al., 2019), a good *in vitro* potential does not always predict relevance *in planta* responses. Furthermore, a predictive linear regression model depicted negative relationships between siderophore ($a = -3.97$; -3.84 and -13.82 for catecholate, hydroxamate and carboxylate siderophores, respectively), IAA ($a = -31.5$) and ammonia ($a = -1.17$) production with plant growth and indirect disease suppression. This phenomenon can be explained by the fact that high production of plant growth-promoting traits such as IAA by bacterial agents can negatively affect plant development. Additionally, many biotic interactions in soil and with plants can lead to the failure of biocontrol agents (Pliego et al., 2011). The aforementioned failure might be ascribed to environmental factors, such as temperature, moisture, humidity, or ultraviolet radiation (Levy et al., 1995). Additionally, inoculation conditions such as carrier liquid, time of inoculation, or spray rate can affect the efficacy of biocontrol agents *in planta*, but this can be managed by developing successful protocols for the application of potential biocontrol agents (Cullen et al., 2011). *In vitro* screening is not necessarily the best way to bioprospect promising biological control agents.

Furthermore, maize seed bacterization remarkably protected seedlings from *F. verticillioides* attack and boosted plant innate immunity in the present study. Some of the biological agents significantly increased PAL- and GPX-specific activities ($p<0.05$). This may be correlated to their best abilities to suppress the development of ear and root rot

diseases. These results suggested that GPX and PAL are implicated in the destressing of infected plants by the best BCA strains. The GPX burst is one of the early responses of host plant cells to pathogen infection (Almagro et al., 2009) and is responsible for ethylene production, which reduces pathogenesis. In addition, peroxidases are important scavengers that act as electron donors, reducing reactive oxygen species (ROS), particularly O_2 and peroxyl radicals, to less or no harmful molecules (Vangronsveld and Clijsters, 1994). Otherwise, the PAL enzyme possesses antimicrobial activity and increases the rate of polymerization of phenolic compounds into lignin-like substances that are deposited in cell walls and papillae and restrict pathogen invasion (Bhardwaj et al., 2014). *C. fruendii* LPR4+ demonstrated the best potential on maize and can constitute a promising candidate for *F. verticillioides* ear and root rot management. However, some caution is needed in the further investigation and application of this species in agriculture because *C. fruendii* species are commonly known as commensal residents in the intestinal tracts of both humans and animals, and some *C. freundii* isolates have been identified as responsible for food poisoning or diarrhea in humans (Bai et al., 2012; Liu et al., 2017). Therefore, further toxicological and safety studies are required for this promising biological agent before any potential application in the field to protect maize against *F. verticillioides* ear and root rot disease.

5. Conclusion

F. verticillioides is a common plant pathogen that infects maize in almost every producing basin across the globe. In Cameroon, for example, the former has been identified as the most serious threat to maize. However, no precautionary measures have been implemented thus far. Our findings support the use of endophytic bacteria as biocontrol agents against maize ear and root rot in Cameroon. Additionally, our data revealed a substantial disparity between the *in vitro* responses and *in planta* efficiency of BCA. The *Bacillus* genus showed considerable inhibitory effects against *F. verticillioides* under laboratory conditions, and its efficacy in the greenhouse was greatly diminished in favor of *Citrobacter*. While highlighting the strain *Citrobacter frundii* as a promising candidate for ear and root rot disease mitigation in maize, our findings highlight the need to revisit the screening processes utilized thus far for the bioprospecting of potent biocontrol candidates. As a result, we estimate that many BCAs with exceptional potentials would have been rejected from several projects in light of their *in vitro* performances. The given situation clearly demonstrates unexplored modes of action offered by bacterial BCAs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Research Article

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Bacterial endophytes inhabiting desert plants provide protection against seed rot caused by *Fusarium verticillioides* and promote growth in maize

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Abstract

BACKGROUND: Fusarium maize ear and root rot disease caused by *Fusarium verticillioides* has become one of the most serious fungal diseases associated with maize production. Due to their abilities to promote plant development and manage diseases, bacterial endophytes provide a more promising approach for treating this vascular disease.

RESULTS: This work was undertaken for the selection and identification of promising isolates as plant growth promoters and biocontrol agents against *F. verticillioides* in maize agroecosystems. A screening procedure consisting of *in vitro* and *in situ* tests was applied to 27 endophytic strains originating from desert plants: *Euphorbia antiquorum*, *Calotropis procera*, and *Alcasia albida*. *In vitro* studies indicated that the bacteria exhibited variable results in biocontrol, endophytism, and plant growth-promoting traits. In addition, *in situ* plant growth promotion and biocontrol experiments allowed the identification of the most promising bacterial endophytes. *In vitro* and *in situ* comparative study results indicated a low correlation. Our data revealed that *in situ* screening must be used as the method of selection of biocontrol agents against Fusarium ear and root rot disease. Based on *in situ* results, seven potent strains were selected and identified as *Bacillus subtilis*, *Bacillus velezensis*, *Bacillus tequilensis*, *Stenotrophomonas maltophilia,* and *Klebsiella pneumoniae*.

CONCLUSION: The results of this study showed that the selected strains seem to be promising candidates to be exploited as biofertilizers and biocontrol agents against Fusarium maize ear and root rot disease.

Supporting information may be found in the online version of this article.

Keywords: endophytic strains; fusarium maize ear and root rot disease; biocontrol; growth promotion

1 INTRODUCTION

Maize has economic importance as a food crop for human, animal consumption, industrial and pharmaceutical purposes worldwide.[1] Maize crops are susceptible to diseases, particularly those caused by pathogenic fungi, of which Fusarium maize ear, kernel, and root rot diseases are caused by *Fusarium verticillioides*. The latter is the most important and the most reported worldwide and in Cameroon.[2] This fungus is a highly destructive soil-borne plant pathogen.[3] The control of *F. verticillioides* is difficult because it can survive in the soil for long periods in the form of resistant structures or as mycelium and conidia on residues from remnant harvest.[4] The issue is far more challenging since the fungus invades the root system and grows endophytically and asymptomatically, causing systemic infection. Infected maize seeds lead to contaminated planting material.[5] In addition, this pathogen produces toxic secondary metabolites called fumonisins that are harmful to animals and human health.[6,7]

Various control strategies were developed to limit the spread of the disease. The use of chemical pesticides as antifungal agents leads to contamination of water, soil, and food crops, and fungal

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resistance development. As a result, there is a high demand for alternative and safe techniques to reduce *F. verticillioides* infection in maize. The use of antagonistic bacteria to control plant pathogenic fungi offers a promising ecologically safe and affordable alternative in comparison to chemical fungicides.[8] Biological control, through the application of plant growth-promoting bacteria (PGPB), offers a substitute for chemical pesticides for the treatment of numerous fungal diseases. Some of these PGPB are endophytes. They survive inside plant tissues without causing any damage. Particular consideration was given to endophytes for the biocontrol of vascular diseases, including *F. verticillioides*.[2,9] In fact, these endophytic bacteria colonize the same ecological niche as the pathogen. This is very advantageous for these potential candidates, as they act against phytopathogens through various mechanisms of action. They induce plant defense mechanisms, produce antagonistic compounds, including antibiotics and lytic enzymes, and compete for space and nutrients.[10] In addition to their direct antipathogenic action, these antimicrobial compounds confer a competitive advantage to biocontrol agents and trigger induced systemic resistance (ISR) in the plant system. Besides, endophytes promote plant growth through nitrogen fixation, phosphate solubilization, siderophores, and phytohormone production, such as indole-3-acetic acid (IAA), and alleviate stress through the modulation of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase expression.[10] A biocontrol agent ensuring both plant growth promotion and inhibition of pathogens would improve plant health, increase plant resistance to pathogen attacks, and decrease harvest losses.[10] Such a combination of traits appears very important in plant protection. In addition, it was suggested that bacteria with a high number of beneficial traits would be promising as improved biopesticides.[11,12]

In our previous study, endophytic bacteria from desert spurge (*E. antiquorum* L.) were investigated for their biocontrol potential against maize root and ear rot caused by *F. verticillioides* under laboratory and greenhouse conditions.[2] A substantial disparity between the *in vitro* responses and *in planta* efficiency of biological control agents against maize ear and root rot was observed. The need to revisit the screening processes utilized for the bioprospecting of potent biocontrol candidates was highlighted. In fact, many biological control agents with exceptional potential would have been rejected in light of their *in vitro* performances.[2] Implementation of large-scale *in vitro* assays will help to select highly active bacterial strains and to detect if they have particular *in vitro* activities, correlated with successful *in planta* protection. In the current study, we aim to (i) evaluate the *in vitro* antagonistic and plant growth promotion potential of isolated endophytic strains originating from *Euphorbia antiquorum*, *Calotropis procera*, and *Alcasia albida*, (ii) assess their plant growth promotion potential and their ability to suppress Fusarium ear and root rot disease in maize *in situ*, and (iii) revisit the screening method of biocontrol agents against maize ear and root rot for the identification of the most promising strains.

2 MATERIALS AND METHODS

2.1 Bacterial isolates

A total of 27 bacterial isolates were investigated for their potential to promote growth and reduce fungal maize infection. The strains were isolated from three plants growing under drastic dryness conditions, identified as *Euphorbia antiquorum*, *Calotropis procera*, and *Alcasia albida* (Table S1), each recognized for its particularly good growth in desert areas, specifically in the hot Far North Region of Cameroon (Latitude: 12° 5′ 13.4988″ N; longitude 15° 0′ 53.3952″ E). Bacteria were isolated from leaves, stem bark, twigs, roots, or fruits depending on the plant (Table S1). The isolates, purified in LB medium, were stored at −80 °C for long-term conservation. The endophytic bacterial strain *Bacillus velezensis* C2 isolated from the crown tissue of a tomato plant with proven antifungal properties against the vascular pathogen *Verticillium dahliae* was used as a positive control for the different assays.[9] All experiments described below in this section were carried out at least in triplicate.

2.2 Antimicrobial potential assessment

2.2.1 *Antifungal potential*

The inhibitory potential of the 27 bacterial candidates was evaluated against mycelial growth of six fungal strains genetically characterized as *F. verticillioides* FV, *Alternaria alternata*, *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium culmorum*, *Botrytis cinerea* and three pathogenic fungal isolates, morphologically identified as *F. verticillioides* (Fv1, Fv2, and Fv3). Briefly, 7-day-old fungal cultures (10^5 conidia mL^{-1}) were evenly spread on the surface of PDA plates. Thereafter, bacterial strains were streaked on the surface of the plates using sterile toothpicks. The plates were incubated at 25 °C for 5 days. Mycelial growth inhibition of the fungi, indicated by clear halos around the bacterial colonies, was determined according to the following formula:

$$\text{Inhibition ratio (IR)} = [(G_T - G_C)/G_C] \times 100. \quad (1)$$

where G_T is the total diameter of the halo and bacterial colony, and G_C is the diameter of the bacterial colony as performed previously.

2.2.2 *Antibacterial activity*

The inhibitory potential of the isolates was evaluated against three bacterial phytopathogens, *A. tumefaciens* strains C58 and B6[13] and *Erwinia amylovora*.[14] The pathogenic strains were first evenly inoculated on the surface of agar plates. Then, the endophytic bacteria were inoculated on the surface of LB agar plates using sterile toothpicks. After incubation for 24 h at 30 °C, antibacterial compound production was indicated by a clear zone of inhibition around the bacterial growth.

2.3 Hydrolytic enzyme production assessment

2.3.1 *Protease production*

Skim milk agar medium was used to reveal the capacity of the bacterial agents to produce proteases for the hydrolysis of fungal cell wall proteins.[15] The appearance of clear halos after 2 days of incubation around the bacterial colonies revealed enzymatic degradation. Halos diameters were measured to determine the enzyme activity (EA) according to the following formula:

$$EA = (D_T - D_C)/D_C. \quad (2)$$

where D_T is the total diameter of the halo and bacterial colony, and D_C is the diameter of the bacterial colony.

2.3.2 *Chitinase production*

The capacity of the bacterial strains to produce chitinase, acting against chitin, the most important component of the fungal cell wall, was studied.[16,17] The bacterial strains were inoculated on a colloidal chitin-based culture medium. After 2 days of incubation

at 30 °C, clear halos around the bacterial colonies were measured, and the enzyme activity was calculated using the same formula as described before.

2.3.3 *Glucanase production*

The capacity of the bacterial strains to produce β-glucanase was studied in barley flour agar plates at 30 °C for 2 days.[18] The appearance of halo zones following Congo red (0.1%) application was a positive response for β-glucanase activity.

2.4 Plant growth-promoting trait assessment

2.4.1 *Phosphate solubilization*

The phosphate solubilization potential was assessed and consisted of bacterial culture in NBRI-BPB medium (National Botanical Research Institute Phosphate Solubilization Media with bromophenol blue [0.025%]).[19] Medium discoloration around the bacterial colony after 7 days of incubation was considered a positive result.

2.4.2 *Nitrogen fixation*

The nitrogen fixation potential was assessed by culturing the strains on a nitrogen-free minimal medium (mannitol NFMM). The growth of bacterial strains after 4 days of incubation at 30 °C indicated the nitrogen fixation potential.

2.4.3 *Siderophore production*

Chrome azurol S medium (CAS medium) was used to study siderophore production by the studied strains.[20] Briefly, all tested strains were spotted on CAS-agar plates in triplicate and incubated for 3 days at 30 °C. Siderophore production was indicated by the formation of an orange halo around the colony.

2.4.4 *IAA production*

Indole-3-acetic acid production was determined in LB medium supplemented with L-tryptophane (1 mg mL^{-1}).[21] After 72 h of incubation at 30 °C at 120 rpm, the cell-free supernatant was mixed with ortho-phosphoric acid and Salkowski reagent. The mixture was kept in the dark for 30 min at 30 °C. The absorbance at 530 nm was measured, and the IAA produced was quantified against an IAA (Sigma–Aldrich) standard curve.

2.4.5 *ACC deaminase gene amplification*

The genomic DNA of isolates was extracted according to standard protocols.[22] The amplification of the Acc deaminase gene was performed using the primers F-accd (AAGAGGGGCATTACCACTTTATTA) and R-accd (CGCCTTCCCAATCRCCATACAT).[23] PCRs contained 1× PCR buffer, 0.2 mM dNTP, 0.5 mM of each primer set, 1.5 U of Taq DNA polymerase (Promega, France), and 50 ng of genomic DNA. Amplifications were performed in a minicycler (Applied Biosystems) with an initial denaturation step at 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 1 min, and extension

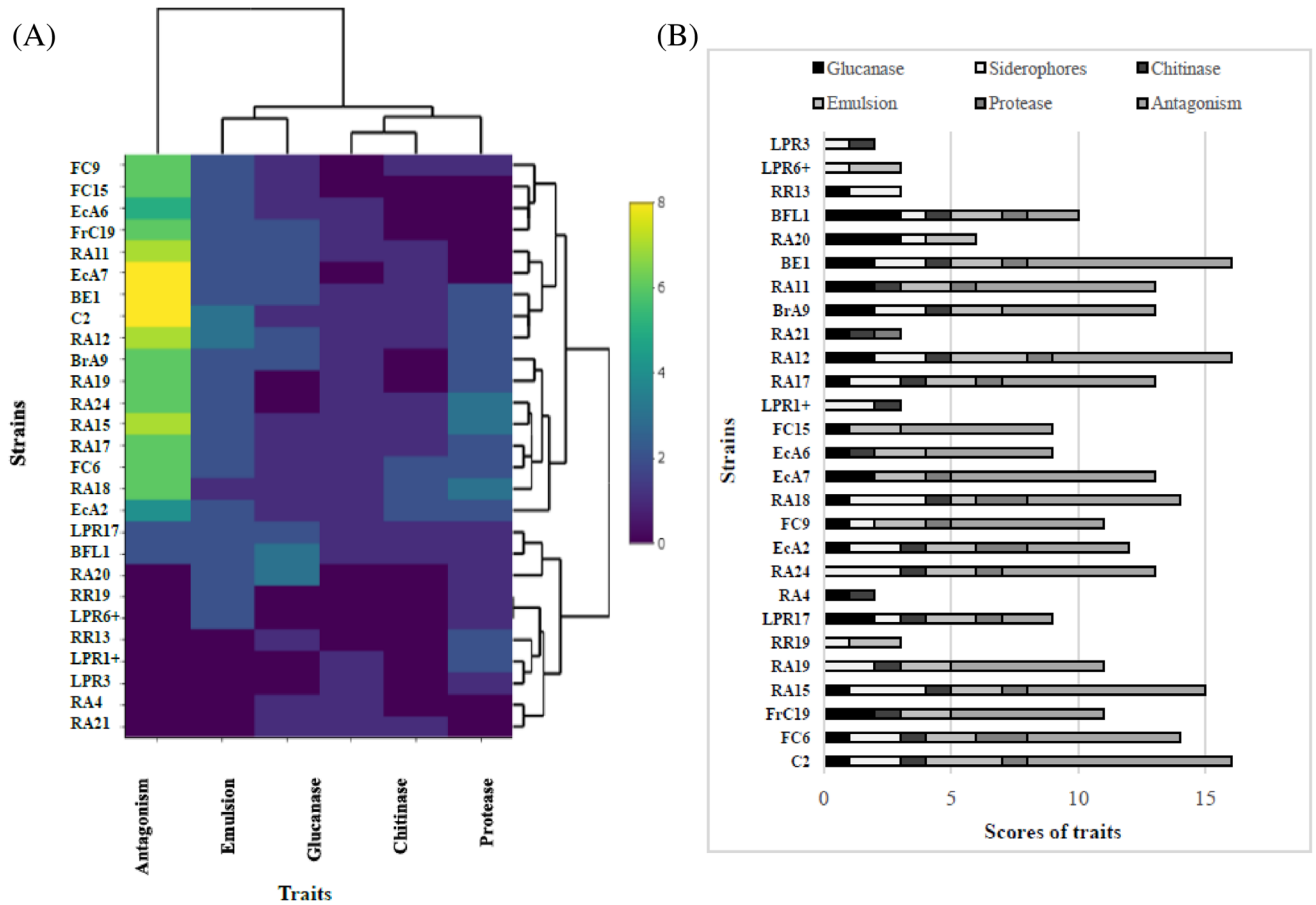

Figure 1. (a) Two-way hierarchical clustering of strains and biocontrol *in vitro* traits. The analysis was performed using the *in vitro* antimicrobial activity (antagonism), emulsification potential, enzymatic activities (glucanases, chitinases, and proteases), and siderophore production as attributes. The color scale from purple to yellow shows the gradually increasing intensity of the traits. (b) Assessments of biocontrol *in vitro* traits.

at 72 °C for 90 s; and a final extension step at 72 °C for 7 min. Each amplification reaction was electrophoretically analyzed using 1.5% agarose gel. PCR was scored positive when a band of the appropriate size was observed.

2.5 Endophytism determinants assessment

2.5.1 Cellulase production

Carboxymethyl cellulose (CMC)-based medium was used to detect the production of cellulases.[24] Bacteria were spot inoculated on plates that were incubated at 30 °C for 8 days. The diameter of the clear zone around the bacterial colony was then measured.

2.5.2 Pectinase production

Pectinase activity was assessed in a pectin-enriched medium.[25] Five days after bacterial culture, the activity was revealed by cetyl methyl ammonium bromide (CTAB), and hydrolysis diameters were measured.[25]

2.5.3 Colonization (motility assays)

Swimming and swarming motilities were studied on LB medium with agar concentrations of 0.3 and 0.7%, respectively.[26] Diameters of bacterial colonies were recorded after 48 h of incubation at 30 °C.

2.5.4 Emulsification activity

The emulsification activity of bacterial strains was performed to assess their emulsion-forming and stabilizing capacities.[27] Equal volumes (2 mL) of mineral oil and cell-free culture of the bacterial strain were added to a test tube, vortexed at high speed for 2 min, and allowed to stand for 24 h. The emulsification index (E24) was then determined as follows:

$$E24 = (\text{height of the emulsion layer})/(\text{height of the total layer}) \times 100. \quad (3)$$

All measurements were performed in triplicate.

2.5.5 Biofilm formation

Quantitative estimation of biofilm formation by the isolates was determined.[28] Each bacterial colony was cultured in liquid LB medium for 12 h at 30 °C and 200 rpm. Then, 150 µL of each culture adjusted to an OD_{595nm} of 0.01 were introduced in an ELISA plate and incubated under stationary conditions at 30 °C for 48 h. After incubation, 100 µL of crystal violet (0.1%) were added, and the plate was incubated for 20 min. Excess crystal violet solution was then discarded, the wells were washed with PBS (1×) twice, and 200 µL of DMSO were then introduced. After 20 min, the formed biofilms were quantified at 620 nm.

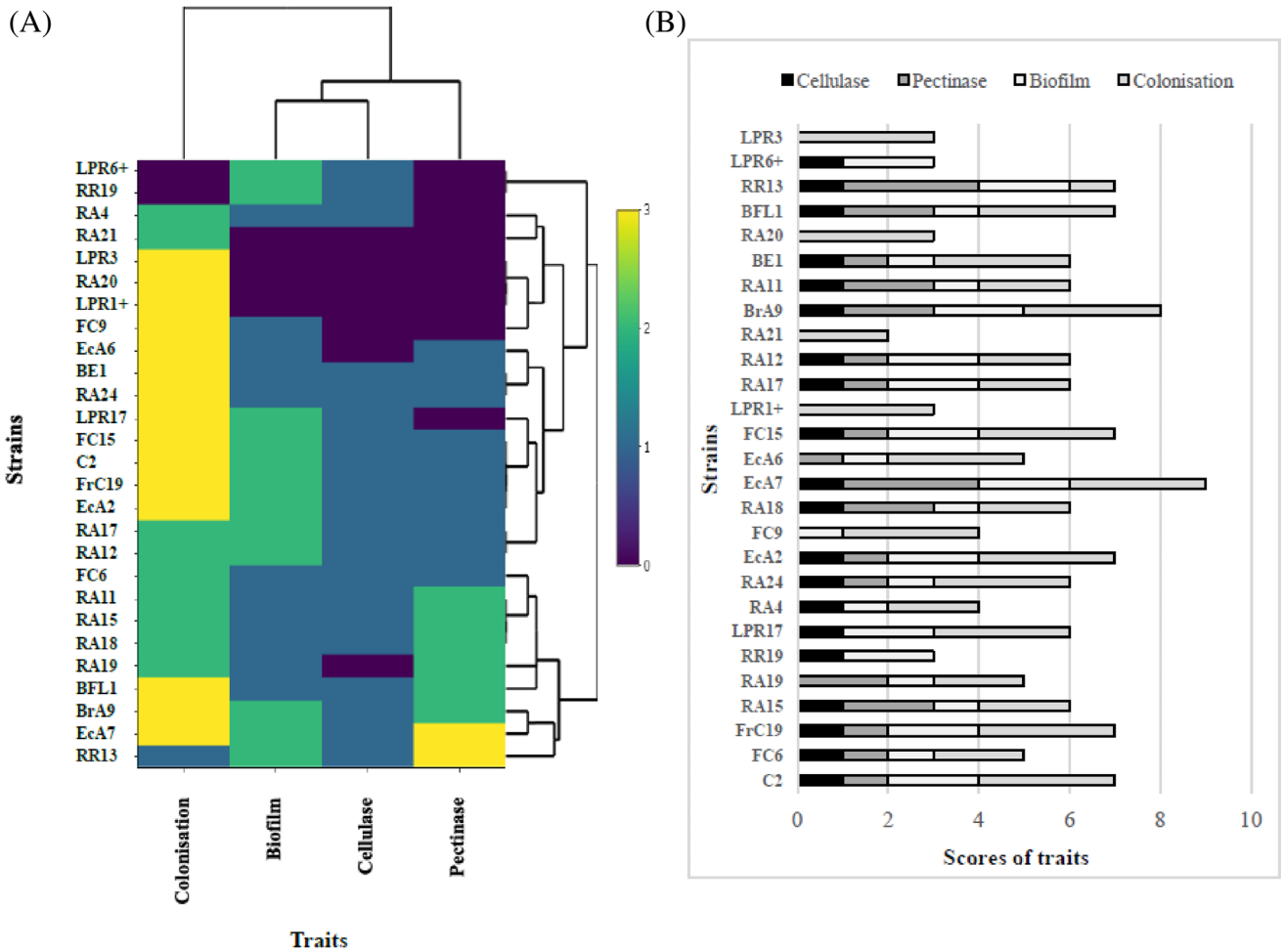

Figure 2. (a) Two-way hierarchical clustering of strains and endophytism *in vitro* traits. The analysis was performed using swimming and swarming motilities (colonization), biofilm formation, cellulase and pectinase enzymatic activities as attributes. The color scale from purple to yellow shows the gradually increasing intensity of the traits. (b) Assessments of endophytism *in vitro* traits.

2.6 Maize seed germination and protection

2.6.1 Seed treatments

Maize seeds (variety BENEDICT CMS (Cameroon Maize Selection) 8704) used in this study were provided by the Institute of Agricultural Research for Development (IARD, Yaoundé). The seeds were surface disinfected with 70% ethanol for 3 min followed by 5% sodium hypochlorite for 5 min and vigorously rinsed with sterile distilled water. Seeds were inoculated with bacterial inoculum (10^8 CFU mL^{-1}) with shaking for 6 h. Sterile distilled water was used as a negative control. Thereafter, seeds were dried and inoculated with *F. verticillioides* (2×10^6 conidia mL^{-1}) for 1 h. The seeds were then placed in Petri dishes (five seeds per dish and three dishes per treatment) and incubated in the dark at 23 °C for 10 days.[29]

2.6.2 Disease parameters

At the end of the incubation period, disease-related parameters were recorded as the germination percentage according to the following formula[30]:

$$\text{Germination\%} = (\text{Number of germinated seeds}/\text{Total number of seeds}) \times 100. \quad (4)$$

The plant height was measured to determine the vigor index as previously described.[31]

$$\text{Vigor index} = \text{Germination\%} \times \text{Total height of the plant}. \quad (5)$$

The disease severity was calculated according to the 0–5 severity scale,[32] where 0: healthy seeds; (i) no visible mycelium on seed surface, presence of tanned lesions on roots; (ii) seed surface partially covered by mycelium, presence of tanned lesions on roots; (iii) seed surface partially covered by mycelium, presence of tanned to brown lesions on roots; (iv) seed surface completely covered by mycelium, presence of tanned to brown lesions on roots; and (v) seed surface completely covered by mycelium, presence of reddish-brown lesions on roots. The following formula was used for disease severity determination[32]:

$$\text{DS} = [(\sum \text{Severity scale} \times \text{Number of seeds in the scale})/(\text{Total number of seeds} \times \text{Highest scale})] \times 100. \quad (6)$$

In addition, the percentage of protection was determined as follows[33]:

$$\text{Protection}\,(\%) = 100 - \text{DS}. \quad (7)$$

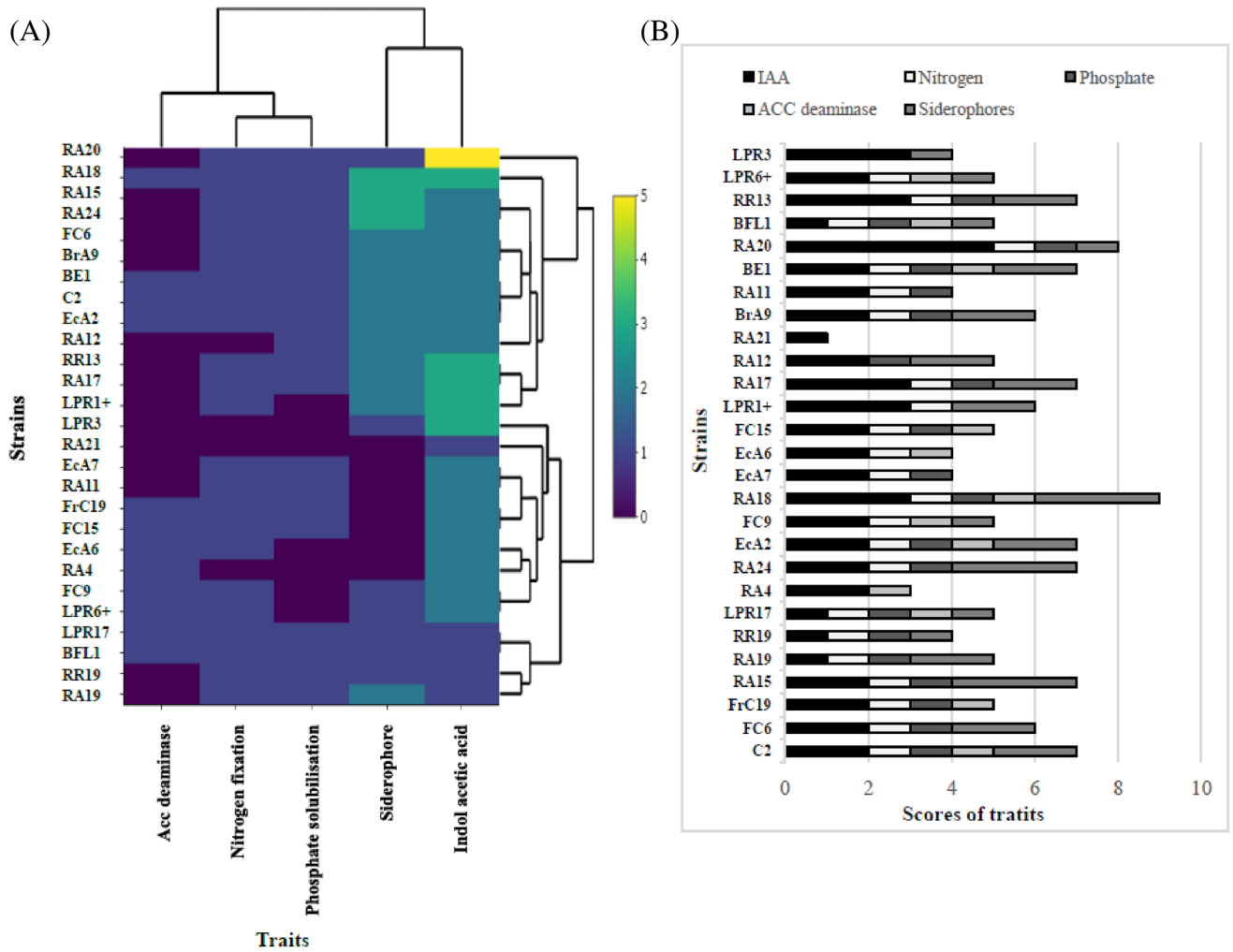

Figure 3. (a) Two-way hierarchical clustering of strains and PGP *in vitro* traits. The analysis was performed using Acc deaminase, nitrogen fixation, phosphate solubilization, siderophore production, and IAA production as attributes. The color scale from purple to yellow shows the gradually increasing intensity of the traits. (b) Assessments of PGP *in vitro* traits.

2.7 Assessment of beneficial traits

All bacteria were tested to elucidate all the beneficial traits. Then, an assessment system was established to identify the best isolates with high beneficial traits among the 27 strains. For each attribute, intervals were defined, and a scale notation was adopted. Phosphate solubilization, chitinase and cellulase production, nitrogen fixation, and ACC deaminase gene amplification were scored as 0 in the absence of production, growth, and amplification, respectively, or 1 in the opposite case. Regarding antimicrobial activity, the score was assigned according to the number of pathogens inhibited by the antagonist (the scores varied from 0 to 12 related to the inhibition of the nine fungi and the three bacteria). Protease production was graded from 0 to 2 based on the ratio used for enzymatic activity determination with 0: <0.5, 1: 0.5–1.0, and 2: >1.0. Siderophore production was also graded according to the diameter of the orange halo around the bacterial colony 0: <0.2 cm, 1: 0.2–0.5 cm, 2: 0.5–1.0 cm, and 3: >1.0 cm. For glucanase production, the scales were defined as 0: <5, 1: 5–10, 2: 10–15, and 3: >15. IAA production was graded based on the obtained concentration with 1: <10 μg mL^{-1}, 2: 10–20 μg mL^{-1}, 2: 20–30 μg mL^{-1} and 3: >30 μg mL^{-1}. For colonization (motility assays), zero to three scales indicated the spread of colony diameter with widths of 0.0–0.5, 0.5–5.0, 5.0–7.5, and ≥7.5 cm, respectively. Regarding biofilm formation, the studied strains were classified based on the obtained OD values with 0: <1; 1: 1–2 and 2: >2. Pectinase activity was graded according to the diameter of inhibition with 0: <1.0, 1: 1.0–2.5, 2: 2.5–5.0, and 3: >5.0. For emulsification activity, the scales were defined as 0: 0.0, 1: 0.1–50.0, 2: 50.0–60.0, and 3: >60.0%. In the same way, germination and vigor index were also graded differently regarding PGP *in situ* and biocontrol *in situ*. Protection was also graded as follows: 1: <65, 2: 65–85, and 3: >85%.

The biocontrol *in vitro* trait score for each strain was considered the sum of points of the antimicrobial potential, emulsification, enzymatic activities (glucanases, chitinases, and proteases), and siderophore activity. That of the endophytism *in vitro* trait was the sum of points of swimming and swarming motilities (Colonization), biofilm formation, and cellulase and pectinase activities. The sum of points of Acc deaminase, nitrogen fixation, phosphate solubilization, and siderophore and IAA production

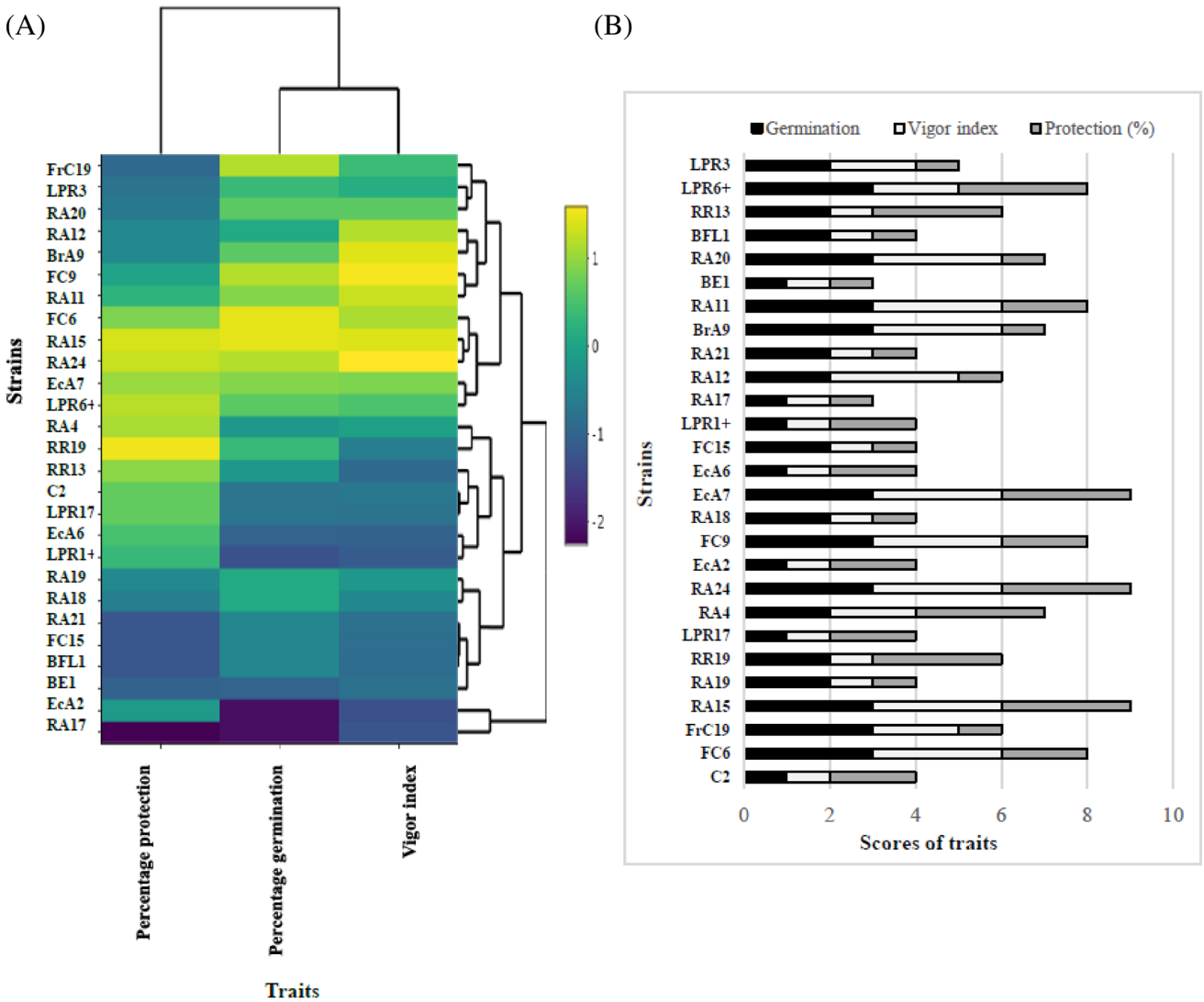

Figure 4. (a) Two-way hierarchical clustering of strains and biocontrol *in situ*. The analysis was performed using the percentage of protection, the percentage of germination, and the vigor index as attributes. The color scale from purple to yellow shows the gradually increasing intensity of the traits. (b) Assessments of biocontrol *in situ* traits.

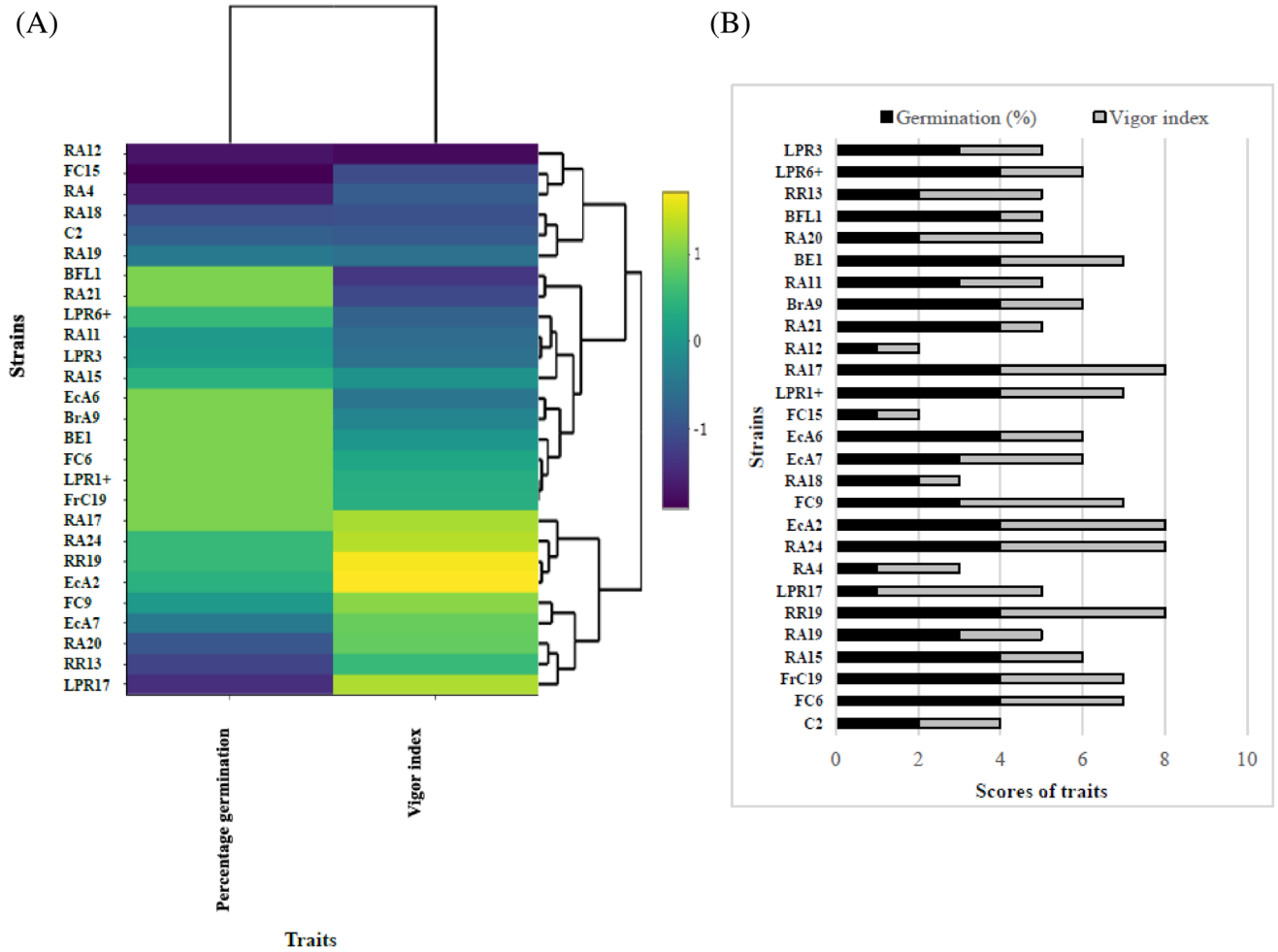

Figure 5. (a) Two-way hierarchical clustering of strains and PGP *in situ*. The analysis was performed using the percentage of germination and vigor index as attributes. The color scale from purple to yellow shows the gradually increasing intensity of the traits. (b) Assessments of PGP *in situ* traits.

was considered the PGP *in vitro* trait score for each strain. For the PGP *in situ* score, the sum of points of the percentage of germination and vigor index was determined. The biocontrol *in situ* score for each strain was calculated by adding those of the percentage of protection, the percentage of germination, and the vigor index.

2.8 Molecular identification of the promising strains

The gene encoding the 16S rRNA gene was amplified using FD1 and RD1 primers[34] following the PCR amplification conditions described previously.[35] Amplified PCR products were purified using a DNA purification kit (FAVORGEN BIOTECH CORP®), sequenced using an ABI 3130 DNA sequencer (Applied Biosystems, Foster, CA, USA), and identified using the EzBioCloud 16S database.[36] The sequences were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/). Accession numbers obtained from GenBank for deposited partial nucleotide sequences are listed in Table S2.

2.9 Statistical analysis

The results were graphically presented using the R studio program. Two-way hierarchical cluster analysis based on Ward's method was performed to reveal the clusters of strains and the studied traits with similar patterns. Correlation analysis was also performed for the studied traits to assess the relationship between them using the R studio program. The latter was also used to perform principal component analysis for the studied traits and strains to assess their relationship.

3 RESULTS

3.1 Biocontrol *in vitro* traits

The antimicrobial potential of the isolates was analyzed against nine phytopathogenic fungal and three phytopathogenic bacterial strains on solid media. The extent of antimicrobial activity varied among the isolates (Fig. 1(a)). Examples of antimicrobial activities are shown in Fig. S1. Seven bacterial strains exhibited antifungal activity against all phytopathogenic fungi: C2, RR19, RA24, RA18, EcA7, RA12, and BE1. Among these latter, only strain BE1 showed antibacterial activity against the three bacterial phytopathogens and strain EcA7 against *A. tumefaciens* B6 and *E. amylovora*. Six strains did not show any antifungal activity.

Stable emulsions were obtained with most strains (Fig. S1). Only five strains did not show any emulsification: RA4, LPR1+, RA21, RA13, and LPR3. The best strains were C2 and RA12, with an emulsification index higher than that of Tween 20 (data not shown).

The enzymatic activities varied among the isolates (Fig. 1(a)). Examples of enzymatic activities are shown in Fig. S1. Of the

tested isolates, 12 strains showed the three enzymatic activities. Strong glucanase activity was observed for BrA9, RA11, and BE1 and strong protease activity was observed for RA18 and RA24 (data not shown). Glucanase, chitinase, and protease activities were not observed in four, seven, and nine strains, respectively. Strains RR19 and LPR6+ did not show any enzymatic activity.

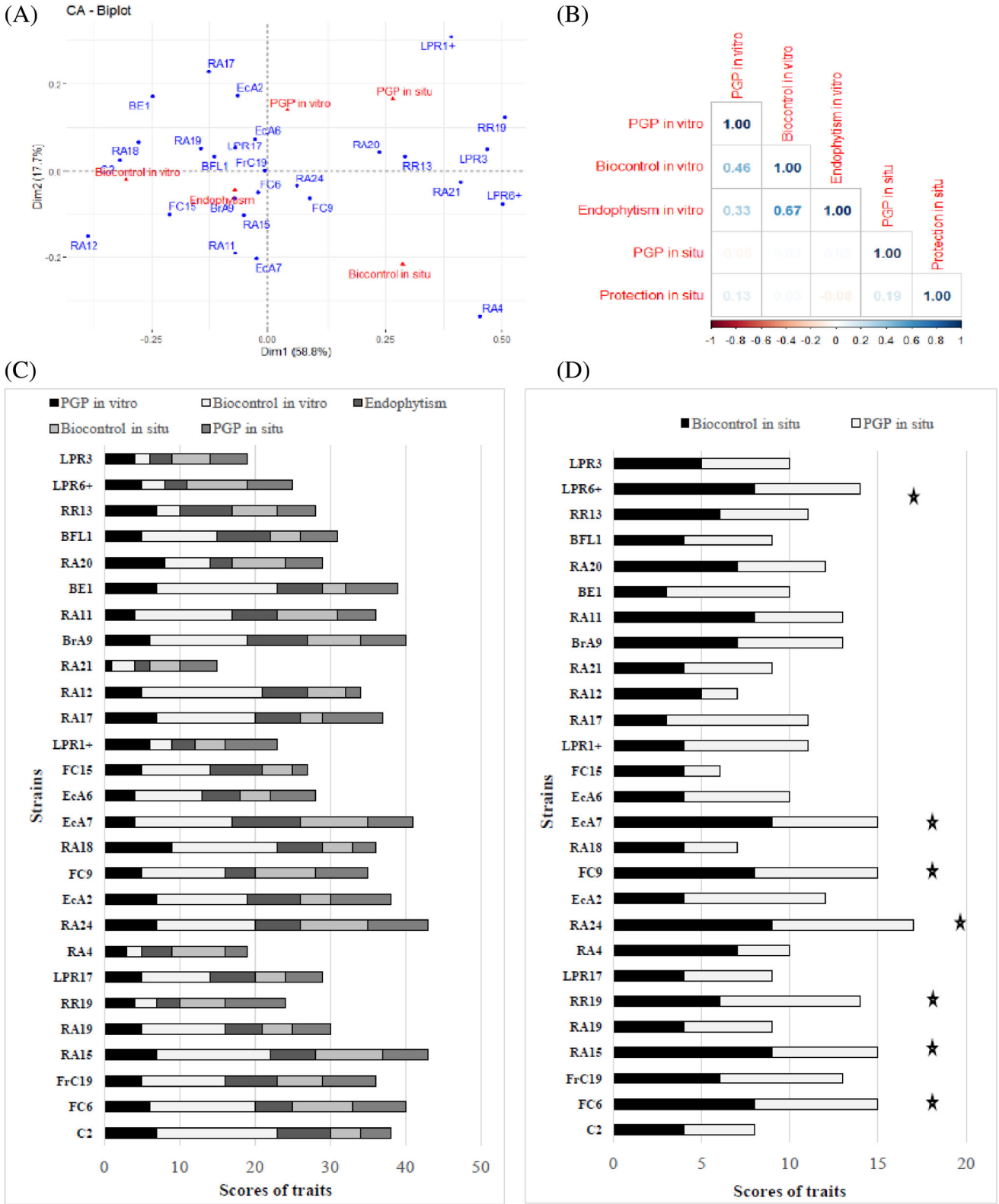

Figure 6. (a) Principal component analysis profiles based on the traits and strains. The percentage of variation accounted for by each axis is indicated in parentheses. (b) Correlation analysis of the studied trait assessment of their relationship. (c) Assessments of the total *in vitro* and *in situ* traits. (d) Assessments of the total *in situ* traits. ★: selected promising strains.

Except for seven strains that did not exhibit any siderophore activity, the 20 remaining strains indicated a large clear orange halo zone around the colonies on CAS agar medium. Strains RA15, RA18, and RA24 showed the highest production (Fig. 1(a)).

Taken together, the assessed biocontrol trait *in vitro* score attributed to each strain was also compared. Strains C2, RA12, and BE1 had the maximum assessed value of 16 points, followed by RA15 (15 points) and FC6 and RA24 (14 points) (Fig. 1(b)).

3.2 Endophytism *in vitro* traits

The endophytism *in vitro* trait results of the 27 isolates are shown in Fig. 2. Fifteen of the 27 strains (55.5%) exhibited high potential for swimming and swarming motility (colonization), and only three strains were nonmotile. Regarding biofilm formation, 12 strains (44.4%, 12/27) were high biofilm producers, 11 were moderate producers, and four did not show any biofilm. In addition, cellulase and pectinase enzymatic activities were found to be variable among the isolates (Fig. 2(a)). A total of 74.1% (20/27) and 74.1% (20/27) of the strains were cellulase and pectinase producers, respectively, and 59.3% (16/27) of the strains produced both enzymes.

The assessed endophytism *in vitro* trait scores showed that the best strains were EcA7 (9 points), followed by BrA9 (8 points), RR13, BFL1, FC15, EcA2, FrC19, and C2 (7 points) (Fig. 2(b)).

3.3 PGP *in vitro* traits

The PGP *in vitro* traits of all 27 isolates are shown in Fig. 3. A total of 44.4%, 85.2%, and 74.1% of the strains exhibited amplification of the Acc deaminase gene, nitrogen fixation, and phosphate solubility potentials, respectively. In addition, all the isolates were siderophore producers (Fig. S2). Regarding IAA production, the highest production was observed for strain RA20, with a value of 117.49 μg mL^{-1}, followed by isolates RA18, LPR1+, RA17, RR13, and LPR3, with values ranging from 20 to 30 μg mL^{-1}.

The PGP traits, expressed as scores, showed that the best strains were RA18 (9 points), followed by RA20 (8 points), and strains RR13, BE1, RA17, EcA2, RA24, RA15, and C2 (7 points) (Fig. 3(b)).

3.4 PGP *in situ*

The isolates exhibited variable percentages of germination and vigor indices on maize seedlings (Fig. 4(a)). The best isolates regarding these two parameters were RA17, EcrA2, RA24, and RR19 (8 points), followed by BE1, FC9, FrC19, and FC6 (7 points) (Fig. 4(b)).

3.5 Biocontrol *in situ*

The growth-promoting activity and the protection provided by the isolates during the challenge with the pathogen were studied on maize seedlings (Fig. 5, Fig. S3). The isolates exhibited variable percentages of protection, percentage of germination, and vigor indices (Fig. 5(a)). The best isolates according to the assigned scores were EcrA7, RA24, and RA15 (9 points), followed by LPR6+, RA11, FC9, and FC6 (Fig. 5(b)).

3.6 Selection and molecular identification of promising strains

Principal component analysis was performed to assess possible correlations between traits and to select the best isolates (Fig. 6 (a)). The obtained results do not show a clear distinction between isolates according to the studied traits. In addition, correlation studies showed a weak link between *in vitro* and *in situ* traits (Fig. 6(b)). As a consequence, the best strains were selected based on *in situ* PGP and biocontrol traits. The representation of all traits expressed as scores is shown in Fig. 6(b). The number of scores varied between 15 and 43, where 13 isolates had scores higher than 35 and only three were below 20 (Fig. 6(c)). When assessing only the *in situ* PGP and biocontrol traits, we showed that seven strains had scores higher than 13: RA24 (17 points); EcA7, FC9, RA15, and FC6 (15 points); and LPR6+ and RR19 (14 points) (Fig. 6(d)).

Strains RA24, EcA7, FC9, and RA15 were identified as *Bacillus subtilis*, FC9 as *Bacillus velezensis*, and FC6 as *Bacillus tequilensis*. In addition, LPR6+ and RR19 were identified in a previous work as *Stenotrophomonas maltophilia* and *Klebsiella pneumoniae*, respectively.[37]

4 DISCUSSION

Endophytes are regarded as the most adapted, ecofriendly, and cost-effective strategy to fight against crop pathogens. However, environmental conditions prevailing nowadays represent a limit to the success of biocontrol agents.[38] In this way, taking endophytes from desert plants, which grow in low-nutrient, high-stress environments, and transferring them to crop plants may be a more fruitful alternative.[39] Some bacteria from *Euphorbia antiquorum* L. had previously demonstrated their biocontrol potential against *F. verticillioides* through some biological assays.[2] However, some contradictions were noticed between *in vitro* and *in situ* evaluations. In fact, *in vitro* assays are conducted to select biological agents based on understanding the possible mechanism used by the biological agents against the pathogen. Nevertheless, when biological agents are associated with plants, they can exhibit various modes of action, which may or may not involve a direct effect on the pathogen. So, establishing a good screening procedure and understanding the mode of action involved in plant protection are essential for optimum disease control.[40] In our study, significant *in vitro* screening procedures have been established to discover highly active biocontrol bacteria. Additionally, the correlation between *in vitro* and *in situ* potentials through assays conducted on maize seeds and young plants (Data not shown) has been clarified. Hence, this study investigates the capacity of endophytic biocontrol agents isolated from three plants growing under drastic dryness conditions to control *F. verticillioides* ear rot and promote the growth of maize.

A large number of our tested isolates has demonstrated the ability to inhibit the mycelial growth of pathogenic fungi and bacteria through direct confrontation. This antagonistic potential could be justified by various mechanisms like the ability to produce broad-spectrum antibiotics. In fact, antibiosis is the most often used biological control mechanism against phytopathogens. A broad range of antibiotics like cyclic lipopeptides, polyketides, and volatile compounds have been identified from *Bacillus* bacterial agents.[41] Beside their antagonistic potential, they also confer a competitive advantage to biocontrol agents and trigger induced systemic resistance (ISR) in the plant system.[42] The studied bacteria also demonstrated the ability to produce biosurfactants through emulsification with promising applications as antimicrobial agents,[43] siderophores, and hydrolytic enzymes, known for their different antimicrobial potentials. Siderophores synthesized by biocontrol agents exhibit biological activity through direct antibiosis against bacterial and fungal phytopathogens along with their well-known mechanism of competition through iron scavenging.[3,44] Besides, the synthesis and release of hydrolytic

enzymes like glucanases, chitinases, and proteases have been widely reported as one of the most interesting mechanisms employed by biocontrol agents. In fact, these enzymes are involved in cell wall component lysis. The latter is mainly constituted by glycoproteins, β-glucan, and chitin, causing fungal death.[45,46] Many studies also reported the antagonistic potential of bacterial strains against *F. verticillioides via* direct inhibition or excretion of hydrolytic enzymes.[47,48] Almost all the tested bacteria were able to produce at least one of the evaluated hydrolytic enzymes, revealing their ability to act on the pathogen's cell wall. All these antagonistic characteristics confirm the previous findings, where significant inhibitions of *F. verticillioides* growth were observed in direct confrontation, along with the production of three types of siderophores and almost one hydrolytic enzyme by bacterial strains derived from *Euphorbia antiquorum* L.[2]

The bacterial candidates exhibited various colonization factors like swimming and swarming motilities, biofilm, and cellulase and pectinase enzymatic activities. Indeed, colonization plays a crucial role in plant protection by biocontrol agents as their effectiveness is enhanced when they are associated with the plant. The swimming and swarming motilities refer to the ability of bacterial strains to move on surfaces or liquid environments, respectively, using rotating flagella.[49] This mechanism allows the bacteria to move out to colonize larger areas and invade host tissues to achieve a more significant impact. The importance of bacterial motility in plant colonization has been widely demonstrated.[50,51] Swimming and swarming motilities also help the bacteria move toward sources of nutrition, avoid harmful sources, protect against the action of antibiotics, avoid competing with other organisms, and participate more effectively in cooperative processes like the formation of biofilms.[52] Biofilms are resistant structures with protected growth modality that allows bacteria to survive in harsh environments. The biofilm formation is an added value for plant colonization. In fact, evidence suggests that inoculation with biofilm-forming bacteria leads to better plant colonization and growth promotion compared to non-biofilm-forming inoculants.[53] Biofilm also contributes to biocontrol by creating a mechanical barrier preventing pathogen infection.[54] Bacterial plant colonization can also be mediated by the release of cellulase and pectinase hydrolytic enzymes which damage the cell wall leading to the colonization of the root system through migration in the intercellular spaces.[55]

In vitro, all the bacterial strains were able to produce at least one trait involved in plant growth stimulation. These traits include nitrogen fixation, phosphate solubilization, production of siderophores, production of the auxin IAA, and expression of the gene involved in the ACC deaminase synthesis. These findings corroborate the results obtained in our previous work, where all the 27 bacterial strains from *Euphorbia antiquorum* demonstrated positive traits related to promoting plant growth.[2] The different properties and modes of action of these traits have been widely discussed in this aforementioned article and many others.[2,56-58] In fact, the ability of bacterial strains to produce plant growth-promoting traits is a crucial criterion for an effective biocontrol agent. These traits not only accelerate plant development but also serve as an indirect mechanism by which bacterial agents counteract the effects of fungal pathogens.[59] This involves mechanisms such as compensation of damaged cells or acquisition of important precursors for metabolic pathways in the plant. For example, siderophores produced by biocontrol agents improve plant growth by scavenging iron in iron-deficient environments. This action also renders the iron inaccessible to pathogens, thereby impeding their development under competitive conditions.[60]

A high number of the strains has significantly improved maize seed germination and growth under greenhouse conditions. Notably, endophytes isolated from roots and stem bark of *Acacia albida* (RA17, EcA2, and RA24) demonstrated the most important activities as revealed by *in vitro* production of PGP traits. Interestingly, the most active strains were derived from the roots and stem bark of the same plant. Indeed, *Acacia albida*, a typical plant of desertic areas in Africa, possesses the ability to improve crop yields.[61] However, there are limited studies on the biological potential of bacterial endophytes associated with this plant.[62] Surprisingly, the capacity of the bacterial agents to stimulate maize seed germination was not directly correlated with their ability to produce plant growth-promoting traits. In fact, some strains with high plant growth-promoting traits *in vitro* showed relatively weaker effects on seed germination and plant growth, while others exhibited the opposite pattern. For instance, strain RA18 demonstrated the highest score for *in vitro* growth promotion but was among the least active in terms of *in situ* results. This discrepancy between *in vitro* and *in situ* findings might be attributed to the fact that *in vitro*-based screening strategies did not consider the environmental and host-antagonist-pathogen interaction factors.[2,63]

The bacterial candidates also demonstrated a good ability to protect maize seeds against Fusarium rot by reducing the development of the infection and stimulating seed germination and growth even under infected conditions. However, there was no correlation between the antimicrobial potential observed *in vitro* and the *in situ* seed protection, which has also been reported in other studies.[2,64] In fact, biocontrol agents can use various modes of action to protect plants against pathogenic infections. These may include hyperparasitism and antibiosis, where the agents directly interact with the pathogens.[65] Moreover, mechanisms such as resistance induction or priming plants do not involve direct interaction with the targeted pathogen.[66] In such cases, the *in vitro* performance may not be directly associated with the *in situ* potential.[2] Additionally, certain culture conditions, such as the culture medium, can impact the production of metabolites that may be produced minimally or not at all in the natural environment.[2]

All the bacterial candidates tested for the different *in vitro* traits were used to assess their ability to protect maize seeds against rot caused by *F. verticillioides* and to improve seed germination. *In vitro* active and non-active strains were all assessed *in situ* to ascertain the correlation between *in vitro* and *in situ* profiles. Seven strains exhibited the highest *in situ* potential regarding plant growth promotion and biocontrol of maize ear and root rot disease. These strains were identified as *B. subtilis*, *B. velezensis*, *B. tequilensis*, *S. maltophilia* and *K. pneumoniae*. *Bacillus* strains are known to produce a wide range of metabolites which are beneficial for plants in terms of growth promotion and defense activation. Previous studies highlighted the importance of *Bacillus* species as effective biocontrol agents in the management of maize ear and root rot.[2,3,67] Additionally, all *Bacillus* strains belong to the *B. subtilis* species complex, which is recognized for its agronomic importance as phytopathogenic antagonist, plant growth promoter, and inducer of systemic resistance. These strains are known for their ability to produce diverse antimicrobial compounds that prevent the proliferation of other microorganisms, form spores that confer resistance to adverse

environmental conditions, and exhibit high adaptability to the soil-root agroecosystem and competitive advantages.[68]

Besides, *S. maltophilia* strains are also recognized as promising agents acting as biofertilizers and biocontrol agents against fungal diseases.[69,70] Similar to *S. maltophilia, K. pneumoniae* isolates attract particular attention due to their ability to promote plant growth through phosphate solubilization, phytohormone production, and increased nutrient uptake by association with roots as endophytes.[71] However, it is important to note that these species (*S. maltophilia* and *K. pneumoniae*) are also associated with healthcare-associated infections and multidrug resistance.[72,73] Therefore, further studies must be conducted to assess the safety of the tested strains before considering their potential application in agriculture.

Finally, considering the various *in vitro* screening assays and *in situ* performances, it is evident that the selection criteria of promising biocontrol agents should be reviewed. These criteria should take into account several factors such as the type of microorganism, the potential mechanisms which can be mediated by the type of microorganism, the envisaged application of the biocontrol agents, and the targeted pathogen.[2,74,75]

5 CONCLUSION

This work was undertaken following a previous investigation to confirm the selection process of promising bacterial isolates as protective agents against *F. verticillioides* and growth promoters in maize. The results obtained from multiple *in vitro* assays demonstrated the isolates' ability to produce various antimicrobial products and growth-promoting traits. Furthermore, *in situ* investigations revealed the protective potential of the biological agents and their ability to enhance maize seed germination. However, as reported in our previous work, there was no direct correlation between the *in vitro* performances and the *in situ* potentials, despite the wide range of observed activities. This highlights the need to reassess the selection process for bacterial candidates, taking into consideration the specific type of microorganism and the mechanism of action typically associated with this group of isolates. Based on the obtained results, seven potent endophytic strains were selected for further studies. These strains belong to *B. subtilis*, *B. velezensis*, *B. tequilensis*, *S. maltophilia*, and *K. pneumoniae* species. They demonstrated a particular ability to protect maize seeds against *F. verticillioides* rot and to improve seed germination. Therefore, these strains could be further investigated to develop a biopesticide for the management of Fusarium ear and root rot disease in maize.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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